Succinic acid production from softwood with genome-edited Corynebacterium glutamicum using the CRISPR-Cpf1 system

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Abstract

Corynebacterium glutamicum is a useful microbe to produce succinic acid, a bio-based platform chemical, under anaerobic condition. The knock-out mutant of lactate dehydrogenase 1 gene (ΔldhA-6) and co-expression of succinic acid transporter (Psod:sucE- ΔldhA) were generated by using CRISPR-Cpf1 genome editing system. HAPC (hydrogen peroxide and acetic acid) pretreatment is a highly efficient method for enzymatic hydrolysis of softwood and the hydrolysate was used for production of succinic acid. In the 1?5% hydrolysate (Pinus densiflora), the best condition for ΔldhA mutant to produce succinic acid from the hydrolysate was confirmed to ferment 4% hydrolysate, resulted in 14.82 g L-1 succinic acid production for 6 h, which reached to 2.47 g L-1 h-1 productivity. No production of acetic acid and lactic acid was detected during the fermentation. The co-expression transformant, [Psod:sucE- ΔldhA], produced 17.70 g L-1 succinic acid in 6 h, presenting a productivity of 2.95 g L-1 h-1 on the 4% hydrolysate. In the fed-batch system, 39.67 g L-1 succinic acid was produced for 48 h. The yield of succinic acid from reducing sugars in the hydrolysate is approximately 56.71%, while the yield of succinic acid from glucose alone as the main substrate is approximately 84.4%. These results indicated that the production of succinic acid from softwood has potential applications in alternative biochemical processes, and minimizing the loss of sugars during enzymatic hydrolysis and fermentation can lead to more economic benefits in succinic acid production from lignocellulosic biomass.

1. Introduction

Succinic acid recently garnered increasing attention as a promising alternative biochemical to replace petroleum-derived compounds due to its wide range of potential industrial application in the fields of pharmaceuticals, agriculture, and the food industry among others. Notably, succinic acid has been ranked as the most valuable chemical among the top 12 bio-based high-value-added chemicals according to the US Department of Energy (DOE).[1] Succinic acid, which belonging to the four-carbon dicarboxylic family, is an intermediate of the citric acid or glyoxylate cycle during glucose metabolism. Several microbes such as *Actinobacillus succinogenes* ,*Mannheimia succiniciproducens* , *Escherichia coli* ,*Yarrowia lipolytica* , and *Corynebacterium glutamicum* are known to produce succinic acid through anaerobic fermentation.[2–5]

C. glutamicum is a gram-positive soil bacterium that has been used for production of amino acids and other value-added metabolites. Succinic acid and lactic acid are the main excreted metabolites in the glucose metabolic pathway of wild-type *C. glutamicum* under anaerobic conditions, and the synthesis of succinic acid increases when the production of lactic acid is disrupted by the knock-out of lactate dehydrogenase 1 gene.[6] A previous study characterized the succinic acid transporter (SucE) of *C. glutamicum* with the authors reporting that, unlike the Dcu family of succinic acid transporters present in *E. coli* , the exported succinic acid was not imported from the medium.[7]The transcription level of *sucE* gene under anaerobic condition was also determined to be 2.2–fold higher than under aerobic conditions.[8] A metabolically engineered *C.*

glutamicum strain (lactate dehydrogenase 1 knock-out ($\Delta\lambda\delta\eta A$) and over-expression of pyruvate carboxylate (*pyc*) was reported to produce up to 146.4 g L^{-1} of succinic acid in a fed-batch condition from glucose alone, within 46 h, and under anaerobic conditions.[5] These results highlight the promising potential of *C. glutamicum* as a succinic acid producer. However, there is a lack of intensive research on succinic acid production from lignocellulosic biomass containing with glucose, xylose, arabinose, mannose and other components.

Classic homologous recombination has been developed to generate site-directed mutagenesis, gene deletion, simultaneous heterogeneous gene expressions and a homogeneous target gene knock-out in the *C. glutamicum* genome; moreover, this approach has been applied to enhance the production of amino acids and metabolites.[9-11] However, the efficiency of homologous recombination is known to be extremely low during the first and second crossover gene recombination, thus, extensive PCR screening must first be conducted to identify the desired recombinant colonies.

The CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9) genome editing system has been developed to provide simple and precise nucleotide editing, specific-gene deletions, and heterogeneous gene insertions into the genomic DNA of microbial, yeast, and human cells.[12–14] However, CRISPR-Cas9 or dCas9 (deactivated Cas9) cannot be used to edit the genome of *C. glutamicum* due to the toxic metabolites secreted by this microbe. In contrast, Cpf1, which was identified as a single-strand RNA-guided endonuclease belonging to the class 2 CRISPR-Cas system, was instead found to efficiently achieve these nucleotide substitutions, insertions, and deletions in *C. glutamicum* .[15]Therefore, CRISPR/Cpf1 genome editing can be used to reinforce succinic acid production through the precise deletion of the *ldhA* target gene and promote the expression of the genes of interest in *C. glutamicum* .

Lignocellulosic biomass has been evaluated as a sustainable and alternative sugar resource to corn-based sugar due to its abundance and high amount of carbohydrates.[16] Furthermore, biorefineries are considered environmentally green facilities that can potentially replace petroleum-based industries by using convertible sugars obtained from biomass in the fermentation process to produce biochemicals such as bioethanol, lactic acid, and succinic acid.[17–19] However, the structural complexity of lignocellulosic biomass enforces the highest cost input to the pretreatment and enzymatic hydrolysis in the overall bioconversion process.

Softwood is composed of ray parenchyma cells, resin canals, and tracheids. Among these structures, tracheids account for approximately 91% of the softwood xylem.[20] The macro- and microfibril structures of tracheids, and their composition of lignin, hemicellulose, and cellulose affect the pretreatment efficiency, although the extent to which depends on the method and condition. Woody plants including pine wood are more recalcitrant than herbaceous agricultural biomass such as corn stover, kenaf, rapeseed straw, and rice straw, when they are pretreated under popping and steam explosion.[21–25] Organosolv and dilute acid techniques are more effective for the hydrolysis of hardwoods (poplar and eucalyptus) than softwoods (pine and spruce).[16, 25] Sulfite pretreatment on aspen, eucalyptus, spruce, and red pine led to efficient hydrolysis rates.[26, 27] HPAC pretreatment conducted in this study, delignification of pine wood with hydrogen peroxide and acetic acid, removes lignin disturbances on cellulase and reduces cellulose recalcitrance, resulting in the highly efficient enzymatic digestion of pine wood.[28]

Succinic acid has been produced using metabolically engineered *C. glutamicum* and pure glucose as a carbon source. However, few studies have explored the applicability of lignocellulosic biomass, including corn cobs hydrolysate[29], for the production of succinic acid. Lignocellulosic biomass must first be pretreated prior to its saccharification and fermentation. Toxic by-products derived from lignin or hemicellulose are released during its pretreatment and are known to hinder the fermentation efficiency of the microbes.[30] Although few studies have assessed the applicability of lignocellulosic biomass and the value it added to the bioconversion process, the fermentation of hydrolysates from lignocellulosic biomass is expected to differ from that of pure glucose. Therefore, our study sought to characterize the production of succinic acid from softwood and analyze the patterns of succinic acid and the other metabolites produced by a CRISPR/Cpf1 generated ldhA mutant ($\Delta\lambda\delta\eta A$-*6*), and aimed to enhance succinic acid production in a fed-batch system by utilizing a co-expression transformant (Psod:sucE-$\Delta\lambda\delta\eta A$).

2. Material and methods

2.1 Construction of CRISPR-cpf1 vector for genome editing

The pJYS3_ ΔcrtYF plasmid for genome editing with CRISPR-cpf1 was purchased from Addgene () (Jiang et al., 2017).[15] The plasmid was modified to obtain the pJYS3_Amp_MCS vector using the primers noted in additional file 2: Table S1 to substitute kanamycin in over the ampicillin selection marker genes for the sub-cloning selection in *E. coli* (Fig. 1). A double target DNA system was constructed with the target DNA 1 primer set (crRNA + 24 bp target DNA + rmB T1 terminator) and target DNA 2 primer set (J23119 promoter + crRNA + 24 bp target DNA + sacB1 terminator). The pJYS3_Amp_DT vector was obtained by sequentially inserting the target DNA 1 and 2 primer sets at the HindIII-BamH1 and Xba1-Apa1 restriction sites in the pJYS3_Amp_MCS1 vector (Fig. 1B). The DNA fragments of the lactate dehydrogenase 1 promoter (LdhAp) and terminator (LdhAt) were obtained from the genomic DNA of *C. glutamicum* with the sense/anti-sense primer pairs of LdhAp and LdhAt, respectively.[31] The genomic DNA isolation and PCR procedures are described in section 2.3. The fragments were sequentially inserted into the pCold 1 vector (Cat. No. 3362, TaKaRa, Japan) at the Sac1-Kpn and HindIII-Xba1 sites for LdhAp and LdhAt as homologues arms, respectively. The kanamycin gene from pJYS3_ ΔcrtYF was amplified and inserted into the Kpn1-Xho1 restriction site between LdhAp and LdhAt in the vector. The [LdhAp]-[Kn^r]-[LdhAt] construct was amplified with the LdhAp-sense/LdhAt-anti-sense primers pairs, and inserted into the pJYS3_Amp_DT vector at the Xma1-Apa1 site. Finally, the pJYS3_Amp_DT_[LdhAp-Kn^r-LdhAt] vector was constructed.

Over-expression of succinic acid transporter gene onto *Δλδηα*mutant was conducted to improve succinic acid production. The genomic DNA of *C. glutamicum* was extracted following the procedure outlined in section 2.3. The gene encoding the succinic acid transporter (sucE) and the ribosomal S12 protein gene (*rpsL*) were subsequently isolated and subcloned into the region between the homologous arms on the pCold vector (Fig. 1C). To obtain streptomycin resistance, the *rpsL* gene was mutated by substituting AAG (Lys^{43}) to AGG (Glu).[32] The variant was denoted to *rpsLm* in this study. The construction [LdhAp]-[Psod:sucE]-[Pro4:*rpsLm*]-[LdhAt] on pCold vector was amplified and inserted into the pJYS3_Amp_DT vector (Fig. 1D), and transformed into *Δλδηα κνοςκ-ουτ μυταντ οφ ˜. γλυταμιυμ*following the procedure outlined in section 2.2-2.3.

2.2 Strain and transformation

The ATCC 13032 *C. glutamicum* strain was obtained from the Korean Agricultural Culture Collection (KACC). *C. glutamicum* competent cells were prepared as previously described by Ruan et al. (2015).[32] Briefly, the cells were cultured in LHB solid media (20 g L^{-1} LB broth, 18.5 g L^{-1} brain heart infusion (BHI), 18 g L^{-1} agar). A single colony was inoculated into 5 mL BHI media (0.2 g $L^{-1}K_2HPO_4$, 0.3 g $L^{-1}NaH_2PO_4$, 0.5 g $L^{-1}MgSO_4{\cdot}7H_2O$, 10 g $L^{-1}(NH_4)_2SO_4$, 37 g L^{-1} BHI, pH 7.2) and cultured at 30 °C for 12 h. The microbes were harvested and re-cultured in 20 mL NCM (1.0 g L^{-1} yeast extract, 5 g L^{-1} tryptone, 5 g L^{-1} glucose, 0.3g L^{-1} trisodium citrate,17.4 g $L^{-1}K_2HPO_4$, 0.05 g $L^{-1}MgSO_4{\cdot}7H_2O$, 91.1 g L^{-1}sorbitol, 11.6 g L^{-1} NaCl, pH 7.2) at 30 °C for 4 h. After harvesting, the microbes were rinsed three times with ice-cold 10% glycerol. The microbes were then centrifuged and resuspended with 2 mL ice-cold 10% glycerol after which 90 μL of cells were aliquoted into microcentrifuge tubes. The competent cells were stored at -80 °C.

Plasmid DNA (5–10 μL) was added to 90 μL of competent cell solution and transferred to a 2 mm electroporation cuvette (Cat. No. Z706086-50EA, Sigma-Aldrich, USA). Electroporation was performed with a MicroPulser system (BioRad) 1.8 kV for 5 ms. After electroporation, 900 μL of liquid BHIS media (18 g L^{-1} BHI, 91 g L^{-1}sorbitol) was added and resuspended, after which the cells were immediately incubated for 6–15 min at 46 °C. The cells were then plated on LBHIS (5 g L^{-1} tryptone, 5 g L^{-1}NaCl, 2.5 g L^{-1} yeast extract, 18.5 g L^{-1} BHI, 91 g L^{-1} sorbitol, 18 g L^{-1} agar, pH 7.2) containing 50 μg mL^{-1} kanamycin or 30 μg mL^{-1}streptomycin, and incubated at 30 °C until colonies appeared.

2.3 Genomic DNA isolation and PCR analysis

The colonies on the selection media were inoculated into 5 mL LB medium, and incubated at 30 °C for

12 h to extract genomic DNA. Then, the cells were harvested and suspended in 100 μL extraction buffer (200 mM lithium acetate, 1% SDS), after which they were incubated at 70 °C for 10 min (Looke et al., 2011). Afterward, 300 μL of 96% ethanol was added to the tubes and the samples were vigorously mixed in a vortex mixer. Genomic DNA and cell debris were precipitated by centrifugation at 15,000×g for 10 min, and the pellets were dissolved in 100 μL distilled water. The cell debris was separated by centrifugation at 15,000×g for 10 min, and 1 μL of the supernatant was used for PCR analysis of the transformants and gene cloning of the LdhAp and LdhAt homologous arms from *C. glutamicum* , using the primers in additional file 2: Table S1. Transformants were analyzed with the LdhAp (-1042) sense, Kn^r anti-sense, and Kn^r sense/LdhAt (+1029) primer pairs (PCR 1 and PCR 2, respectively). The over-expression strains appearing on streptomycin solid media were selected by PCR analysis with LdhAp (-1042) sense and sucE anti-sense primer.

2.4 Pretreatment of pine wood

Pine wood (*Pinus densiflora* , diameter: 13 cm) was chopped into approximately 0.25 cm (width) × 0.35 cm (height) × 4.5 cm (length) chips, and 100 g L^{-1} of the wood chips were soaked in hydrogen peroxide (H_2O_2): acetic acid (CH_3COOH) solution (1:1 ratio, HPAC solution).[33] The wood chip sample was delignified in a water bath at 80 °C for 2–3 h. The delignified wood chips were then strained and thoroughly washed with water until the HPAC solution was completely removed. Finally, the sample was freeze dried and stored at room temperature.

2.5 Enzyme preparation and hydrolysis

Cellulase was produced using the Rut-C30 strain of *Trichoderma reesei* .[22] The activity of the cellulase stock on filter paper was measured to be 50 FPU mL^{-1}. Xylanases derived from *Thermomyces lanuginosus* (Cat.X2753-50G, St. Louis, MI, USA) was purchased from Sigma-Aldrich. β-glucosidase of*Aspergillus niger* (Lot 141001, Wicklow, Ireland) was obtained from Megazyme. One unit of xylanase was defined as the enzyme concentration that released 5 g L^{-1} of reducing sugars from 1% beechwood xylan (X4252-100G, Sigma-Aldrich) at 50 °C for 10 min. β-glucosidase, 20 μg mL^{-1}, was completely hydrolyzed to glucose in 6.85 g L^{-1} cellobiose over 10 min at 50 °C, this amount was defined as one unit.

The HPAC-pretreated pine was weighed to prepare 1–5, and 10% (g v^{-1}) in 100 mL citric acid buffer (10 mM, pH 5.5) and hydrolyzed with 20 FPU cellulase g^{-1} biomass and auxiliary enzymes (200 units L^{-1} xylanase and 100 units L^{-1} β-glucosidase). In the case of the small scale volume, 2% HPAC-pretreated pine was prepared in 1 mL citrate buffer (10 mM, pH 5.5) with 5-100 FPU g^{-1} biomass alongside 2 units of xylanase and 1 unit of β-glucosidase. All the reactions were performed at 50 °C for 12–96 h. The solutions were centrifuged at 13000 rpm for 10 min to obtain a clear hydrolysate. The concentrations of fermentable sugars were measured using a DNS assay and HPLC analysis. [21, 33] To prepare the feedstock for fed-batch system, 20% HPAC-pretreated pine was hydrolyzed with 20 FPU cellulase g^{-1} biomass along with the auxiliary enzymes at 50 °C for one week. The supernatant was clarified through filtration and then incubated more for 24 h. The concentration of reducing sugar in the hydrolysate was measured as 155.08 g L^{-1}.

All of the hydrolysates were stored at -20 °C until used for succinic acid fermentation.

2.6 Succinic acid production from the HPAC-pretreated pine

Mineral salts (0.5 g $L^{-1}KH_2PO_4$, 0.5 g $L^{-1}K_2HPO_4$, 0.5 g $L^{-1}MgSO_4{\cdot}7H_2O$, 6.0 mg $L^{-1}FeSO_4{\cdot}7H_2O$, 4.2 mg $L^{-1}MnSO_4{\cdot}H_2O$, 0.2 mg L^{-1}biotin, 0.2 mg L^{-1} thiamine) were added to the hydrolysates and adjusted to pH 7.5 with 1.0 N NaOH.[5] The Δ*ldhA* -6 mutant of *C. glutamicum* was pre-cultured in 20 mL of LB medium at 30 °C overnight. The cells were subsequently transferred to 0.5-1.5 L LB medium and cultured at 30 degC for 24–48 h (approximately 2.5–3.0 g L^{-1}, CDW). After harvest, the cells (5.36–26.89 g L^{-1}) were added to the 100 mL hydrolysates and 400 mM sodium bicarbonate was added to retain semi-anaerobic condition. The fermentation to produce succinic acid was performed at 200 rpm and 30 degC in a shaking incubator. The pH of the reaction solution was checked and maintained at 7.2–7.5 by adding sodium hydroxide after each sampling.

A fed-batch process was initiated with 4% hydrolysate (initial hydrolysate) and approximately 30 g L^{-1} CDW of [Psod:sucE- Δ*ldhA*] transformant. After 24 h of fermentation, 20 mL of the 20% hydrolysate as a feed was added. The fermentation was conducted using the same method described above. The yield of succinic acid was calculated based on the followed equation:

Yield of succinic acid (%) = S_t / (G_i + G_f) × 100

Where S_t is total amount of succinic acid produced in fed-batch system, and G_i and $G_f$represent the glucose concentration of the initial and feeding hydrolysates, respectively.

2.7 Organic acids analysis

Organic acids in the supernatant after fermentation with Δ*ldhA* -6 mutant were analyzed using an HPLC system equipped with a refractive index (RI) detector (Waters 2414, USA) and a ROA column (7.8 × 300 mm, Phenomenex, Torrance, CA) for the organic acids and an RPM column (4.6 × 300 mm, Phenomenex, Torrance, CA) for the fermentable sugars. The temperatures of the detector and column were maintained at 40 and 65 °C, respectively. The mobile phase was passed through the column at a flow rate of 0.6 mL per min with 5 mM sulfuric acid.

3. Results and Discussion

3.1 CRISPR-Cpf1 mediated *ldhA* knock-out recombination in *C. glutamicum.*

Cpf1 from *Francisella novicida* is a putative class 2 CRISPR effector, similar to the role of Cas9 in *Streptococcus pyogenes* .[34] However, unlike Cas9, Cpf1 cleaves target DNA with a sole single RNA-guided endonuclease and is not coupled to a trans-activating CRISPR RNA (tracrRNA). Additionally, Cpf1 recognizes two or three thymine residues (5'-TTN-3' or 5'-TTTN-3') known as the T-rich protospacer-adjacent motif (PAM) and cleaves the phosphodiester bonds between the 23rd and 24th base in the annealing strand and between the 18th and 19th base from the PAM on the non-annealing strand.[15, 34] Although the CRISPR-Cas9 system was confirmed to be a simple and precise tool for genome editing in several microbes, the system does not work well in *C . glutamicum* . Therefore, the Cpf1 system, adopted in this study, enabled single-strand DNA recombination, endogenous gene deletions, and exogenous gene insertions in *C . glutamicum* .[15]

To obtain a recombinant *C . glutamicum* strain with both a gene deletion (lactate dehydrogenase 1, *ldhA*) and a gene insertion (kanamycin resistance gene, Kn^r), the all-in-one, pJYS3_ ΔcrtYF vector was modified to obtain the pJYS3_Amp_MCS vector (Fig. 1). The CRISPR-Cas9 genome editing system has been used previously to modify *C. glutamicum* . For example, Peng evaluated three factors affecting the recombination efficiency.[13] The authors reported that the recombination efficiency in a gene deletion process was much higher when the length of each homologous arm exceeded 0.1kb, whereas the heterogeneous gene insertion performance improved when the length of a single arm exceeded 0.3 kb. Furthermore, a sgRNA (single guide RNA) including a 20 bp target DNA showed different genome editing efficiencies suggesting that the positions and ratios of the nucleotides in the target DNA sequence must be considered. Therefore, the pJYS3_Amp_DT vector was constructed by incorporating double target DNA sets of the template and non-template strands of the *ldhA* gene into the pJYS3_Amp_MCS vector. The lengths of the left and right homologous arms were 974 and 942 bp, respectively, and they were subcloned into the pCold vector. A kanamycin resistance gene (1.1 kb) was also sub-cloned between the two homologous arms. The homologous arms and kanamycin resistance gene set (LdhAp-Kn^r-LdhAt) were introduced into multi-cloning site one of pJYS3_Amp_DT, which resulted in the pJYS3_Amp_DT_(LdhAp-Kn^r-LdhAt) vector (Fig. 1C). The vector was transformed into *C. glutamicum*at 46 °C for 6–12 min. The transformants appeared on Kn^r LB solid media and we confirmed that the first homologous recombination had occurred with a 100% efficiency rate (Fig. 2 and additional file 1: Fig. S1). One colony was picked and suspended in 1 mL BHIS media. After the second heat shock at 46 °C for 6–12 min, appropriately diluted transformant solution was spread on Kn^r LB media. Twelve colonies out of several transformants were picked and we confirmed that the second homologous recombination occurred with a 50% recombination efficiency (Fig. 2B). Among the transformants, the production of succinic and lactic acids was assessed using line six, a knock-out mutant of the *ldhA* gene (*Δλδηλ-6*), under semi-

anaerobic conditions with pure glucose. As expected, the lactic acid production of *C. glutamicum* was completely blocked (Fig. 2C).

Hydrolysis of HPAC-pretreated pine

Table 1 summarizes the rates of pretreatment and enzymatic hydrolysis depending on lignocellulosic biomass. The differences among them were attributed to three factors that were reported to be responsible for the delay in enzymatic hydrolysis of lignocellulosic biomasses: (1) the structural complexity and solidity of lignin, (2) the structural recalcitrance of cellulose caused by the interaction strength between the β-1,4-glucose chain bundles in cellulose microfibrils, and (3) the inhibition of cellulases by glucose, cellobioses, and xylo-oligomers during enzymatic hydrolysis.[28, 35–40] HPAC pretreatment of pine wood showed high efficiencies in lignin removal and cellulose fiber swelling, which resulted in an 85% hydrolysis rate after 3 h and complete hydrolysis after 9 h with 3 FPU mL^{-1} of cellulase and xylanase in 1% substrate.[21, 28] These observations indicated that there was no structural recalcitrance of cellulose fibers. Additionally, the tracheid structures from HPAC-pretreated pine wood (pit, window-like pits, and lumen diameter) provided a wider accessible surface for cellulases and increased the hydrolysis rate compared to hardwood fibers.[28]

Hemicellulose consisting of galactoglucomannan and arabinoglucuronoxylan coats the microfibril units composed of cellulose chains.[41, 42] The trunks of the Korea red pine (*P. densiflora*) used in this study are composed of 41.9% cellulose, 14.9% galactomannan, and 6.4% xylan (Rahmini et al., 2019).[43] A cellulase solution obtained from *T. reesei* was reported to contain several hemicellulases, including one mannanase and six xylanases. Mannanase (Man5A, 53.6 kDa) hydrolyzes galactoglucomannan, which accounts for 60%–70% of the hemicellulose in softwood. Endo-xylanases cleave the β-1,4-xylosidic bonds within the xylan structures including arabinomethylglucuronoxylan and methylglucuronoxylan, which account for 13%–30% and 5%–15% of the hemicellulose, respectively.[44] Among the aforementioned endo-xylanases, XYNIV (55 kDa) is remarkably effective in the treatment of soluble beechwood, whereas the activity of hemicellulase in the cellulase solution was very low, suggesting that additional xylanases are required to enhance the hydrolysis rate. The xylanase solution from *Thermomyces lanuginosus* containing β-xylanase (Xyn11A, 23kDa) and β-xylosidase (GH43, 38.1 kDa) is considered a surrogate of the small molecular weight xylanases XYN1 and XYN2 of *T. reesei.* Previous studies have reported that additional xylanase enhanced the hydrolysis rates through a synergistic interaction with mannanase (Varnail et al., 2011).[45]

The open structural cellulose surface of HPAC-pretreated pine can be saturated by hydrolytic enzymes such as Cel7A (cellobiohydrolase I) and Cel6A (cellobiohydrolase II) and accounts for 68–78% of the secretome of *T. reesei* Rut-C30.[46] Moreover, these enzymes release cellobiose from solid cellulose fibers, which is a strong inhibitor of Cel7A, while glucose inhibits Cel6A (cellobiohydrolase II) and β-glucosidase.[39]It means that end-product inhibition can be a dominant retardation factor throughout the enzymatic saccharification process. In Fig. 3, the addition of the xylanase cocktail and β-glucosidases of *A. niger* into a 5–10 FPU cellulase g $biomass^{-1}$ showed remarkable enhancement of the hydrolysis rate, increasing it from 39.0–42.1% to 84.0–87.5% for 12 h. HPAC pretreatment on softwood provided rapid saccharification and a small dose of hydrolysis enzymes, which begins to overcome the aforementioned major challenges and reduce the costs of the enzymes and energy supplied throughout the bioconversion process of the lignocellulosic biomass.[47]

The occurrence of the strong end-product inhibition when scaling-up the conditions caused retardation of enzymatic saccharification and contribute to the loss of fermentable sugars, predominantly due to the remaining solid fraction and the existence of oligomers or un-identified sugars (Table 2), compared to the results in Fig. 3. It is inferred that the insufficient dosages of the high cost of β-glucosidase were responsible for the retardation during the large scale hydrolysis. Preparation of low-cost and highly efficient β-glucosidase on cellobiose is key for rapid and economical saccharification.

Succinic acid production from HPAC-pretreated pine

Succinic acid has been produced using various microbes such as *A. succinogenes* , *M. succiniciproducens* , *Y. lipolytica* ,*E. coli* , and *C. glutamicum* (Table 3). Moreover, this process has been enhanced by engineering the genes associated with glucose metabolism (TCA cycle or glyoxylate cycle).[8] For example, the overexpression

of a single gene encoding for pyruvate carboxylase (pyc) significantly increased succinic acid yields in a lactate dehydrogenase 1 knock-out mutant of *C. glutamicum* .[5] Nevertheless, unlike several gene knock-out mutants, the *C. glutamicum* wildtype can be used to produce succinic acid under anaerobic conditions.[48] Table 3 compares the succinic acid production yields of different recombinant *C. glutamicum* strains and other microbes. Interestingly, the production yields of succinic acid from the hydrolysates tended to be much lower than those achieved using pure glucose as a carbon source and showed a wide range of yield depending on the cell-dried weight (CDW, cell concentration) and fermentation time. These results suggest that the carbon sources and the cell concentration are the rate-limiting factors in the biosynthesis of succinic acid (Okino et al., 2008).[5]

The single knock-out mutant of the *ldhA* gene in *C. glutamicum* , *ΔλδηA-6* (10.15–21.19 g L^{-1} CDW), was incubated in 100 mL of 1–5% hydrolysate (Table 2), and the metabolites (succinic acid, lactic acid, and acetic acid) produced under semi-anaerobic condition were analyzed (Fig. 4). The glucose in the 1–4% hydrolysate was almost consumed by the *ΔλδηA-6* mutant after 9 h, and produced 3.64, 7.45, 8.49 and 13.77 g L^{-1} of succinic acid, without lactic acid. Simultaneously, the xylose consumption was entirely delayed at the same interval of time. The *ΔλδηA-6* mutant (CDW: 21.08 g L^{-1}) in the 5% hydrolysate produced higher levels of lactic acid than in the other hydrolysates, the semi-anaerobic or anaerobic fermentation condition of which required to tightly retain to block surging lactic acid production. In some cases, there was a failure to retain the semi-anaerobic conditions, and lactic acid, which is the dominant metabolite, was produced 3.6 times higher than succinic acid in the 5% hydrolysate by the *ΔλδηA-6* mutant (data not shown). Although the *ΔλδηA-6* gene activity was lost in the *ΔλδηA-6*mutant, lactic acid still tended to be produced under the semi-anaerobic condition in the 1–5% HPAC-pretreated hydrolysates over the 9 h period. It is estimated that a minor metabolic pathway related to lactic acid production was stimulated by some derivative in the hydrolysate of the HPAC-pine. Indeed, xylo-oligomers, cello-oligomers, xylose, mannose, and unidentified chemicals are identified as candidate materials responsible for this lactic acid production. However, additional research is required to confirm which material is responsible for lactic acid.

A comparison of the conversion rate to succinic acid (Fig. 5), illustrated that the best condition, among the hydrolysates, was to ferment the 4% hydrolysate with approximately 20 g L^{-1} CDW for 9 h, as it provided 1.58 g L^{-1} h^{-1} productivity with a 98% glucose consumption rate. Cell densities of 10.15 g L^{-1} for 1%, 15.72 g L^{-1} for 2%, and 16.08 g L^{-1} for 3% hydrolysate were required for a 9h complete consumption, while an 88% glucose consumption was shown in the fermentation of 5% hydrolysate. The correlations between cell concentration, succinic acid production, and glucose consumption are summarized in Fig. 6. A more efficient and economical production of succinic acid was attempted from the 4% hydrolysate using higher cell concentration (26.89 g L^{-1} CDW) than the previous experiment, which resulted in 14.82 g L^{-1} succinic acid production over 6 h, showing productivity of 2.47 g L^{-1} h^{-1} and an 86.2% glucose and a 20.0% xylose consumption. It is close to the 2.5 g L^{-1} h^{-1} value, which is the minimum productivity of succinic acid provided from corn-based sugar required to compete with the current market petrol-based succinic acid production.[1] There is still the potential to increase succinic acid production from 4% hydrolysate because remaining sugars available for the conversion include 15% glucose and 80% xylose. To increase efficiency of succinic acid production, we incorporated and overexpressed the succinic acid transporter gene,*sucE* , under the Psod promoter using the CRISPR/cpf1 gene editing system (Fig. 7). The co-expression transformant (Psod:sucE-*ΔλδηA,* 10.00 g L^{-1} CDW) exhibited higher production of succinic acid in 4% pine hydrolysate compared to the*ΔλδηA-6* mutant (10.94 g L^{-1} CDW). In comparison of succinic acid production of the 4, 5, and 10% hydrolysate (containing 27.45, 39.66, and 57.66 g L^{-1} reducing sugars, respectively), the optimal concentration of the hydrolysates in the fermentation with [Psod:sucE- *ΔλδηA*] transformant (28–30 g L^{-1} CDW) was found to be 4%, consistent with our previous results. The productivity of succinic acid was found to be 3.83 g L^{-1} h^{-1} for the 3 h fermentation and 2.95 g L^{-1} h^{-1} for the 6 h fermentation period. In the fed-batch with 4% hydrolysate, the first feeding was carried out after 24 h of the fermentation. This feeding consisted of 20 mL of 20% pine hydrolysate (155.08 g L^{-1}), which was adjusted to be equivalent to the final concentration of the 4% hydrolysate. As a result, the amount of succinic acid produced was doubled, which reached to 39.67 g L^{-1}. Yield of succinic acid from in-put reducing sugars is 56.71 %.

The yield of succinic acid from glucose that is mainly consumed during the fermentation is ˜84.4%. Small amount of acetic acid was measured at the time of feeding, while lactic acid was observed at the late stage of fermentation, furthermore, about half of the xylose remained in the solution. Based on the results of glucose consumption, we fed 20 mL of 20% pine hydrolysate three times at 6, 9, and 24 h. However, the efficiency of succinic acid production decreased immediately after each feeding. These findings emphasize the significance of maintaining a concentration of 4% hydrolysate to achieve high-efficiency of succinic acid production. It seems that xylose accumulation is a limiting factor during the fermentation over 4% hydrolysate, requiring further study on the retardation factors in high concentration of hydrolysate, which is technically important to improve succinic acid production using a fed-batch system.

Succinic acid production has been studied using diverse microbes. There are hitherto some rare cases of succinic acid production using hydrolysates from lignocellulosic biomasses. We conducted the overall process of succinic acid production from lignocellulosic biomass, and suggest an optimization condition in each process for succinic acid production. In this study, the conversion ratio of glucose to succinic acid was higher than in previous studies using engineered *C. glutamicum* strains in which several genes were knocked-out and/or overexpressed (Table 3). This indicates that the single knock-out$\Delta\lambda\delta\eta A$ mutant and co-expression of sucE gene is a sufficient succinic acid producer from glucose. To achieve further economical production of succinic acid from HPAC-pretreated pine, future studies must focus on improving xylose consumption rate and fermentation efficiency particularly in high concentration of hydrolysate, which can deliver economic feasibility to succinic acid production from lignocellulosic biomass.

4. Conclusions

CRISPR-Cpf1 genome editing system is an efficient recombination technology to generate knock-out mutants of the lactate dehydrogenase 1 gene to enhance succinic acid production. The *$\Delta\lambda\delta\eta$A-6* mutant produced 14.82 g L^{-1}succinic acid production over 6 h, equating to a 2.47 g L^{-1} h^{-1} productivity in the fermentation of 4% hydrolysate, with an 86.2% glucose and 20% xylose consumption. Under the same fermentation conditions, the transformant [Psod:sucE- *$\Delta\lambda\delta\eta A$*] showed a productivity of 2.95 g L^{-1} h^{-1} and produced approximately 39.67 g L^{-1} succinic acid over 48 h in the fed-batch process. The yield of succinic acid from the hydrolysate when only considering glucose consumption during the fed-batch fermentation is approximately 84.4%. Xylose consumption was slower than glucose and still remained throughout the fermentation. By diminishing the sugar loss during enzymatic hydrolysis and the fermentation processes, the production of succinic acid will likely be enhanced in the future.

Credit authorship contribution statement

D.S.L. wrote the main manuscript text and performed the experiments. E.J.C. carried out the HPLC analysis and revised the manuscript. Y.S. and J.C. analysed the data and D.T.N. revised the manuscript. D.S.L. and H.J.B. received the funding. H.J.B. coordinated the study, contributed to the analysis of the results and in the improvement of the manuscript. All authors read and approved the final manuscript.

Declaration of Competing Interests

All authors declare that they have no competing interests.

Data Availability

All data generated and analyzed in this study are included in this published article.

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Figure Captions

Fig. 1. Construction of the vector for CRISPR/Cpf1 genome editing in*C. glutamicum* . (A) pJYS3_-Amp_MCS vector derived from pJYS3_ ΔcrtYF was constructed. (B) A double guide crRNAs set was incorporated into the pJYS3_Amp_MCS plasmid, between the HindIII and Xba1 restriction enzyme sites. (C, D) Homologous arms with a selection marker gene (Kn^r, kanamycin resistance gene) and co-expression cassette (Psod:sucE-Pro4:rpsLm) were finally inserted into the JYS3_Amp_DT plasmid between the Xma1 and Apa1 restriction enzyme sites. T1 and T2, target DNA sites on the *ldhA* gene of *C. glutamicum* ; Pro1, AmpR promoter; Pro2, PlacM promoter; Pro3, J23119 promoter; Pro4, Kn^r promoter derived from pJYS3_ ΔcrtYF; Psod, promoter; rmB T1 term and sacB T1 term, terminator regions of the rmB and sacB genes, respectively; sT1, sacB T1 terminator; LdhAp, lactate dehydrogenase 1 (*ldhA*) promoter region; ldhAt, terminator region of *ldhA* gene.

Fig. 2. CRISPR/Cpf1 mediated homologous recombination in *C. glutamicum* . (A) The double target sites were located 243 bp and 683 bp from the start codon of the *ldhA* gene, respectively. The first homologous recombination occurred on the LdhAp region at the first heat shock, and the second crossing-over on the LdhAt region occurred after the second heat shock. (B) The deletion of the *ldhA* gene and simultaneous Kn^r gene insertion were confirmed via PCR analysis (PCR 1 and 2). (C) Confirmation of the complete inhibition of lactic acid production in the *ldhA* gene deletion mutant (*ΔλδηA-6*). Glucose was used as the sole carbon source.

Fig. 3. Synergistic effect of cellulase with xylanase and β-glucosidase. (A) HPAC-pretreated pine with cellulase cocktail solution alone and (B) supplement with xylanases (Xyl) and β-glucosidases (Bgls) were conducted in 1 mL citrate buffer. The incorporation of auxiliary enzymes remarkably increased the hydrolysis rate of the 2% substrate and decreased the dose of cellulase required for optimal hydrolysis (5–25 FPU). The structural recalcitrance of cellulose fiber did not substantially affect the enzymatic hydrolysis of HPAC-pretreated pine.

Fig. 4. Fermentation of the *ΔλδηA-6* mutant with the hydrolysates of HPAC-pretreated pine. The metabolites (succinic acid, lactic acid, and acetic acid) produced by fermentation of the 1% (A), 2% (B), 3% (C), 4% (D), and 5% hydrolysate (E) were analyzed. The cell concentrations were 10.15 (A), 15.72 (B), 16.08 (C), 21.19 (D), and 21.08 (E) g L^{-1} CDW (cell dried weight) for fermentation of the hydrolysates, respectively.

Fig. 5. Productivity of succinic acid from the hydrolysate of HPAC-pretreated pine. Succinic acid productivity involving in glucose consumption rate (A) and cell concentration (B) were analyzed over 9 h. SA, succinic acid (g L^{-1}); Gc, glucose consumption rate; h, hour; Cw, cell dried weight (g L^{-1})

Fig. 6. Succinic acid production correlated to cell concentration. The hydrolysates were fermented with various ranges of cell densities of*ΔλδηA-6* mutant for 6 h, and succinic acid (A), glucose consumption rate

(B), and xylose consumption rate (C) were analyzed.

Fig. 7. Over-expression of succinic acid transporter (*sucE*) gene and succinic acid production in fed-batch system. (A, B) The gene was inserted onto the genomic DNA of *ΔλδηA-6* mutant under Psod promoter regulation through the CRISPR/cpf1 gene editing system. (C) The enhancement of succinic acid production was demonstrated in [Psod:sucE- *ΔλδηA*] transformant (10.00 g CDW) compared to*ΔλδηA* mutant (10.94 g CDW) when using 4% hydrolysate. (D) Comparison of the succinic acid production depending on the concentration of hydrolysates was performed with 28˜30 g L^{-1} CDW of [Psod:sucE-*ΔλδηA*] transformant. (E) A fed-batch system was carried out with an initial concentration of 4% hydrolysate and 30.37 g L^{-1} CDW of [Psod:sucE-*ΔλδηA*] transformant. After 24 h of fermentation, 20 mL of 20% pine hydrolysate was added to the reaction solution, resulting in a final concentration of the 4% hydrolysate. Acetic acid was prone to be released during the first 9 h after feeding, while lactic acid was measured at the later stage of the fermentation. (F) The same volume of the 20% hydrolysate was added at 6, 9, and 24 h. P1, Psod promoter; *sucE* , succinic acid transporter; T1, sacB terminator; P2, Kn^r promoter; *rpsL* m, ribosomal S12 protein mutant gene for streptomycin resistant; arrows, feeding.

Additional files

Additional file 1: Figure S1. CRISPR/Cpf1 mediated homologous recombination. *C. glutamicum* was transformed with the pJYS3_Amp_DT_[Lp-Kn^r-Lt] vector. The vector was incorporated into the genomic DNA after the first cross-linking in the promoter region of the *ldhA* gene. The vector accessories and the*ldhA* gene are arranged in tandem. The selection marker gene (Kn^r) on the vector part (A) and *ldhA* gene (B) was concomitantly detected.

Additional file 2: Table S1. Primers design to construct CRISPR-cpf1 gene editing vector.

Table 1. Hydrolysis rate (%) of softwoods and hardwoods depending on pretreatment methods.

Biomass	Pretreatment	Enzymatic hydrolysis	Enzymatic hydrolysis	Reference
		Substrate (%)	Conversion rate (%)	
Aspen Eucalyptus	Sulfite pretreatment (4% sodium bisulfite with or without sulfuric acid, 180 , 30 min) Sulfite pretreatment Dilute acid	2%	90-95% (20 FPU + 30 CBU ?-glucosidase g^{-1} cellulose, 12 h) 90% (48 h) 82% (48 h)	[26]
Spruce Red pine	Sulfite pretreatment (8-10% bisulfite and 1.8-3.7% sulfuric acid, 180 , 30 min) Dilute acid pretreatment Sulfite pretreatment (8-10% bisulfite and 1.8-3.7% sulfuric acid, 180 , 30 min)	2%	˜90% (14.6 FPU+22.5 CBU ?-glucosidase, 48 h) 40% 98% (14.6 FPU+22.5 CBU ?-glucosidase, 48 h)	[27]

Biomass	Pretreatment	Enzymatic hydrolysis	Enzymatic hydrolysis	Reference
Corn stover Poplar Pine	Steam explosion Organosolv Steam explosion Organosolv Steam explosion Organosolv	2%	65% (Cellulast, 48 h) 70% (Cellulast, 48 h) 52% (Cellulast, 48 h) 70% (Cellulast, 48 h) 37% (Cellulast, 48 h) 52% (Cellulast, 48 h)	[25]
Pine	HPAC (peroxide hydrogen: acetic acid = 1:1, 80 , 2 h)	2%	87.5% (10 FPU Cel+ Xyl+Bgl, 12 h) [a] 74.6% (20 FPU Cel+Xyl+Bgl, 24 h) [b]	This study

[a] small scales volume (1 mL); [b]large scales volume (100 mL)

Table 2. Large scale hydrolysis of HPAC-pretreated pine and the reducing sugar composition

Biomass concentration	Hydrolysis rate	Reaction time (h)	Reducing sugars	Glucose (g L^{-1})	Xylose (g L^{-1})	The others (g L^{-1})
1%	89.30±0.11	12	8.93±0.32	6.07±1.81	1.03±0.25	0.99±0.05
2%	74.55±0.84	24	14.91±0.84	11.30±3.15	1.70±0.50	1.66±0.24
3%	71.65±1.23	48	21.49±1.23	16.44±0.55	2.26±0.82	1.04±0.39
4%	68.62±0.41	72	27.45±0.41	20.71±1.16	3.13±0.87	4.45±2.26
5%	79.31±1.88	96	39.66±1.88	25.01±2.02	3.87±0.78	9.75±1.76

Microbe	**Metabolic engineering**	**Metabolic engineering**	**Carbon source**	**Succinic acid production (g L^{-1})**	**Productivity (g L^{-1} h^{-1})**	**Ref.**
	Knock-out	**Over-expression**				
C. glutamicum	*WT ldhA ldhA+pyc*	- - -	36 g L^{-1} glucose	4.4 g L^{-1} (3 h) 5.0 g L^{-1} (3 h) 2.4 g L^{-1} (3 h)	1.47 1.67 0.80	[6]
C. glutamicum	-	-	337.5 g L^{-1} glucose	93.6 g L^{-1} (fed-batch, 65 h)	1.44	[48]
C. glutamicum	*ldhA*	*pyc*	288 g L^{-1} glucose	146 g L^{-1} (fed-batch, 46 h)	3.20	[5]
C. glutamicum	*ldhA*	*xylA+xylB+ gapA+pntAB*	40 g L^{-1} glucose	23-26 g L^{-1} (18 h)	1.28 –1.44	[49]
C. glutamicum	*ldhA*	*xylA+xylB+gapA*	66.1 g L^{-1} glucose 139.1 g L^{-1} glucose	43.3 g L^{-1} (8 h) 113 g L^{-1} (fed-batch, 48 h)	5.41 2.35	[10]

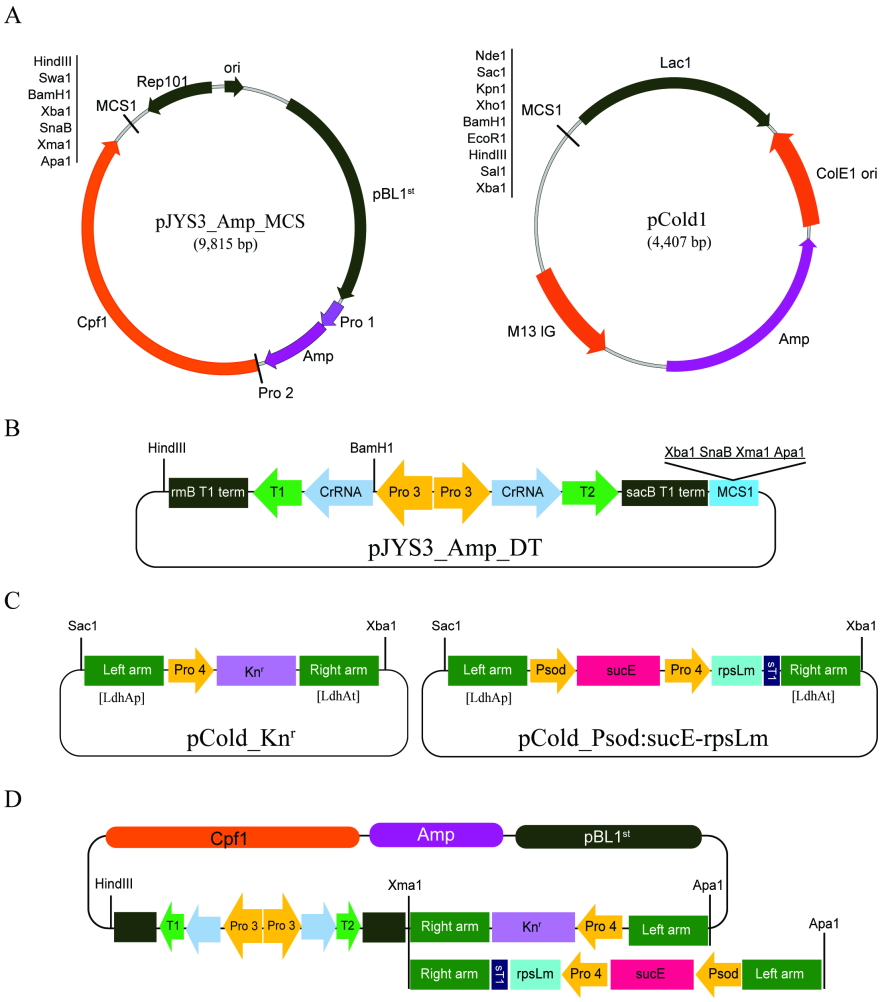
Microbe	Metabolic engineering	Metabolic engineering	Carbon source	Succinic acid production (g L^{-1})	Productivity (g L^{-1} h^{-1})	Ref.
C. glutamicum	*ldhA*	*xylA+xylB+ gapA*	Corn cob hydrolysate (55 g L^{-1} xylose, 4 g L^{-1} glucose)	40.8 g L^{-1} (48 h)	0.85	[29]
C. glutamicum	*ldhA+pqo+cat+ ackA*	*pyc+ppc+gltA+ sucE+aceA+ aceB*	20 g L^{-1} glucose 150 g L^{-1} glucose	18.7 g L^{-1} (12 h) 109 g L^{-1} (fed-batch, 100 h)	1.56 1.09	[8]
C. glutamicum	*ldhA+pta+ack +pqo+cat*	*pyc+fdh+gapA*	20 g L^{-1} glucose + 15 g L^{-1} formate	14.17 g L^{-1} (5 h)	2.83	[50]
C. glutamicum	*ldhA+pta:ackA +actA+poxB+pck+ptsG*	*pyc+ppc+pckG+udhA*	40 g L^{-1} glucose 220 g L^{-1} glucose	9.98 g L^{-1} (24 h) 152.2 g L^{-1} (fed-batch, 160 h)	0.41 0.95	[51]
E. coli	- - -	- *pyc pyc*	20 g L^{-1} glucose 20 g L^{-1} glucose *Cocos nucifera* water	1.08 g L^{-1} (48 h) 1.82 g L^{-1} (48 h) 11.75 g L^{-1} (7.5 h)	0.02 0.03 1.13	[52]
E. coli	*ldhA+ackA+ dhE+pflB+mgsA+ poxB*	-	100 g L^{-1} glucose	43.0 g L^{-1} (72 h)	0.59	[53]
E. coli	*ptsG+pflB+ldhA*	-	5% detoxified softwood dilute sulfuric acid hydrolysates	39.5 g L^{-1} (48 h)	0.82	[54]
A. succinogenes	-	-	80 g L^{-1} DDAP hydrolysate of corn stover	42.6 g L^{-1} (54 h)	0.78	[55]
A. succinogenes	*WT ackA+pflB*	- *pck+mdh+fum*	60 g L^{-1} mimicking the DDAP hydrolysate (6.5 g L^{-1} glucose, 44 g L^{-1} xylose, 3.5 g L^{-1} galactose, 6.5 g L^{-1} arabinose)	25.0 g L^{-1} (72 h) 13.5 g L^{-1} (72 h)	0.35 0.18	[4]

Microbe	Metabolic engineering	Metabolic engineering	Carbon source	Succinic acid production ($g\ L^{-1}$)	Productivity ($g\ L^{-1}\ h^{-1}$)	Ref.
A. succinogenes	-	-	58 g L^{-1} corn straw hydrolysate (Dilute-alkali pretreatment)	45.5 g L^{-1} (48 h)	0.95	[56]
A. succinogenes	-	-	Napier grass hydrolysate (22.5 g L^{-1} glucose, 10.6 g L^{-1} xylose)	17.5 g L^{-1} (22 h)	0.79	[57]
M. succiniciproducens	-	-	Oak wood hydrolysate (22.82 g L^{-1} glucose, 7.4 g L^{-1} xylose)	11.78 g L^{-1} (10 h) 7.98 g L^{-1} (Continuous fermentation, 2.5 h)	1.17 3.19	[2]
Y. lipolytica	*Ylsdh5*	-	200 g L^{-1} glycerol 625 g L^{-1} glycerol	43 g L^{-1} (120 h) 160 g L^{-1} (fed-batch, 400 h)	0.36 0.40	[3]
C. glutamicm	*LdhA LdhA LdhA*	- *sucE sucE*	4% pine hydrolysate 4% pine hydrolysate 4% + 30.7 g L^{-1} pine hydrolysate	14.82 g L^{-1} (6 h) 17.70 g L^{-1} (6 h) 39.7 g L^{-1} (fed-batch, 48 h)	2.47 2.95 0.83	This study

Table 3. Succinic acid production of microbe species.

Knock-out or over-expression genes: *ldhA* , lactate dehydrogenase 1; *pyc* , pyruvate carboxylase; xylA, xylose isomerase; xylB, xylulose kinase; *gapA* , glyceraldehyde-3-phosphate dehydrogenase A; *pntAB* , membrane-bound transhydrogenase; *ecaA* , carbonic anhydrase; *pepc* , phosphoenolpyruvate carboxylase; *pqo* , pyruvate:menaquinone oxidoreductase; *cat* , acetyl-CoA:-CoA transferase; *ackA* , acetate kinase; *ppc* , phosphoen+olpyruvate carboxylase; *aceA* , isocitrate lyase;*aceB* , malate synthase; *sucE* , succinic acid transporter;*gltA* , citrate synthase; *Ylsdh5* , succinate dehydrogenase E;*adhE* , aldehyde/alcohol dehydrogenase; *pflB* , pyruvate formate-lyase; *mgsA* , methylglyoxal synthase; *ptsG* , glucose transporter; *poxB* , pyruvate/ubiquinone oxidoreductase;*pck* , phosphoenolpyruvate caboxylkinase; *fum* , fumarase;*mdh* , malate dehydrogenase; *sucE* , succinic acid transporter

DDAP, diluted acid pretreatment



pJYS3_Amp_DT_[LdhAp-Knr-LdhAt] or [Psod:sucE-rpsLm]

Fig. 1. Construction of the vector for CRISPR/Cpf1 genome editing in *C. glutamicum* . (A) pJYS3_-Amp_MCS vector derived from pJYS3_ ΔcrtYF was constructed. (B) A double guide crRNAs set was incorporated into the pJYS3_Amp_MCS plasmid, between the HindIII and Xba1 restriction enzyme sites. (C, D) Homologous arms with a selection marker gene (Kn^r, kanamycin resistance gene) and co-expression cassette (Psod:sucE-Pro4:rpsLm) were finally inserted into the JYS3_Amp_DT plasmid between the Xma1 and Apa1 restriction enzyme sites. T1 and T2, target DNA sites on the *ldhA* gene of *C. glutamicum* ; Pro1, AmpR promoter; Pro2, PlacM promoter; Pro3, J23119 promoter; Pro4, Kn^r promoter derived from pJYS3_ ΔcrtYF; Psod, promoter; rmB T1 term and sacB T1 term, terminator regions of the rmB and sacB genes, respectively; sT1, sacB T1 terminator; LdhAp, lactate dehydrogenase 1 (*ldhA*) promoter region; ldhAt, terminator region of *ldhA* gene.

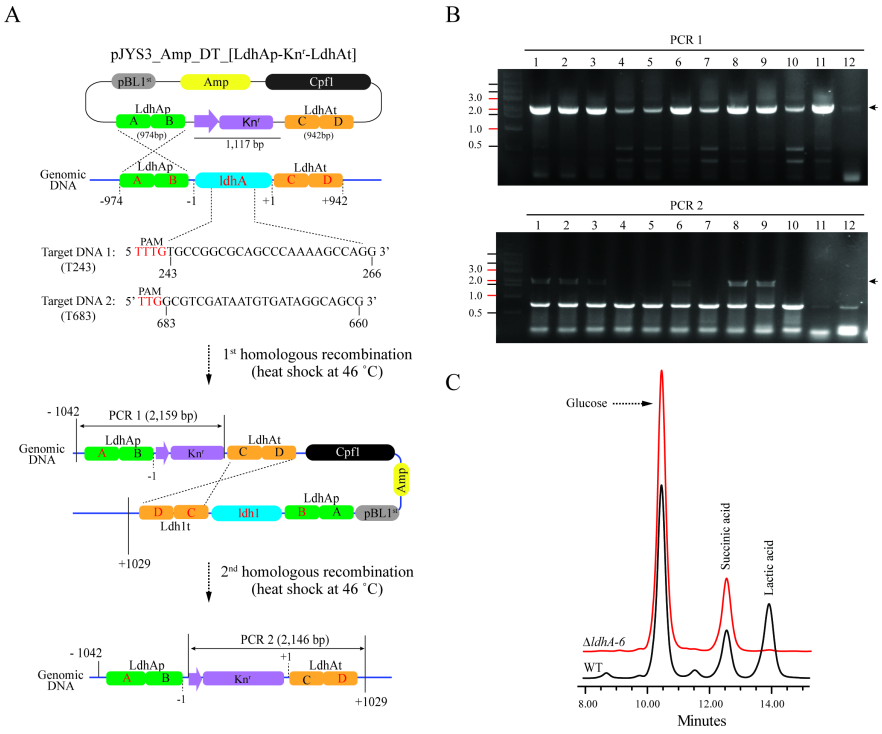


Fig. 2. CRISPR/Cpf1 mediated homologous recombination in *C. glutamicum* . (A) The double target sites were located 243 bp and 683 bp from the start codon of the *ldhA* gene, respectively. The first homologous recombination occurred on the LdhAp region at the first heat shock, and the second crossing-over on the LdhAt region occurred after the second heat shock. (B) The deletion of the *ldhA* gene and simultaneous Kn^r gene insertion were confirmed via PCR analysis (PCR 1 and 2). (C) Confirmation of the complete inhibition of lactic acid production in the *ldhA* gene deletion mutant (*Δλδηλ-6*). Glucose was used as the sole carbon source.

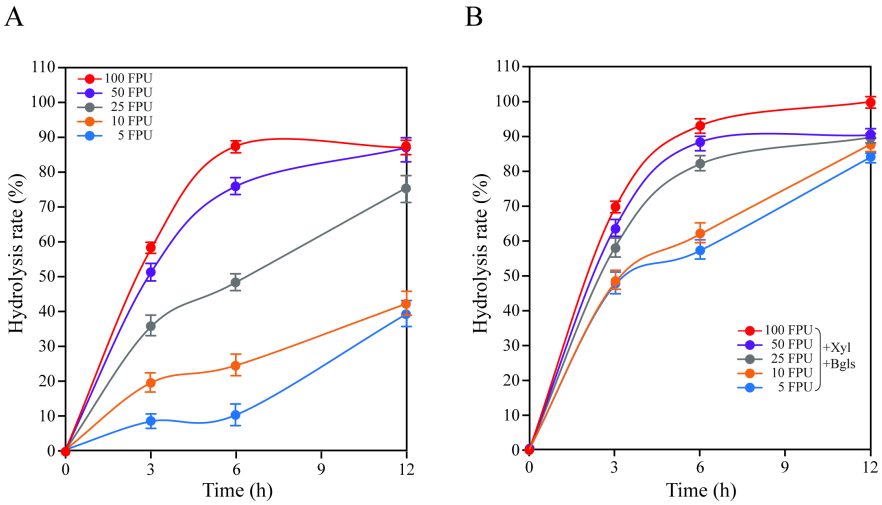


Fig. 3. Synergistic effect of cellulase with xylanase and β-glucosidase. (A) HPAC-pretreated pine with cellulase cocktail solution alone and (B) supplement with xylanases (Xyl) and β-glucosidases (Bgls) were

conducted in 1 mL citrate buffer. The incorporation of auxiliary enzymes remarkably increased the hydrolysis rate of the 2% substrate and decreased the dose of cellulase required for optimal hydrolysis (5–25 FPU). The structural recalcitrance of cellulose fiber did not substantially affect the enzymatic hydrolysis of HPAC-pretreated pine.

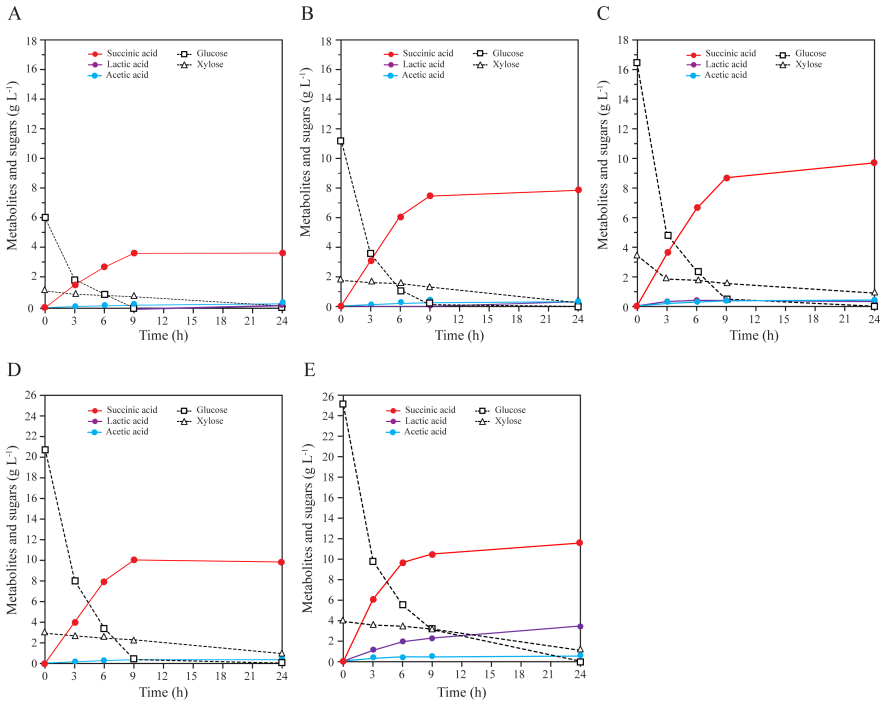


Fig. 4. Fermentation of the *Δλδηα-6* mutant with the hydrolysates of HPAC-pretreated pine. The metabolites (succinic acid, lactic acid, and acetic acid) produced by fermentation of the 1% (A), 2% (B), 3% (C), 4% (D), and 5% hydrolysate (E) were analyzed. The cell concentrations were 10.15 (A), 15.72 (B), 16.08 (C), 21.19 (D), and 21.08 (E) g L^{-1} CDW (cell dried weight) for fermentation of the hydrolysates, respectively.

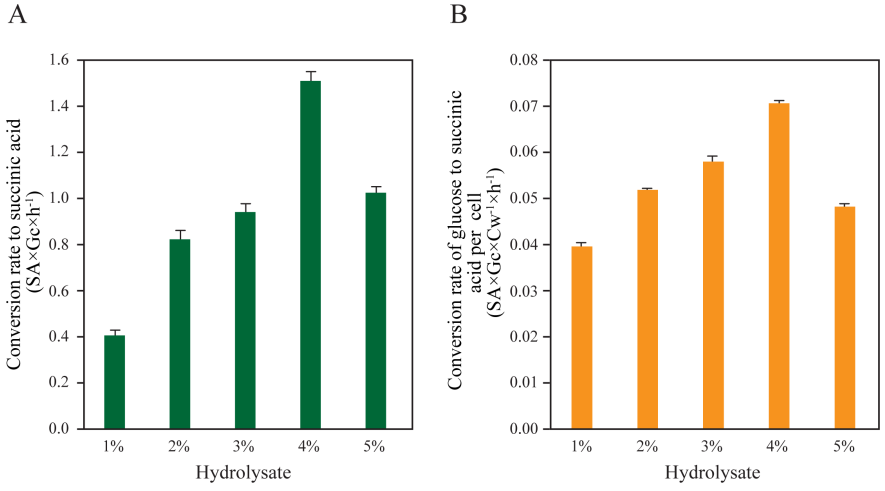


Fig. 5. Productivity of succinic acid from the hydrolysate of HPAC-pretreated pine. Succinic acid productivity involving in glucose consumption rate (A) and cell concentration (B) were analyzed over 9 h. SA,

succinic acid (g L^{-1}); Gc, glucose consumption rate; h, hour; Cw, cell dried weight (g L^{-1})

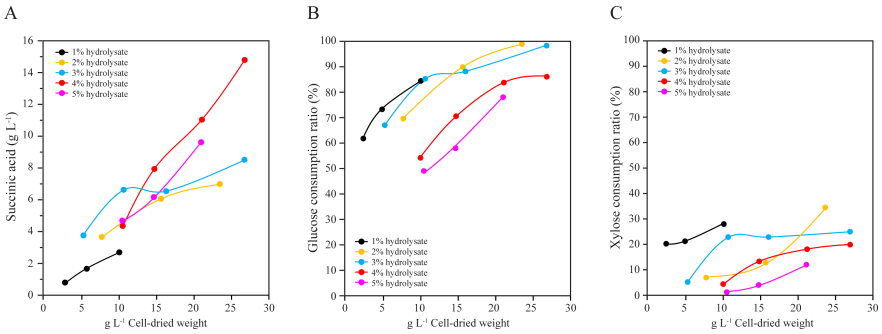


Fig. 6. Succinic acid production correlated to cell concentration. The hydrolysates were fermented with various ranges of cell densities of*ΔλδηA-6* mutant for 6 h, and succinic acid (A), glucose consumption rate (B), and xylose consumption rate (C) were analyzed.

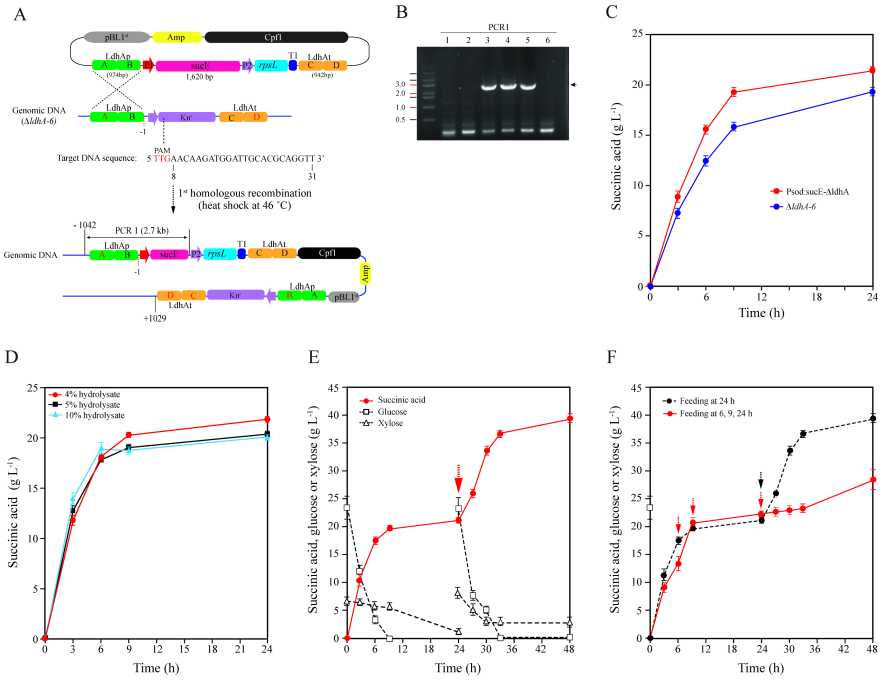
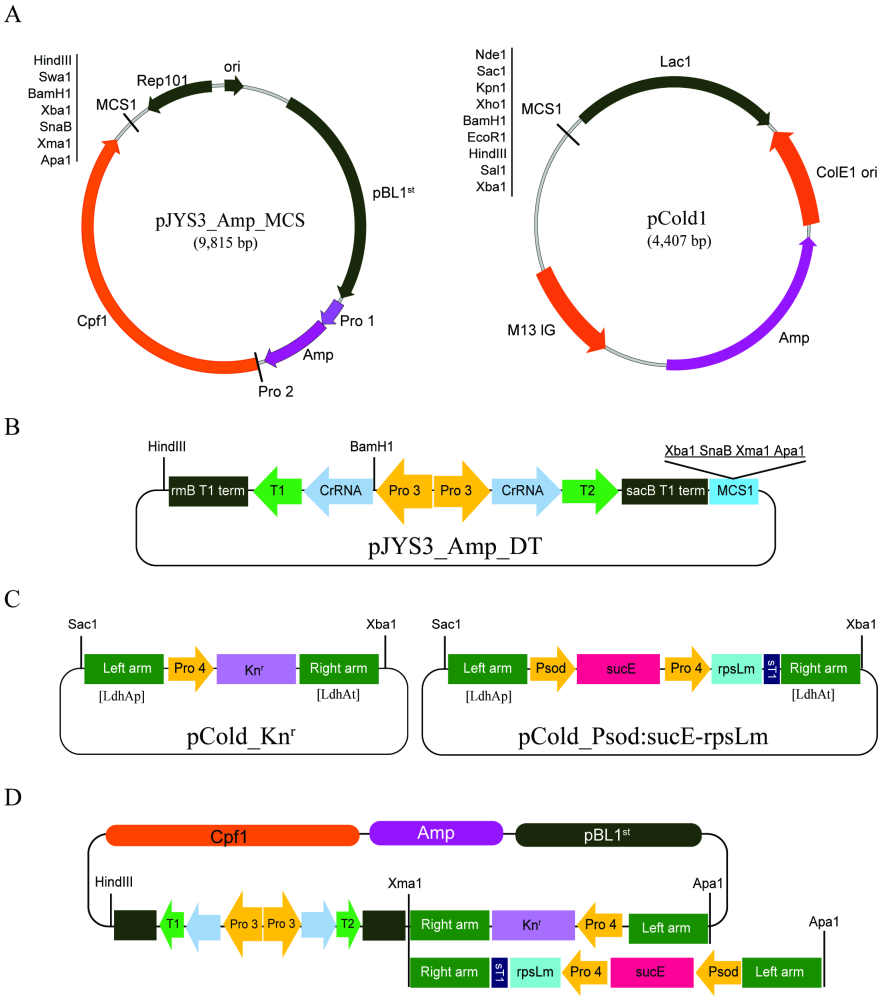


Fig. 7. Over-expression of succinic acid transporter (*sucE*) gene and succinic acid production in fed-batch system. (A, B) The gene was inserted onto the genomic DNA of *ΔλδηA-6* mutant under Psod promoter regulation through the CRISPR/cpf1 gene editing system. (C) The enhancement of succinic acid production was demonstrated in [Psod:sucE- *ΔλδηA*] transformant (10.00 g CDW) compared to*ΔλδηA* mutant (10.94 g CDW) when using 4% hydrolysate. (D) Comparison of the succinic acid production depending on the concentration of hydrolysates was performed with 28˜30 g L^{-1} CDW of [Psod:sucE-*ΔλδηA*] transformant. (E) A fed-batch system was carried out with an initial concentration of 4% hydrolysate and 30.37 g L^{-1} CDW of [Psod:sucE-*ΔλδηA*] transformant. After 24 h of fermentation, 20 mL of 20% pine hydrolysate was added to the reaction solution, resulting in a final concentration of the 4% hydrolysate. Acetic acid was prone to be released during the first 9 h after feeding, while lactic acid was measured at the later stage of the fermentation. (F) The same volume of the 20% hydrolysate was added at 6, 9, and 24 h. P1, Psod

promoter; *sucE* , succinic acid transporter; T1, sacB terminator; P2, Kn^r promoter; *rpsL* m, ribosomal S12 protein mutant gene for streptomycin resistant; arrows, feeding.



pJYS3_Amp_DT_[LdhAp-Kn^r-LdhAt] or [Psod:sucE-rpsLm]