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Succinic Acid Production from Monosaccharides and Woody and Herbaceous Plant Hydrolysates Using Metabolically Engineered *Corynebacterium glutamicum*

Running title: Succinic Acid Production from Biomass

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SUMMARY

Research background. Succinic acid derived from lignocellulosic biomass presents a sustainable alternative for biochemical production, providing eco-friendly substitutes for petroleum-based chemicals. This study aimed to evaluate the impact of variations in hemicellulose content and cellulose fiber structure within the microfibrils of woody and herbaceous plants on the enzymatic saccharification and succinic acid production efficiencies of Psod:SucE12-ΔldhA, a strain overexpressing the succinic acid transporter (SucE).

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Experimental approach. The study investigated the influence of different monosaccharide combinations on succinic acid production, focusing on combinations including mannose compared to glucose alone. Additionally, hydrolysates from various lignocellulosic biomass—bamboo, oak, poplar, pine, and spent coffee grounds—were analyzed to determine the most favorable bioresource for succinic acid production.

Results and conclusions. Monosaccharide combinations containing mannose resulted in 2.20-2.48 times higher succinic acid production than glucose alone, indicating a positive influence of mannose on succinic acid metabolism. Among the lignocellulosic biomass hydrolysates, bamboo, with its higher xylose content compared to woody plants, was the most efficient bioresource for succinic acid production (23.38–24.12 g/L within 24 h), followed by oak, poplar, pine, and spent coffee grounds. Therefore, improving the xylose consumption rate is crucial for enhancing succinic acid production from lignocellulosic biomass and increasing market competitiveness.

Novelty and scientific contribution. This research highlights the potential of lignocellulosic biomass, especially bamboo, as a sustainable feedstock for succinic acid production. The novelty of the study lies in its detailed examination of how hemicellulose content and cellulose fiber structure affect enzymatic saccharification and fermentation. The significant impact of mannose and xylose on succinic acid yields provides key insights for optimizing biomass use in biochemical production. These findings advance bio-based chemical manufacturing, reducing reliance on fossil fuels and enhancing industrial sustainability.

Keywords: succinic acid; lignocellulosic biomass; CRISPR; *Corynebacterium glutamicum*; fermentation

INTRODUCTION

Amidst global concerns over the environmental impacts of CO_2 emissions and microplastics associated with a petroleum-based economy, biochemicals have garnered significant attention as a potential remedy. Particularly, succinic acid, which has diverse applications across various industrial fields, stands out as a key platform chemical in the bio-based economy.

Succinic acid, a member of the four-carbon dicarboxylic acid family, serves as an intermediate in the citric acid and glyoxylate cycles during the conversion of glucose to

adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NADPH) within living cells. Due to its distinct structural properties, it has diverse applications in industries, pharmaceuticals, agriculture, cosmetics, and food. Notably, succinic acid can be polymerized to produce diverse industrial products including polyurethanes. Additionally, it can be converted to 1,4-butanediol (1,4-BDO), serving as a precursor for synthesizing polyesters, polybutylene succinate (PBS), and tetrahydrofuran (THF). After the COVID-19 pandemic bioactive compounds such as vitamins and antioxidants to support immune system and overall health. Succinic acid, with its antioxidant properties, has emerged as a promising bioactive compound in the food industry (*1,2*).

Succinic acid has been reported to be produced by wild type microbes (*e.g*. *Actinobacillus succinogenes*) and recombinant strains of various microorganisms, including *Escherichia coli*, *Corynebacterium glutamicum*, *Mannheimia succiniciproducens*, and *Yarrowia lipolytica* (*3-7*). Bio-based succinic acid production offers several advantages, including the availability of diverse fermentable sugar resources, high fermentation efficiency, and the usability of intermediates and products (*6*). However, challenges persist in terms of cost competitiveness in the market when compared to petroleum-based succinic acid production. A minimum productivity of 2.5 g/(L·h) succinic acid is required, particularly when employing corn starch as a raw material (*8*). Notably, the estimation of greenhouse gas emissions during succinic acid production from lignocellulosic biomass, which is currently confined to laboratory scales, has not yet been reported. While corn starch-based succinic acid production has its merits compared to lignocellulosic biomass-based production, there are some problems associated with the use of corn starch as an alternative carbon source for biochemicals or bioenergy. One issue is the surging price of corn stock due to its excessive utilization, leading to competition with animal feed-stock and food-chains in the market. Other challenges include the extensive need for land, fertilizers, and pesticides for farming corn crop. These reasons explain why the companies producing succinic acid from corn-based carbon sources are predominantly located in China and USA. The drawbacks of corn starch-based bring interest of alternative bioresource toward lignocellulosic biomass that is an abundant, carbon-neutral, and renewable source worldwide (*9*).

C. glutamicum is a gram-positive soil bacterium, well known as an industrial microbe with a wide range of applications including the production of amino acids, organic acids, fuels, and healthcare products (*10*). Briki *et al*. (*11*) reported that wild-type *C. glutamicum* is a natural

overproducer of succinic acid under the optimal transition condition with a volumetric mass transfer coefficient (k_La value) of 5/h. Many researches employing metabolic engineering through knock-out of genes involving lactic and acetic acids metabolism, and overexpression of genes associating with citric acid and glyoxylate cycles has been reported (*3,12,13*). Moreover, the presence of the gene (SucE) encoding the transporter of succinic acid in *C. glutamicum*, characterized to be one-way exporter from the cell into the medium, provides a significant advantage to *C. glutamicum* during succinic acid fermentation (*14*).

Metabolic engineering aimed to enhance succinic acid production of *C. glutamicum* has been conducted using allelic exchange, transposon, and integrase-mediated integration. However, these methods are known for low homologous recombination efficiency and potential for inaccurate genome editing (*15*). CRISPR gene editing system has been proposed as a solution to tackle these challenges. CRISPR-Cpf1 (Clustered regularly interspaced short palindromic repeats and CRISPR nuclease from *Prevotella* and *Francisella1*) was identified as a type V CRISPR with a nuclease consisting of 1,200,500 amino acids (*16*). The enzyme recognizes a protospacer adjacent motif (PAM) site of 5' (T)TTN 3', flanking 24 base pairs of the target genomic DNA, and guides the annealing of crRNA (CRISPR RNA) with the complementary target DNA. The double-strand DNA cleavage can induce the activation of endogenous DNA repair systems, namely non-homologous end-joining (NHEJ) and homologous recombination (HR). The combination of CRISPR-Cpf1 with HR DNA repair system can enable more precise nucleotide or gene substitution, insertion, and deletion (*17*). CRISPR-Cpf1 genome editing system was successfully applied for nucleotide substitutions in the gene encoding arginine repressor (argR), as well as simultaneous insertions and deletion in the gene of C50 carotenoid epsilon cyclase (crtYf) of *C. glutamicum*.

Most of the previous research using genetically engineered *C. glutamicum* for succinic acid production utilized pure glucose as sole carbon source (*17*), except for a few cases from corn cobs hydrolysate (*13*). Lignocellulosic biomass is a good carbon source for succinic acid production. Lignocellulosic biomass, depending on the types of wood and herbaceous plants, is composed of various monosaccharides such as glucose, xylose, mannose, galactose, arabinose, etc (*18-20*) The impact of these monosaccharides and the hydrolysates from lignocellulosic biomass on the production of succinic acid and other metabolites during the fermentation of *C. glutamicum* remains largely unexplored. In this study, the metabolically engineered *C. glutamicum* strains created through CRISPR/Cpf1 gene editing system were

applied to produce succinic acid from monosaccharides (glucose, xylose, and mannose), hydrolysates of three woody plants (pine, oak, and poplar), and two herbaceous plants (bamboo and spent coffee grounds). It elucidates the effect of monosaccharides on succinic acid fermentation, and determines the optimal biomass and hydrolysate concentration for efficient succinic acid production.

MATERIALS AND METHODS

Preparation of wood materials and chemicals

Pine, poplar, oak and bamboo were obtained from the arboretum in Chonnam National University, Gwangju in South Korea. Pine (*Pinus densiflora*, diameter: 35 cm), poplar (*Populus deltoids*; diameter: 20 cm), and oak (*Quercus acutissima*; diameter: 25 cm) wood were cut into chips measuring approximately 0.25 cm×0.35 cm×5.0 cm (width, height and length, respectively) using a saw. Bamboo shoots (*Phyllostachs pubescens*) were cut into 5 cm columns and then vertically divided into eight segments (*21*). Spent coffee grounds were sourced from Starbucks in Gwangju, South Korea.

All chemicals were purchased from Sigma-Aldrich, Merck (St. Louis, MO, USA).

Pretreatment and enzymatic hydrolysis

The wood samples were individually soaked in the hydrogen peroxide-acetic acid (HPAC) solution containing 30 % hydrogen peroxide and acetic acid (1:1, v/v) and then incubated at 80 °C for 3–4 h (*17*). The wood fibers were filtered using an iron mesh tray and then washed with water several times until the lignin-HPAC solution was completely removed. Subsequently, spent coffee grounds were individually treated with 1 M NaOH, 6 % H_2O_2, and a combination of both at room temperature for 24 h. After washing with water, the HPAC-pretreated woody and herbaceous samples were freeze-dried and stored at room temperature.

The HPAC-pretreated samples (1–4, 5, 10 and 20 %) were hydrolyzed with 20 FPU/g biomass cellulase (Cellic CTec2, Novozymes, Bagsvaerd, Denmark) at 50 °C for 5 d. The resulting hydrolysates were centrifuged to separate fiber debris and soluble sugars and then filtered using Whatman No. 1 paper. The concentration of reducing sugars in each hydrolysate was measured using the DNS assay (involving 10 g/L dinitrosalicylic acid, 2 % NaOH, 0.5 g/L sodium sulfite, Rochell salt ($KNaC_4H_4O_6 \cdot H_2O$), and 2 mL/L phenol). The hydrolysis ratio was calculated using the following equation.

$$\text{Hydrolysis ratio} = Rs/(Ts \cdot 1.05) \cdot 100 \quad /1/$$

where Rs is the reducing sugar (g) in the hydrolysate, Ts is the total monosaccharide (g) in biomass.

Preparation of Corynebacterium glutamicum strain for transformation

The strain of *C. glutamicum* (ATCC 13032) was obtained from the Korean Agricultural Culture Collection (KACC). The cells were cultured in LB liquid media (20 g/L LB broth) at 30 °C for 24~48 h for competent cell preparation. The microbes were harvested and re-cultured in 100 mL NCM (1.0 g/L yeast extract, 5 g/L tryptone, 5 g/L glucose, 17.4 K_2HPO_4, 0.05 g/L $MgSO_4 \cdot 7H_2O$, 0.3 g/L trisodium citrate, 91.1 g/L sorbitol, 11.6 g/L NaCl, pH 7.2) at 30 °C for 4 h. After centrifuging, the cells were rinsed three times with ice-cold 10 % glycerol, and resuspended with ice-cold 10 % glycerol. The cells (90 µL) were aliquoted into microcentrifuge tubes and stored at - 80 °C.

Plasmid DNA (5–10 µL) was added to a tube containing 90 µL of competent cell solution and transferred to a 2 mm electroporation cuvette (Sigma-Aldrich, Merck, St. Louis, MO USA). Electroporation was performed with a MicroPulser system (BioRad) 1.8 kV for 5 ms. BHIS liquid solution (18 g/L BHI, 91 g/L sorbitol), 900 µL, was added and transferred to microcentrifuge tubes. The cells were immediately incubated for 6–15 min at 46 °C. The cells were then plated on Luria-Bertani supplemented with brain heart infusion and sorbitol (LBHIS, 2.5 g/L yeast extract, 5 g/L tryptone, 5 g/L NaCl, 18.5 g/L BHI, 91 g/L sorbitol, 18 g/L agar, pH 7.2) containing 50 µg/mL kanamycin or 30 µg/mL streptomycin, and incubated at 30 °C until colonies appeared.

CRISPR-Cpf1 mediated recombination of Corynebacterium glutamicum

The CRISPR-Cpf1 vector (pJYS3_ΔcrtYF), obtained from Addgene Co. (Watertown, MA, USA), was modified by replacing the kanamycin resistance gene with an ampicillin resistance marker gene and adding a multiple cloning site with restriction enzymes. The modified vector pJYS3Am_MCS, is illustrated in Fig. 1. Information regarding the primers used in this study is presented in Table S1. The promoter and crRNA linked to the 24-bp long target DNA sequence (T53 and T683) on the lactate dehydrogenase A (*ldhA*) gene were sequentially and individually incorporated to generate pJYS3Am_ldhA-dT. pCold vector was used to construct the homologous recombination cassette, [left arm-Pro4:Kn^r-Right arm] (Fig. 1 and Fig. S1). Finally, the homologous recombination cassette was introduced into the pJYS3Am_ldhA-dT vector at the sites of Xma1–Apa1, generating pJYS3Am_ldhA-dT-[$ldhA_{pro}$-

Pro2:Kn^r-ldhA$_{gen}$]. The CRISPR-Cpf1 vector was transformed into the wild-type competent cells of *C. glutamicum* using an electroporation machine (MicroPulser, BioRad) set at 1.8 kV for 5 ms (*22*). Subsequently, the knock-out mutant of ldhA (*ΔldhA-L6*) was selected, and the first and second homologous recombination events were confirmed through PCR analysis (described in *Genomic DNA isolation and PCR analysis*).

The succinic acid transporter–overexpressing strain, *Psod:SucE-ΔldhA*, was generated by transforming the single-target CRISPR vector, pJYS3Am_Kn-sT, containing the homologous recombination set [LdhA$_{pro}$-Psod:SucE-rspLm-LdhA$_{gen}$], into the *ΔldhA-L6* knock-out strain (Fig. 2). The succinic acid transporter (SucE) and ribosomal S12 protein gene (*rpsL*) were isolated from wild type *C. glutamicum* (described in *Genomic DNA isolation and PCR analysis*). The AAG (Lys^{43}) sequence on the *rpsLm* gene was replaced with AGG (Glu), thereby generating the streptomycin resistance gene, *rpsLm*. The SucE and *rspLm* genes were subcloned downstream of the Psod and Kn^r promoters on the pCold vector, respectively, resulting in the pCold homologous recombination set: pCold [LdhA$_{pro}$-Psod:SucE-rspLm-LdhA$_{gen}$]. The 4.95 kb-long homologous recombination set was PCR amplified and introduced into the pJYS3Am_Kn-sT vector to generate pJYS3Am_Kn-sT [LdhA$_{pro}$-Psod:SucE-rspLm-LdhA$_{gen}$]. Transformation was performed following the previously described procedure, and two transformants (*Psod:SucE-ΔldhA*) were selected on solid LB media supplemented with 30 μg/mL streptomycin.

Genomic DNA isolation and PCR analysis

The colonies of *C. glutamicum* obtained on LB (Bioshop, Canada) or selection media were inoculated into 5 mL of liquid LB media and then incubated at 30 °C overnight. Subsequently, the cells were harvested, suspended in 100 μL extraction buffer (0.2 M lithium acetate, 1 % SDS), and incubated at 70 °C for 10 min. Then, genomic DNA was precipitated by adding 300 μL ethanol and vortexing the samples. Next, cell debris and genomic DNA were separated from the liquid phase through centrifugation at 15 000×*g* for 10 min. The precipitate was dried at room temperature and then dissolved in 100 μL of distilled water. Then, the cell debris was removed by centrifuging at 15 000×*g* for 10 min, and 1 μL of the supernatant was used to isolate the left and right arms of the LdhA, SucE, and rspL genes or to verify the selection of the transformants through PCR analysis using the primers listed in Table S1.

Succinic acid production from the hydrolysates

The colonies of the *ΔldhA-L6* knock-out strain or the *Psod:SucE-ΔldhA* overexpression strain line 12 (Psod:SucE12-*ΔldhA*), obtained on the selection media, were separately inoculated into 20 mL of liquid LB media and incubated at 30 °C for 24 h. Subsequently, the cells were transferred into 1 L of liquid LB media, and incubated at 30 °C for 2 d. Then, the harvested cells (approximately 10 g/L cell dry mass) were used to produce succinic acid from the hydrolysates of pine, poplar, oak, bamboo, and spent coffee grounds. Mineral salts (0.5 g/L K_2HPO_4, 0.5 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 4.2 mg/L $MnSO_4 \cdot 7H_2O$, and 6.0 mg/L $FeSO_4 \cdot 7H_2O$) and vitamins 0.2 mg/L biotin, 0.2 mg/L thiamine were added to each of the hydrolysates, and the pH was adjusted to 7.5. The cells were suspended in 100 mL of each hydrolysate, taken in a cylindrical bottle (volume: 700 mL) and finally, 0.4 M sodium bicarbonate was added to each suspension. Each cylindrical bottle was sealed with a screw cap, and fermentation was performed at 200 rpm and 30 °C in a shaking incubator. Sampling was conducted after 3, 6, 9, and 24 h (k_La), which can provide information regarding the operation parameters during the experiment, was calculated using the following equation (*11*):

$$k_La = 0.024 \cdot N^{1.16} \cdot V^{-0.83} \cdot d_0^{0.38} \cdot d^{1.92} \quad /2/$$

where N is the shaking frequency (rpm), V is the percentage of filling volume, d is the maximal shake flask diameter (cm), and d_0 is the shaking diameter. The k_La value for this experiment was determined to be 9.6/h. The concentrations of metabolites and monosaccharides were analyzed through high-performance liquid chromatography (HPLC).

For comparing the succinic acid production efficiency based on reducing sugar concentrations, the reducing sugar sets (RS20, RS30, RS40 and RS50. Corresponding to the hydrolysate concentrations of 20, 30, 40 and 50 g/L, respectively) were prepared through the dilution of hydrolysates from pine, oak, poplar, bamboo, and spent coffee grounds, respectively. Similarly, succinic acid was also produced using the strain Psod:SucE12-*ΔldhA* (cell dry mass 10 g/L).

Fermentation of the monosaccharides using the Corynebacterium glutamicum strains

The *ΔldhA-L6* and Psod:SucE12-*ΔldhA* strains (10 g/L cell dry mass) were individually employed to ferment 10 and 20 g g/L glucose (G10 and G20), 20 g/L xylose (X20), and 20 g/L mannose (M20) in separate 50 mL conical tubes (diameter: 3 cm) containing 10 mL mineral salts solution. Similarly, the following combinations of monosaccharides were also fermented: 20 g/L glucose + 10 g/L xylose (G20X10), 20 g/L glucose + 10 g/L mannose (G20M10), 20 g/L glucose + 5 g/L xylose + 5 g/L mannose (G20X5M5), 20 g/L glucose + 5 g/L xylose + 10 g/L

mannose (G20X5M10), 20 g/L glucose + 10 g/L xylose + 5 g/L mannose (G20X10M5), and 20 g/L xylose + 10 g/L mannose (X20M10). All conical tubes were sealed after inoculation with the respective strain and fermented at 200 rpm and 30 °C in a shaking incubator. Sampling was conducted after 3, 6, 9, and 24 h. The concentrations of metabolites and monosaccharides were measured through HPLC analysis. k_La was calculated as specified in Eq 2. The k_La value was 16.3/h for this experiment. The yield and conversion efficiency of succinic acid were calculated using the following equations:

$$\text{Yield}=(\text{succinic acid/sugar}) \times \cdot 100 \quad /3/$$

$$\text{Conversion efficiency}=(\text{succinic acid/sugar consumed}) \cdot 100 \quad /4/$$

The amount of succinic acid measured at 24 h after fermentation was used for the calculation.

Measurement of metabolites and monosaccharides

The concentrations of organic acids, glucose, and xylose were measured using an HPLC system (2702 Autosampler; Waters, Milford, MA, USA) equipped with a refractive index (RI) detector (Waters 2414, USA). A reversed-phase octadecylsilane (ROA) column (7.8 mm×300 mm; Phenomenex, Torrance, CA, USA) was used for analyzing the organic acid, glucose, xylose and mannose contents in the fermented solution, whereas an RPM column (4.6 mm×300 mm; Phenomenex) was used for analyzing the monosaccharides contents in the hydrolysates. The mobile phase, consisting of 5 mM sulfuric acid, was passed through the column at a flow rate of 0.6 mL per min. The temperatures of the column and detector were set at 65 and 40 °C, respectively.

Analysis of the chemical composition of lignocellulosic biomass

The monosaccharides, including glucose, xylose, and mannose, in the HPAC-pretreated biomass (pine, poplar, oak, bamboo, and spent coffee grounds) were quantified using gas chromatography, following a previously established method (*21*). Each raw and HPAC-pretreated biomass were treated with 0.25 mL of 72 % (v/v) sulfuric acid (H_2SO_4) for 45 min at 30 °C and was diluted with distilled water to 4 % (v/v) H_2SO_4. The hydrolysis step was performed at 121 °C for 1 h, and a solution containing a known amount of myo-inositol was used as an internal standard. The solution was then neutralized with ammonia water. One mL of 2 % (w/v) sodium borohydride resolved in dimethyl sulfoxide and 0.1 mL of glacial (anhydrous) acetic acid (18 M) were added in order to degrade the sodium tetrahydroborate.

Then, 0.2 mL of methyl imidazole and 2 mL of anhydrous acetic acid were sequentially added, and 5 mL of deionized water was then added and extracted with 2 mL of dichloromethane. The samples were analyzed via GC (GC-2010; Shimadzu, Otsu, Japan) using a DB-225 capillary column (30 m×0.25 mm i.d., 0.25 μm film thickness, J&W; Agilent, Folsom, CA, USA) operated with helium. The operating conditions were as follows: injector temperature of 220 °C, flame ionization detector (FID) at 250 °C, and an oven temperature of 110 °C for 1.5 min with a constant increase of 10 °C/min to 220 °C.

RESULTS AND DISCUSSION

Generation of Corynebacterium glutamicum recombinants

Under oxygen deprived or anaerobic conditions, wild-type *C. glutamicum* primarily excretes lactic acid, which is approximately 2.5 times more abundant than succinic acid during the fermentation of pure glucose at an optimal k_La of 9.6/h for 50 h (*11*). Therefore, the gene encoding lactate dehydrogenase A (ldhA) was first targeted and blocked using the CRISPR/Cpf1 genome editing system to enhance succinic acid production (Fig. S2). The two target DNAs on the ldhA gene and the homologous recombination cassette ($LdhA_{pro}$-Kn^r-$LdhA_{gen}$) were inserted into the pJYS3Am_ldhA-dT vector, constructing the all-in-one vector, pJYS3Am_ldhA-dT-[$LdhA_{pro}$-Kn^r-$LdhA_{gen}$] (Fig. 1 and Fig. S1). This vector was transformed into the wild-type *C. glutamicum* to create a ldhA gene knock-out mutant *ΔldhA-L6* strain (Fig. 1). After confirming the PCR1 fragment for the first homologous recombination, the colony of No. 6 was incubated at 46 °C to induce the second homologous recombination. It was confirmed that the deletion in the genomic DNA corresponded to a region approximately 94 bp from the start codon of the ldhA gene, and the expression of the ldhA gene was successfully blocked through a frame shift. During the fermentation of pure glucose, the *ΔldhA-L6* strain excreted succinic acid as the main organic acid due to the absence of lactic acid in the media.

The succinic acid transporter of *C. glutamicum*, SucE, is a unidirectional transport system responsible for exporting succinic acid to the external environment (*14*). It offers a significant advantage in boosting succinic acid production through gene overexpression when compared to other succinic acid transporter system, such as the Dcu family in *E. coli*. Notable, extracellular succinic acid has been reported to inhibit both glucose consumption rate and succinic acid production (*3*). Considering the potential inhibitory effect of intracellular succinic acid on succinic acid metabolic pathways, including glycolysis, the glyoxylate cycle, and the citric acid cycle, it can be hypothesized that a stronger excretion of succinic acid may lead to

an increase in the glucose-to-succinic acid conversion rate. To explore this hypothesis, the single-target DNA sequence of the kanamycin resistance gene located in the genomic DNA of the *ΔldhA-L6* strain and the homologous recombination set were subcloned to generate the CRISPR/Cpf1 genome editing vector, pJYS3Am_Kn^r-sT [LdhA$_{pro}$-Psod:SucE-rsplm-LdhA$_{gen}$] (Fig. 2). Consequently, a SucE overexpressing strain regulated by the constitutive Psod promoter, namely, the Psod:SuceE12-*ΔldhA* strain was generated.

Allelic exchange through homologous recombination has been effectively utilized in *C. glutamicum* for metabolic engineering purposes (*15*). The successful deletion of a gene from the genomic DNA involves sequential first and second crossovers in the process of homologous recombination. Notably, the spontaneous process of gene deletion and insertion is more successful when the size of the insertion gene is approximately 1.0 kb or below. Thus, spontaneous gene deletion (95 bp of LdhA gene) and insertion (1.1 kb of kanamycin resistance gene) were observed in the *ΔldhA-L6* mutant under serial heat shock and crossovers. However, in the Psod:SuceE12-*ΔldhA* transformant, colonies exhibiting complete crossovers could not be selected. It is presumed that Psod:SucE12-*ΔldhA* transformant, containing a 2.3 kb insertion, predominantly undergoes the first crossover or infrequently achieves the second crossover recombination, posing a significant challenge in the selection of colonies with complete crossovers.

Saccharification of lignocellulosic biomass

Lignocellulosic biomass encompasses a diverse array of carbohydrates, and their composition can vary based on the species, and even within the same plant. This variability can be attributed to variations in growing condition, growth stage, and tissue position within the plant (*23*). Cellulose, a major component, consists of glucose, whereas hemicellulose is a heterogeneous mixture of various sugars, including xylose, mannose, galactose, arabinose, and others. In softwood, hemicellulose comprises arabinoglucuronoxylan and galactoglucomannan, responsible for 8 and 18 % of the cell wall components, respectively, whereas in hardwood, hemicellulose consists of glucuronoxylan and glucomannan, accounting for 15–30 % and 2–5 % of the cell wall components, respectively (*24*). The hemicellulose in Korean red pine (*P. densiflora*) comprises 14.6 % galactomannan and 6.4 % xylan (*25*). The cell wall of herbaceous plants is composed of a primary cell wall rich in pectin, which contributes to the releases of more diverse sugars in the hydrolysates (*26*). The diversity of cell wall components within lignocellulosic biomass facilitates the determination of sugar

preferences in *C. glutamicum* during succinic acid fermentation. In this study, the *ΔldhA* strain served as a standard, representing the baseline response to various sugars derived from lignocellulosic biomass. To represent different plant species, we selected five biomass: pine wood for softwood, poplar and oak wood for hardwood, and bamboo and spent coffee grounds for herbaceous plants. The carbohydrate compositions of the biomass are presented in Table 1 (*23,24,27-31*). Xylose is the main component of hemicellulose in poplar, oak, and bamboo. In pine and spent coffee grounds, mannose is the main component of hemicellulose accounting for 17.53 and 44.57 % of the carbohydrates, respectively. Additionally, both pine and spent coffee grounds exhibit higher levels of galactose compared to the other biomass. The HPAC-pretreated pine, poplar, oak, bamboo, and spent coffee grounds were enzymatically hydrolyzed with a cellulase cocktail solution (20 FPU/g biomass) at 50 °C for 5 d, resulting in hydrolysis ratios ranging from 80 to 98 %, with the exception of spent coffee grounds (Table 2).

Succinic acid production from lignocellulosic biomass using the knock-out strain ΔldhA

The efficiency of succinic acid production from lignocellulosic biomass through fermentation with *C. glutamicum* is affected by various factors, including the cell concentration, k_La, anaerobic condition, and optimal glucose concentration (*11,12*). Under semi-anaerobic conditions, with a cell dry mass of 10 g/L and a k_La value of 9.6/h, fermentations using *ΔldhA-L6* were conducted on 1 % hydrolysates derived from various lignocellulosic biomass (in g/L: pine 8.93, poplar 9.27, oak 8.44, bamboo 9.93 and spent coffee grounds 8.52 L) to analyze the effects of the sugar composition on succinic acid productivity under unsaturated sugar conditions (Fig. 3). The glucose in the hydrolysates of poplar, oak, bamboo, and spent coffee grounds were depleted within 3 to 6 h and converted into succinic acid. The productivity at the initial stage of each hydrolysate exhibited an efficient conversion rate to succinic acid in the following order: poplar > oak = bamboo >> pine > spent coffee grounds. Under saturated conditions with 5 % hydrolysates, the patterns observed in the hydrolysates of poplar and spent coffee grounds were opposite to those seen in the previous results: oak = spent coffee grounds ≥ bamboo > poplar = pine. The by-products, lactic acid and acetic acid, were first detected at the 24-hour fermentation checkpoint with 5 % hydrolysate. Lactic acid was primarily observed during the fermentation of pine and bamboo hydrolysates. Although produced in small amounts, acetic acid appeared to be generated in proportion to the pattern of succinic acid production. It seems that the production of these by-products does not affect succinic acid

metabolism. Based on the results in Fig. 3g, succinic acid production was found to be highest in oak hydrolysate, followed by hydrolysates of bamboo and poplar. The optimal concentration of hydrolysate for succinic acid production was determined to be 4 %, considering economic factors such as electricity, enzyme dosage, and sugar utilization efficiency. Although xylose exhibited a much slower consumption rate than glucose, a comparison of the results of two hardwoods and bamboo (all of which contain xylose-rich hemicellulose) with those of softwood and spent coffee grounds demonstrated no correlation between xylose and succinic acid fermentation. Next, we investigated which types of derivatives or monosaccharides influence succinic acid production during fermentation with *C. glutamicum*.

Effects of monosaccharides on succinic acid production

To demonstrate the correlation between monosaccharides and succinic acid fermentation, two transgenic strains, *ΔldhA-L6* and *Psod:SucE12-ΔldhA*, were utilized to ferment various combinations of pure monosaccharides, such as glucose, xylose, and mannose (Fig. 4). During the initial stages (3–6 h) of G10 and G20 fermentation, the succinic acid transporter overexpressing strain, *Psod:SucE12-ΔldhA*, exhibited succinic acid conversion rates that were approximately 2.01–4.91 and 1.20–1.49 times higher than that in the *ΔldhA-L6* strain, lasting approximately 1.91 and 1.72 times over 24 h, respectively (Fig. 4). The *Psod:SucE12-ΔldhA* strain showed much higher succinic acid production compared to the *ΔldhA-L6* strain in the sole presence of mannose (M20). Notably, when mannose was used in combination with glucose (G20M10), the *Psod:SucE12-ΔldhA* strain exhibited a remarkable enhancement in succinic acid production during the initial stages (approximately 2.54–3.38 times higher production compared to that in the *ΔldhA-L6* strain). Additionally, the enhancement was 1.32–1.61 times higher than that observed with glucose alone (G20) in the same strain, *Psod:SucE12-ΔldhA*, persisting approximately 2.12 times over 24 h, demonstrating theoretical succinic acid productivity similar to that obtained for 40 g/L glucose. The succinic acid fermentation of xylose with glucose also presented patterns similar to those observed with mannose. All values related to the consumption and conversion of glucose, xylose, and mannose to succinic acid were much higher in the *Psod:SucE12-ΔldhA* strain than in the *ΔldhA-L6* strain (Fig. 4c and Fig. 4f). Overall, the results indicated that the succinic acid transporter and the monosaccharides xylose and mannose have a positive impact on succinic acid production when combined with glucose.

The triple combinations of glucose, xylose, and mannose (G20X5M5, G20X10M5, and G20X5M10) exhibited 2.05–2.21 times higher succinic acid production over 24 h compared to that of G20 in the *Psod:SucE12-ΔldhA* strain, with the glucose in these triple combinations being completely consumed by 9 h (Fig. 5). Lactic acid was not detected in any of the combinations, but the acetic acid levels were notably higher when the metabolism of succinic acid production was more active.

Succinic acid production exhibited a significant increase in the *Psod:SucE12-ΔldhA* strain compared to the *ΔldhA-L6* strain. Thus, the enhancement of metabolism of succinic acid production by the transporter and monosaccharides was corroborated.

Optimization of succinic acid production from woody and herbaceous plants

Succinic acid production using of *C. glutamicum*-derived strains and hydrolysates from lignocellulosic biomass as a carbon source has not yet been reported. In our analysis of succinic acid production using hydrolysates obtained from woody and herbaceous plants, we observed that a higher concentration of hydrolysate does not lead to a proportional increase in succinic acid production (Fig. 3). Additionally, we investigated the effect of monosaccharides (glucose, xylose, and mannose) on the rate of succinic acid conversion by the recombinant strains. We observed a positive tendency toward succinic acid production, except in two cases involving the absence of glucose or the sole presence of xylose. Although a small amount of xylose was consumed in some cases, it did not disturb glucose consumption and conversion to succinic acid in either strain. However, the factor limiting the hydrolysate concentration during the fermentation process remains unknown. To address this issue, we initially focused on glucose, exploring the effects of concentrations ranging from 20 to 50 g/L during fermentation with the Psod:SucE-*ΔldhA* strain. The optimal glucose concentration was determined to be 30 g/L, as it exhibited the most favorable balance between glucose consumption and succinic acid production, serving as a limiting factor when the concentration exceeded 30 g/L (Fig. S2).

In the enzymatic saccharification process of lignocellulosic biomass, variations in the final concentration of reducing sugars are often observed. These variations can be attributed to various factors, including pretreatment and substrate conditions, enzyme dosage and reactions, and the method employed for reducing sugar measurement. To enhance the accuracy of determining the final concentration of reducing sugars, reducing sugar sets (RS20, RS30, RS40, and RS50) derived from pine, poplar, oak, bamboo, spent coffee grounds, and

their mixture were prepared and used to estimate the efficiency of succinic acid production with Psod:SucE12-*ΔldhA* (Fig. 6). The composition of glucose and xylose + mannose in RS20–RS–50 remained consistent, accounting for approximately 75 and 24 % in pine, 77 and 22.73 % in poplar, 80.05 and 19.95 % in oak, 75.56 and 24.44 % in bamboo, 50.81 and 49.19 % in spent coffee grounds, and 73.02 and 26.98 % in the mixture, respectively. However, the residues remaining, after the enzymatic saccharification of the woody and herbaceous substrates caused changes in the sugar compositions compared to the data in Table 1. It can be assumed that the highly compact and recalcitrant cellulose chains in pine, poplar, oak, and bamboo plants contributed to the changes in glucose concentration (*10*).

A substantially high amount of unhydrolyzed residues remained after the enzymatic hydrolysis of spent coffee grounds. These residues were inferred to encompass mannan polymer, lipophilic fractions, ethanol- and water-soluble compounds, and compounds soluble in 1 % NaOH (*31*). In this study, a mannanase, Man5A, was identified in the cellulase cocktail solution from *Trichoderma reesei* (*32*). The small amount of Man5A in the cellulase cocktail may contribute to the relatively lower release of mannose from spent coffee grounds.

During the fermentation of reducing sugars derived from woody plants, hardwood exhibited a more favorable tendency for succinic acid production compared to softwood (Fig. 6). The optimal concentrations of the hydrolysate for succinic acid production were found to be RS50 for pine, RS30 for poplar, and RS40 for oak. The hemicellulose in pine wood comprises six times higher amounts of mannose than that in hardwood, with a xylose to mannose ratio of approximately 1:2. Based on the results from G20X5M10 in Fig. 5, the monosaccharide composition of pine hydrolysate is theoretically more conducive to succinic acid production. Additionally, the side pathway for acetic acid is more pronounced during hardwood fermentation than during softwood fermentation, implying that more acetyl-CoA is utilized for acetic acid production during hardwood fermentation. Despite these factors, the succinic acid production from pine wood (13.45–18.80 g/L succinic acid for 24 h) was lower than that from xylose-rich hardwoods (17.69–21.09 g/L succinic acid for 24 h) and bamboo (21.41–24.12 g/L succinic acid for 24 h).

Bamboo, among herbaceous plants, has a higher cellulose content when compared to softwood and hardwood (Table 1). Moreover, its unique microfibril structure, comprising lower lignin content (10 %) and hemicellulose with higher xylose content, is advantageous during pretreatment and enzymatic hydrolysis (*21*). These properties make bamboo a valuable bioresource for the production of bioenergy and biochemicals. The xylose-to-mannose ratio of

HPAC-pretreated bamboo was determined to be 15:1 (Table 1), and the hydrolysate comprised 75.56 % glucose and 24.44 % hemicellulose derivatives. Thus, bamboo hydrolysate is more abundant in xylose compared to the others studied materials. When applied to succinic acid fermentation, 21.4–24.12 g/L succinic acid was produced from the RS30–RS50 bamboo hydrolysates over 24 h (Fig. 6). Among the lignocellulosic biomass examined in this study, bamboo hydrolysates exhibited the highest succinic acid production. Furthermore, succinic acid production from the RS20 bamboo hydrolysate was comparable to that from the RS40 pine hydrolysate.

Spent coffee grounds have much higher lignin and mannan-based hemicellulose contents compared to pine, oak, poplar, and bamboo (Table 1). However, their lower cellulose content, indicating a limited glucose supply, is a disadvantage for the bioconversion process. However, spent coffee grounds are useful for analyzing and comparing the patterns of succinic acid production in lignocellulosic biomass due to their high mannose content. After enzymatic hydrolysis, the glucose-to- mannose ratio in the hydrolysate changed from 1:1.8 to 1:1. However, despite the much higher mannose content in the hydrolysate, the succinic acid production in spent coffee grounds was observed to be the lowest among the woody and herbaceous plants (Fig. 6). Compared to the data in Fig. 3, the overexpression strain *Psod:SucE-ΔldhA* showed a 36.27 % decrease in succinic acid production at 4–5 % and RS40–RS50 when compared to the *ΔldhA* strain. Similar patterns were observed during the fermentation of the RS20–RS50 mixture.

In the case of mixture fermentation, the acetic acid-to-succinic acid ration was much higher in the mixture (0.38–0.53) than in bamboo (0.23–0.27), indicating that acetic acid production is more active in the mixture fermentation. In particular, lactic acid was first observed at 24 h during the fermentation of the mixture.

Lignocellulosic biomass serves as a sustainable carbon source for alternative energy and chemical production. HPAC pretreatment is efficient in removing lignin and promoting the swelling of cellulose fibers in the microfibril structure of lignocellulosic biomass. Thus, it facilitates efficient enzymatic hydrolysis, while cellulose is a supplier of glucose, hemicellulose supplies a diverse range of sugars, including xylose, mannose, arabinose, and galactose. Xylose and mannose, derived from hemicellulose, contribute to the high efficiency of glucose–succinic acid metabolism in the *Psod:SucE-ΔldhA* strain. However, these derivatives tend to be consumed more slowly than glucose during succinic acid fermentation, leading to a

higher residual proportion of xylose and mannose. In addition, acetic acid is a major by-product, with its production pattern mirroring that of succinic acid from hydrolysates. The genes acetate kinase (ackA) and acetyl-CoA synthase (acsA), which influence acetic acid metabolism during the fermentation, have been engineered to improve succinic acid production. It is evident that further metabolic engineering of xylose and acetic acid conversion to succinic acid represents the next challenge for achieving even more efficient succinic acid production from poplar, oak, and bamboo.

CONCLUSIONS

The engineered *C. glutamicum* strains were employed for succinic acid production from various monosaccharide combinations and lignocellulosic biomass. The Psod:SucE-*ΔldhA* strain exhibited a 12–91 % increase in succinic acid production compared to the *ΔldhA* strain when utilizing pure monosaccharide sources and their combinations. Notably, mannose was identified to induce efficient succinic acid production. Among the lignocellulosic biomass, bamboo emerged as an optimal carbon source for succinic acid production, followed by hardwoods (oak and poplar), softwood, and spent coffee grounds.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

SUPPLEMENTARY MATERIALS

All supplementary materials are available at: www.ftb.com.hr.

AUTHORS' CONTRIBUTION

Dae-Seok Lee was actively involved in investigation, methodology, and writing the paper. Eun Jin Cho contributed to investigation, data analysis, and visualization. Seryung Kim performed part of the analyses for this work. Dien Thanh Nguyen also performed part of the analyses for this work. Hyeun-Jong Bae contributed to the conception of the work, project administration, funding acquisition, and provided critical revision and final approval of the version to be published.

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Table 1. The chemical composition of lignocellulosic biomass

	Cell wall content/%			Carbohydrate						
	Cellulose	Hemi-cellulose	Lignin	Glu g/kg, (%)	Xyl g/kg, (%)	Ara g/kg, (%)	Rham g/kg, (%)	Man g/kg, (%)	Gal g/kg, (%)	Sum g/kg, (%)
Softwood (*27*)	45–50	25–35	18–25	67.06	9.49	2.41	-	17.18	3.86	100
Pine (*28*)	42.10	21.80	24.12	65.88	9.23	2.66	-	17.53	4.69	100
HPAC-pretreated pine	51.2	8.3	-	489.19± 46.46 (86.00±0.22)	10.99±1.42 (1.93±0.09)	0.54±0.10 (0.10±0.02)	- -	66.31±5.78 (11.66±0.14)	1.76±0.15 (0.31±0.05)	568.80±53.44 (100.00)
Hardwood(*24, 29*)	44–55	24–40	18–25	67.82	26.19	0.80	-	3.59	1.60	100
Poplar (*28*)	42.90	20.25	23.60	67.93	25.73	0.55	-	2.65	0.80	100
HPAC-pretreated poplar	54.4	5.9	-	543.52±43.76 (90.31±0.57)	38.68±6.09 (6.40±0.47)	1.21±0.38 (0.20±0.05)	-	18.64±1.98 (3.09± 0.10)	- -	602.05±52.12 (100.00)
Oak (*24*)	39.71	28.77	23.99	64.47	29.87	0.63	-	3.14	1.89	100
HPAC-pretreated oak	52.4	4.4	-	523.91±62.09 (92.19± 0.12)	34.48±4.71 (6.06± 0.14)	1.01±0.16 (0.18±0.02)	- -	5.83±0.34 (1.03±0.07)	3.07±0.55 (0.54±0.12)	568.29±67.69 (100.00)
Herbaceous plants (*30*)	28~40	19~35	7–32	-	-	-	-	-	-	-
Bamboo (*23*)	50.81	38.73	10.47	427.59 ±18.59 (56.84)	300.23± 9.84 (39.84)	15.47±0.73 (2.05)	2.17±0.39 (0.29)	4.36±2.83 (0.58)	3.68± .97 (0.49)	753.52±17.53 (100.00)
HPAC-pretreated Bamboo	67.77	7.06	-	677.75± 62.55 (90.54± 0.39)	64.10± 4.15 (8.58± 0.32)	2.40±0.34 (0.32±0.06)	-	4.13±0.30 (0.55±0.05)	-	748.37± 66.40 (100.00)
Spent coffee grounds (*31*)	9.90	33.40	38.60	22.86	2.54	5.08	-	44.57	24.94	100
Pretreated-spent coffee grounds	17.58	32.28	-	175.52±9.25 (35.22±0.35)	0.00	0.94±0.04 (0.19±0.00)	-	308.33±12.36 (61.89±0.19)	13.47±0.16 (2.71±0.15)	498.26±1.49 (100.00)

Table 2. Enzymatic hydrolysis ratios of different lignocellulosic biomass

Substrate concentration/%	Pine	Poplar	Oak	Bamboo	Spent coffee grounds
1	92.11±1.38	86.57±1.81	78.28±0.12	93.23±1.33	79.08±1.82
2	94.02±2.01	93.17±1.28	84.74±2.52	96.19±1.93	78.74±3.94
3	90.53±1.59	95.99±1.30	80.91±1.49	98.50±3.43	66.71±2.56
4	83.86±3.33	94.34±4.97	87.68±3.24	91.48±1.59	59.66±4.50
5	79.17±3.02	90.65±2.07	87.14±2.19	90.66±4.19	53.88±7.60

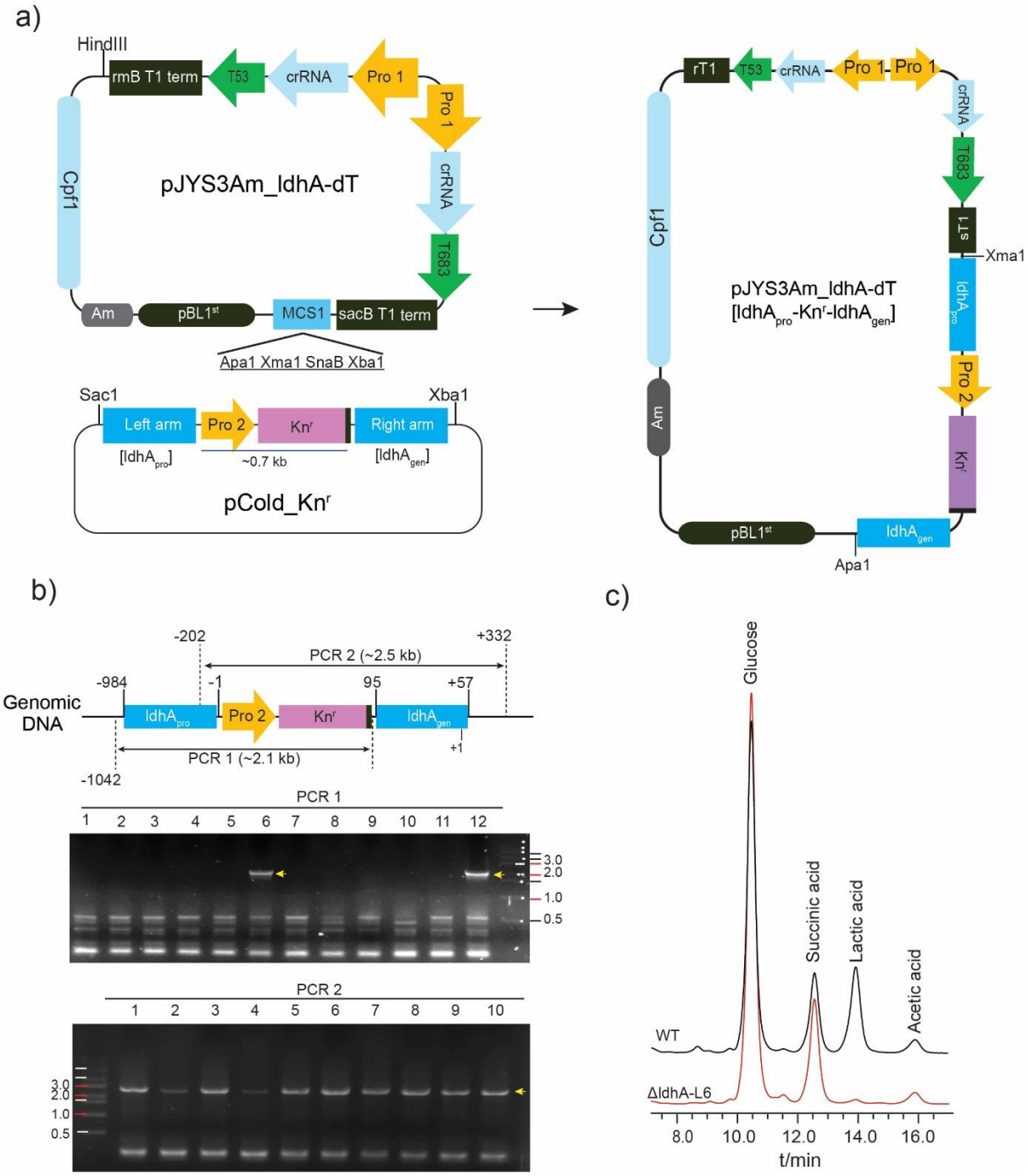

Fig. 1. Construction of a homologous recombination vector for CRISPR/Cpf1 genome editing: a) the double target DNA sets (T53 and T683) with crRNAs were introduced into the pJYS3Am vector. The homologous arms consisted of the promoter region of the ldhA gene (left arm) and

the open reading frame of ldhA (right arm). A spontaneous deletion and overexpression vector, pJYS3Am_ldhA-dT [$ldhA_{pro}$-Kn^r-$ldhA_{gen}$], was constructed, b) when the 1st and 2nd homologous crossovers were complete, a 95 bp-long region of the ldhA gene was deleted. PCR 1 and 2 show the complete homologous recombination, and c) the *ΔldhA-L6* strain was employed for fermentation with pure glucose and the activity of ldhA was completely blocked. Kn^r=kanamycin resistance gene, Am=ampicillin resistance gene, rmB T1 term and sacB T1 term, terminator regions of the rmB and sacB genes, respectively; Pro 1, J23119 promoter; Pro 2, Kn^r promoter, MCS=multiple cloning sites

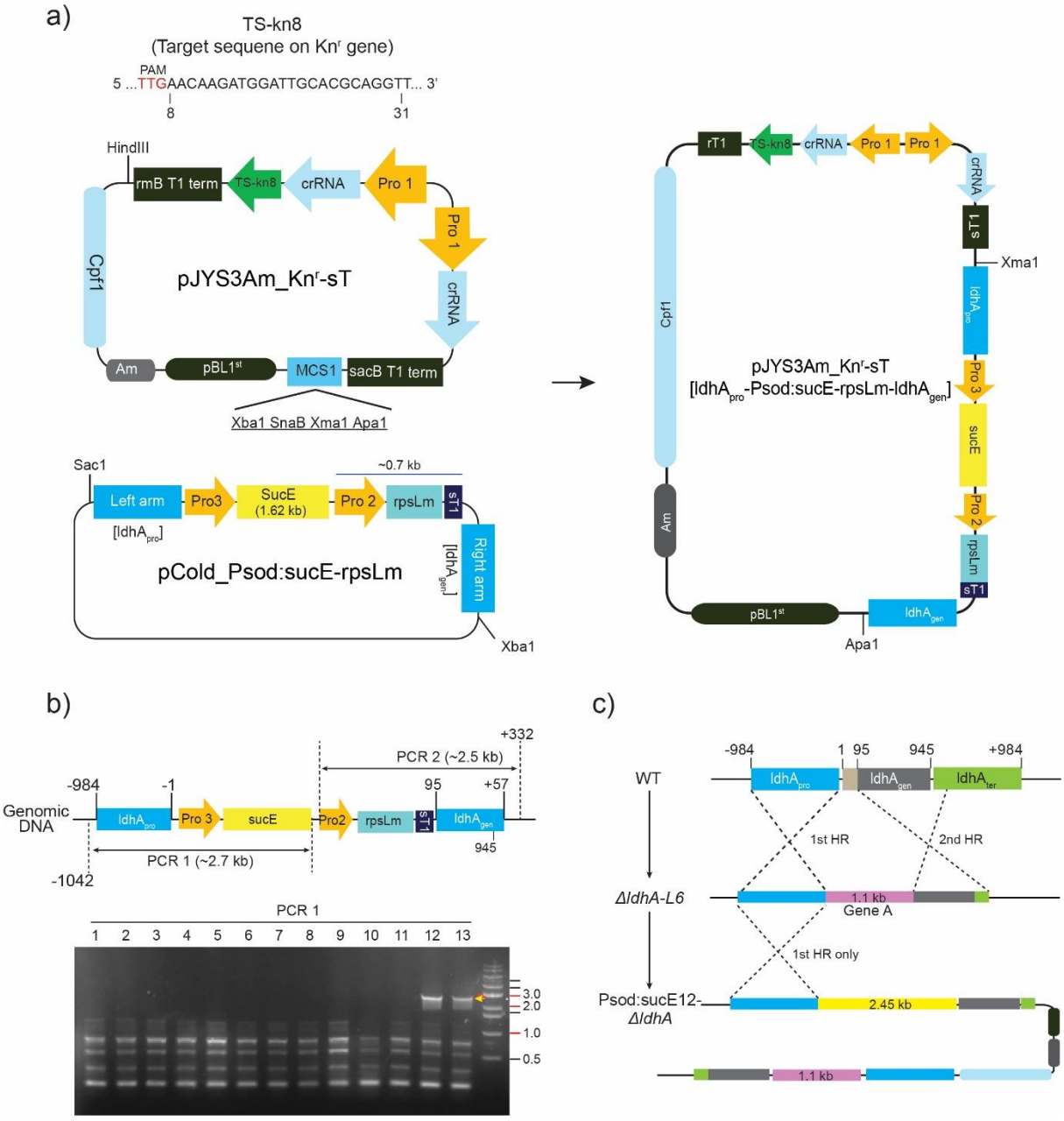

Fig. 2. Overexpression of succinic acid transporter (SucE) using a CRISPR/Cpf1 vector containing a single-target DNA set: a) the target site was located at 8 bp from the start codon of the Kn^r gene in the *ΔldhA-L6* strain. The SucE and rpsLm genes between the left and right arms were subcloned to construct the CRISPR/Cpf1 single target vector pJYS3Am_Kn^r_sT [ldhA$_{pro}$-Psod:SucE-rpsLm-ldhA$_{gen}$]. The expression of the rpsLm gene is regulated by the Kn^r promoter, providing streptomycin resistance to the *ΔldhA-L6* strain, b and c) the overexpression strain Psod:SucE12-*ΔldhA* was created, and the occurrence of the 1st

homologous crossover was confirmed. However, the 2nd crossover was not observed. Pro3=Psod promoter, rpsLm=ribosomal S12 protein mutant gene, rT1 and sT1, rmB T1 and sacB T1 terminators, respectively; HR, homologous recombination

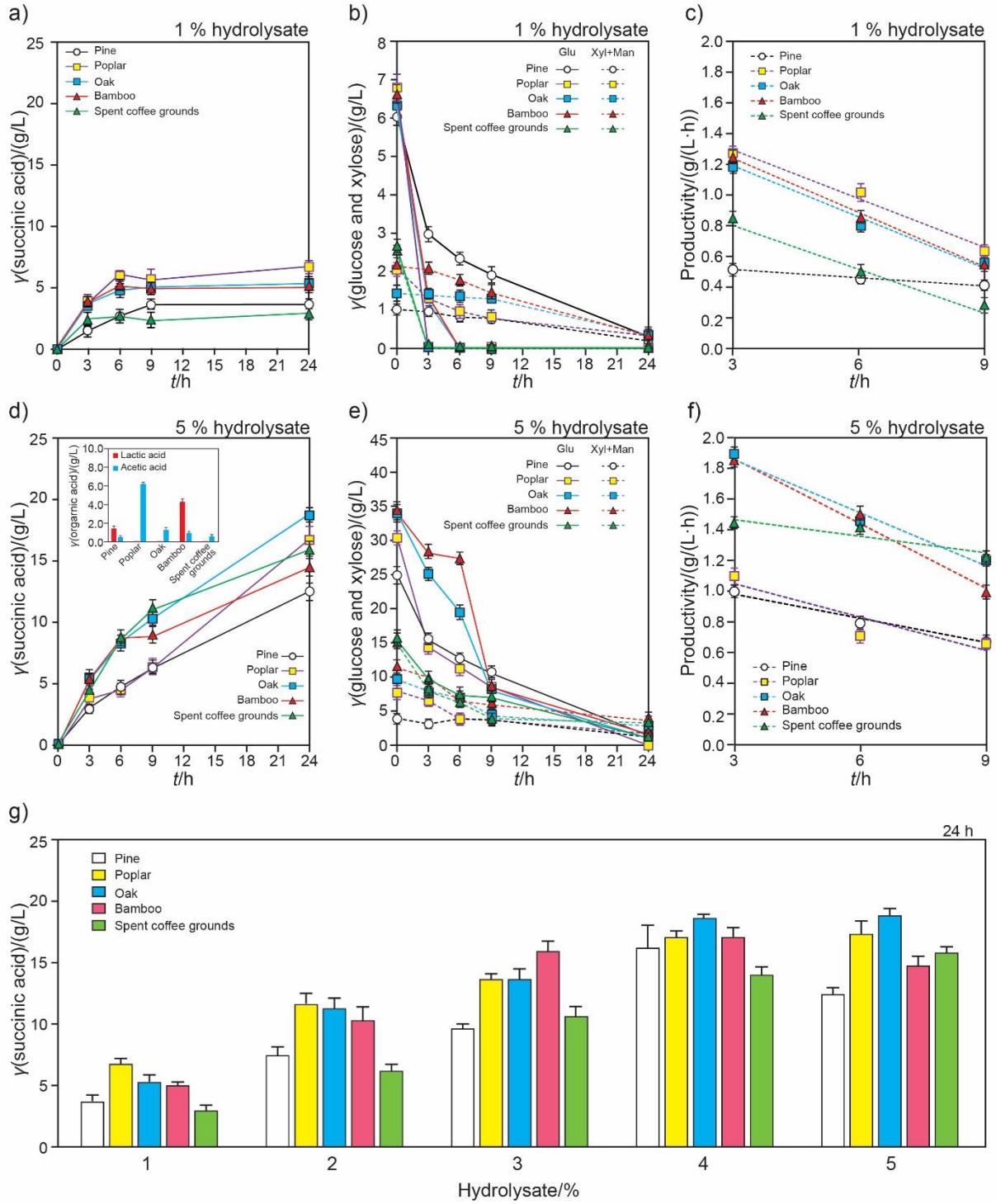

Fig. 3. Succinic acid production from woody plants (pine, poplar and oak) and herbaceous plants (bamboo and spent coffee grounds) using the *ΔldhA-L6* strain. a) succinic acid production using 1% hydrolysate under unsaturated sugar conditions, b) monosaccharide consumption of 1% hydrolysate under unsaturated sugar conditions, c) productivity of 1%

hydrolysate under unsaturated sugar conditions, d) succinic acid production using 5% hydrolysate under saturated sugar conditions, e) monosaccharide consumption of 5% hydrolysate under saturated sugar conditions, f) productivity of 5% hydrolysate under saturated sugar conditions, and g) a comparison of succinic acid production patterns depending on various sugar concentrations and compositions derived from lignocellulose biomasses after 24 hours of fermentation

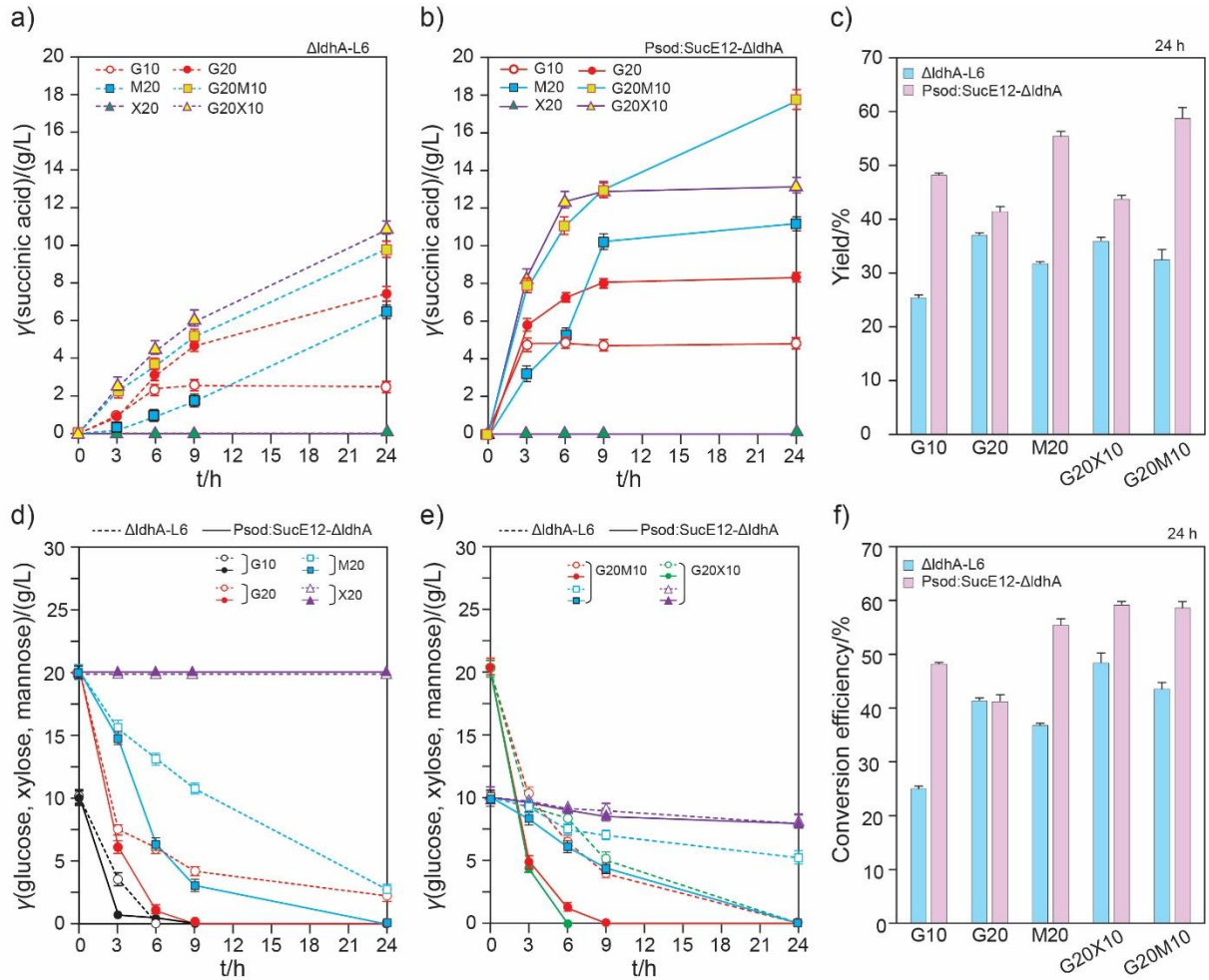

Fig. 4. Succinic acid production using the engineered *Corynebacterium glutamicum* strains on monosaccharides. Succinic acid production by: a) *ΔldhA-L6* and b) Psod:SucE12-*ΔldhA* was observed during the 24 h fermentation of monosaccharides and their combinations. Dotted line: *ΔldhA-L6*, solid line: Psod:SucE12-*ΔldhA*, c) the yield after 24 h of fermentation using monosaccharides. (d and e) monosaccharide consumption when suing the sole monosaccharide and two-monosaccharide combinations, respectively. Dotted line: *ΔldhA-L6*,

solid line: Psod:SucE12-*ΔldhA*, circle: glucose, square: mannose, triangle: xylose, f) the conversion efficiency after 24 h of fermentation using monosaccharides. G10 and G20: 10 and 20 g/L glucose, respectively; X5, X10 and X20: 5, 10 and 20 g/L xylose, respectively; M5, M10, and M20: 5, 10 and 20 g/L mannose, respectively

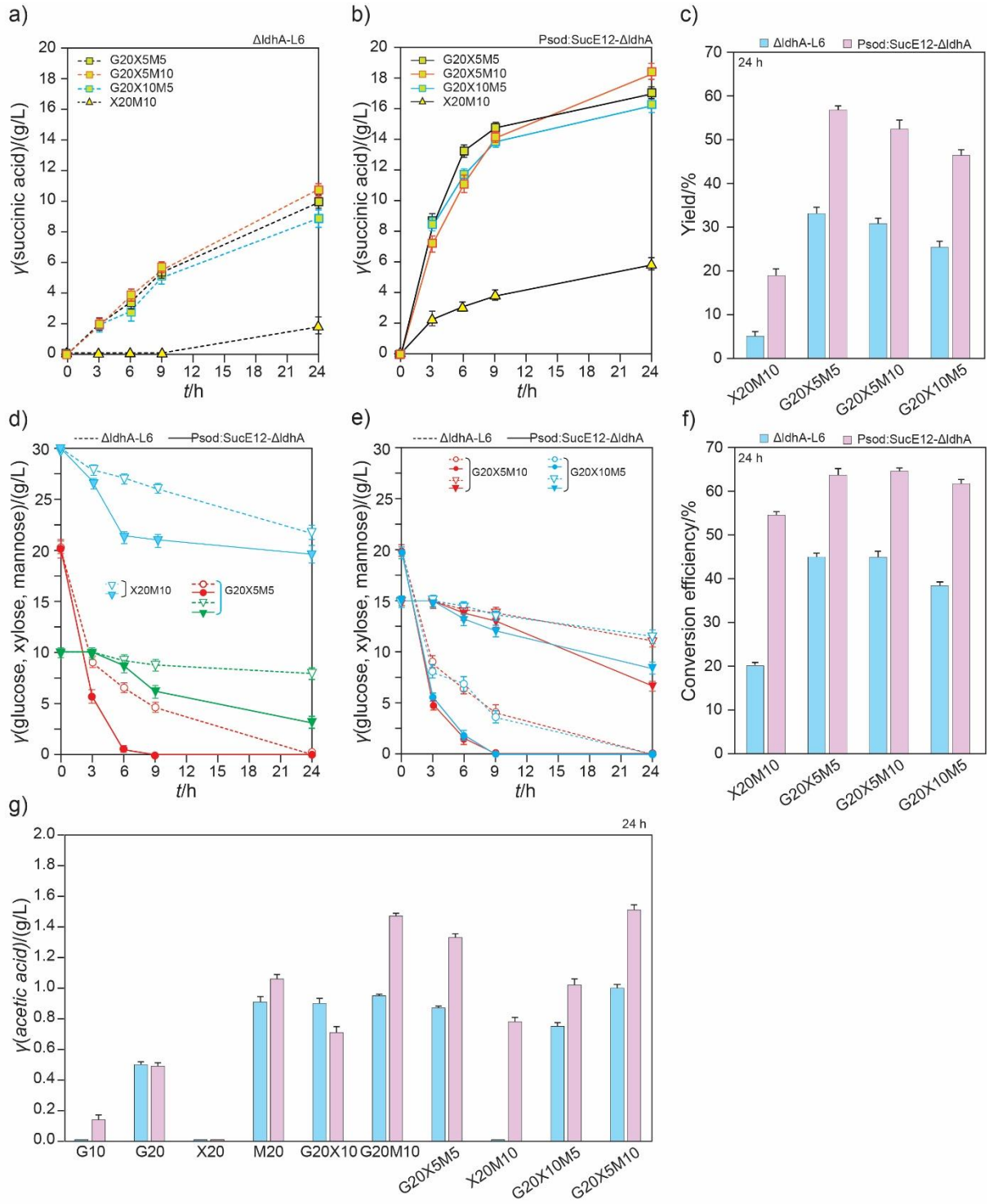

Fig. 5. Comparison of succinic acid production and monosaccharide consumption by the engineered *Corynebacterium glutamicum* strains: a) succinic acid production by *ΔldhA-L6* and b) Psod:SucE12-*ΔldhA* was observed during the 24 h fermentation of the three-

monosaccharide combinations. Dotted line: *ΔldhA-L6*, solid line: Psod:SucE12-*ΔldhA*, c) the yield of the three-monosaccharide combination, d and e) monosaccharide consumption when using different monosaccharide combinations. Dotted line: *ΔldhA-L6*, solid line: Psod:SucE12-*ΔldhA*, circle: glucose, reverse-triangle: xylose+mannose, f) the conversion efficiency of the three-monosaccharide combination

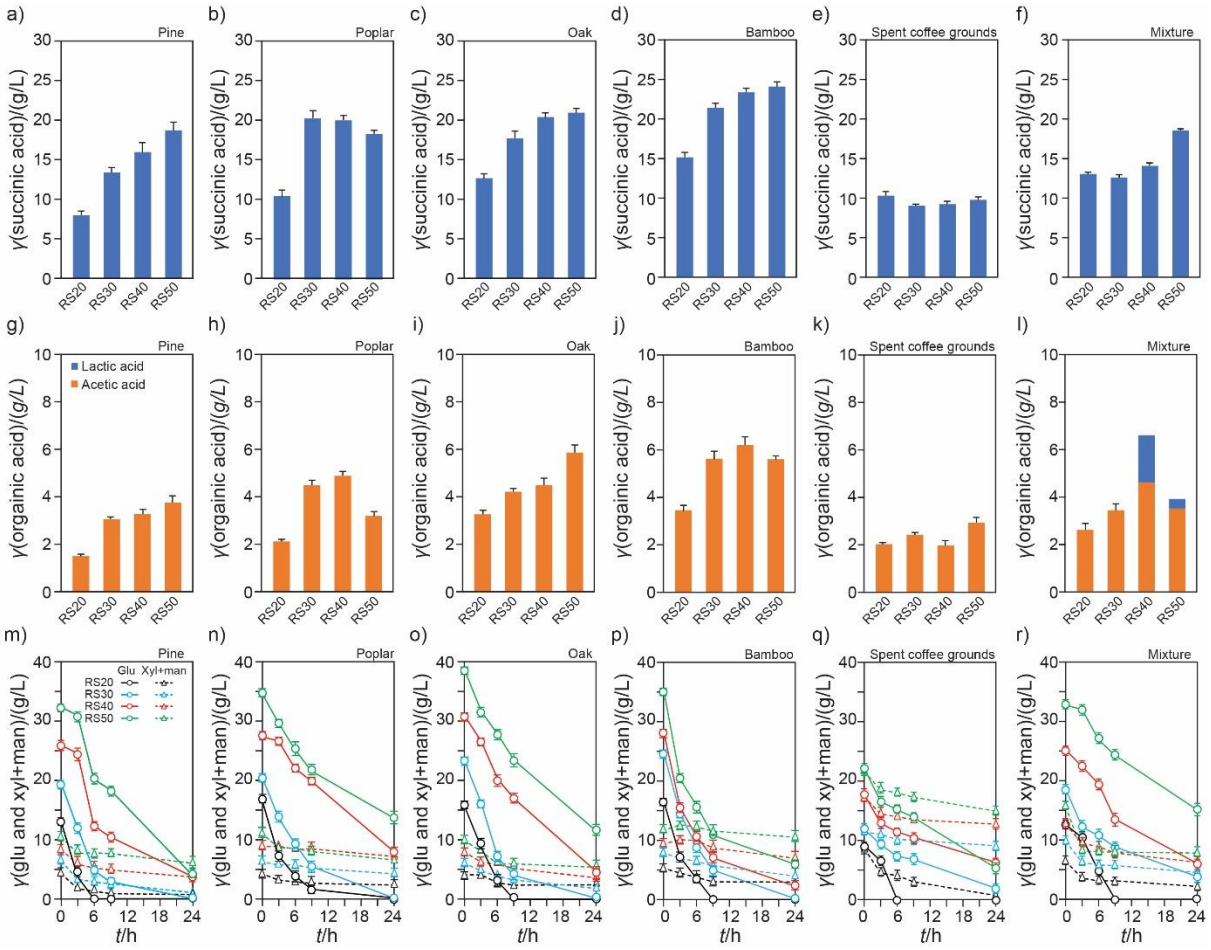

Fig. 6. Succinic acid production from woody and herbaceous plants using the Psod:SucE12-*ΔldhA* strain: Succinic acid production using hydrolysates from: a) pine, b) poplar, c) oak, d) bamboo, e) spent coffee grounds, and f) a mixture was analyzed after 24 hours of fermentation. The production of organic acids, including acetic and lactic acids, using hydrolysates from: g) pine, h) poplar, i) oak, j) bamboo, k) spent coffee grounds, and l) a mixture was analyzed after 24 hours of fermentation. No lactic acid was detected during the 24 h fermentation, Sugar consumption using hydrolysates from: m) pine, n) poplar, o) oak, p) bamboo, q) spent coffee grounds, and r) a mixture was assessed after 24 hours of fermentation. All data were based on the results of the 24h fermentation. Glu, glucose; Xyl, xylose; Man, mannose

SUPPLEMENTARY MATERIAL

Table S1. Primers to generate recombinant *C. glutamicum* using CRISPR-cpf1 gene editing vector

pJYS3Am_MCS	**Fragment 1 (Cpf1 gene)** Sense: 5' CTTTAGGTCTCCCATGGTCATGACCTGAGCTGTTTACAATT 3' Anti-sense: 5 GGTTCGTTCTCATGGCTCACGC 3' **Fragment 2 (Rep101+ pBL1st)** Sense: 5' CCTGCAGGCATGCAAGCTTAAGAG 3' Anti-sense: 5' GAAATGGTCTCCCATGGTGACCGCCTCGATGATCGCC 3' **Fragment 3 (Amp gene)** Sense: 5' CTTTAGGTCTCCCATGGAAAGGGCCTCGTGATAC 3' Anti-sen: 5' CCAATTCCACACATGGTACCACACGATGATTAATTGTAAACAGCTCAGGTCATGCTGA CAGTTA CCAATGCTTAATCAGTG 3'
Target DNA 1 set (crRNA + target DNA)	Sense: 5' CCTGCAGGCATGCAAGCTTAAGAG 3' Anti-sense: 5' GAAATGGTCTCGGATCCGAATTTCTACTGTTGTAGATGAGTTGCATACGCAT ACGCACTGCAATTTAAATAAAACGAAAGGCTCAGTCG 3'
Target DNA 2 set (crRNA + target DNA)	Sense: 5' GAAATGGTCTCACTAGTTTGACAGCTAGCTCAGTCCTAGGTATAATTCTAGAGAATTTC TACTGTTGTAGATGCGTCGATAATG 3' Anti-sense: 5' CTTTAGGTCTCGGGCCCATCGGCATTTTCTTTTGCGTTTCCCGGGCGCTGCCTAT CA CATTATCGACGCATCTACAACAG 3' Experiment: After annealing two primers, the short DNA fragments were synthesized. The PCR products were digested with restriction enzymes and inserted into the pJYS3Am_MCS vector.
pCold 1 vector	Construction of homologous arms and kanamycin selection marker gene.
LdhAp (Left arm)	Sense: 5' GAAATGGTCTCGGGCCCGCTCCGGCTCCCACGGCTGCTC 3' Anti-sense: 5' GAAATGGTCTCGGTACCTTTCGATCCCACTTCCTGATT 3'
LdhA (Right arm)	Sense: 5' CTTTAGGTCTCAAGCTTATCACCTTGCGATCATCGACAT 3' Anti-sense: 5' CTTTAGGTCTCACTAGTGTTGGCAGCGCAAGTGTTCCA 3'
Kn^r gene set (Promoter + gene + terminator)	Sense: 5' CTTTAGGTCTCGGTACCGGAAGCGGAACACGTAGAAAGC 3' Anti-sense: 5' GAAATGGTCTCCTCGAGGGTCATGATTCCGCGAACCCA 3'
LdhAp (-1042)	Sense: 5' CCGACAACACTGCACGGCCCTGCGA 3'
LdhAp (-202)	Sense: 5' CAATTCTGCAGGGCATAGGTTGG 3'
LdhA (+332)	Anti-sense: 5' CGCAGAGGAGAGGCGTTGCTGCAGG 3'
Promoter sequence	**Amp^r promoter:** 5' CGTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATACA TTCAAATATGTATCCGCTCATGAGACAATAACCCTGATAAATGCTTCAATAATATTGAAAAAGGAA GAGT 3' **PlacM promoter:** 5' TGAGCTGTTTACAATTAATCATCGTGTGGTACCATGTGTGGAATTGGAAAGGACTTGAACG 3' **J23119 promoter:** 5' TTATACCTAGGACTGAGCTAGCTGTCAA 3' **Kn^r promoter:** 5' GGAAGCGGAACACGTAGAAAGCCAGTCCGCAGAAACGGTGCTGACCCCGGATGAATGTCAG CTACTGGGCTATCTGGACAAGGGAAAACGCAAGCGCAAAGAGAAAGCAGGTAGCTTGCAGTG GGCTTACATGGCGATAGCTAGACTGGGCGGTTTTATGGACAGCAAGCGAACCGGAATTGCCA GCTGGGGCGCCCTCTGGTAAGGTTGGGAAGCCCTGCAAAGTAAACTGGATGGCTTTCTTGCC GCCAAGATCTGATGGCGCAGGGGATCAAGATCTGATCAAGAGACAGGATGAGGATCGTTTCG 3'

	Psod promoter: 5’ TGCGGAAACCTACGAAAGGATTTT 3’
Terminator sequence	**rmB T1 terminator:** 5’ ATTTGTCCTACTCAGGAGAGCGTTCACCGACAAACAACAGATAAAACGAAAGGCCCAGTCTTTCGACTGAGCCTTTCGTTTTATTT 3’ **SacB T1 terminator:** 5’ AAACGCAAAAGAAAATGCCGAT 3’ **Kn^R terminator** 5’ GCGGGACTCTGGGGTTCGCGGAATCATGACC3’
rpsL gene	5’ ATGCCAACTATTCAGCAGCTGGTCCGTAAGGGCCGCCACGATAAGTCCGCCAAGGTGGCTACC GCGGCACTGAAGGGTTCCCCTCAGCGTCGTGGCGTATGCACCCGTGTGTACACCACCACCCCT**AAG**AAGCCTAACTCTGCTCTTCGTAAGGTCGCTCGTGTGCGCCTTACCTCCGGCATCGAGGTTTCCGCTTACATCCCTGGTGAGGGCCACAACCTGCAGGAGCACTCCATGGTGCTCGTTCGCGGTGGTCGTGTTAAGGACCTCCCAGGTGTCCGTTACAAGATCGTCCGTGGCGCACTGGATACCCAGGGTGTTAAGGACCGCAAGCAGGCTCGTTCCCG CTACGGCGCGAAGAGGGGATAA 3’ (Under line: point mutagenesis site, AAG →AGG)
Kanamycin resistance gene target DNA	5’ GAAATGGTCTCGGATCCGAATTTCTACTGTTGTAGATAACAAGATGGATTGCACGCAGGTTAATTTAAATAAAACGAAAGGCTCAGTCG 3’
sucE	5’ CTTTAGGTCTCGAATTCATGAGCTTCCTTGTAGAAAATC 3’ 5’ GAAATGGTCTCAAGCTTGATAAGTAGGAACAACAACGTTTG 3’

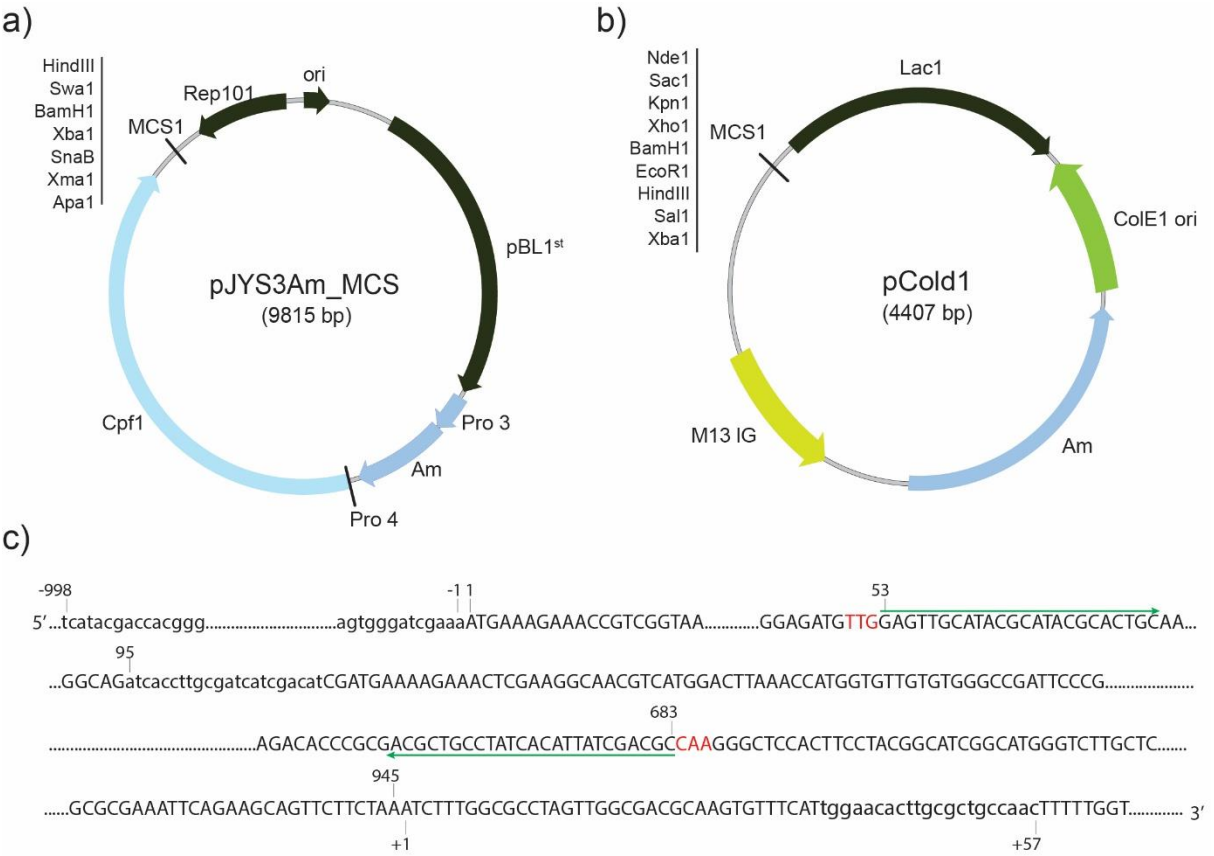

Fig. S1. The vectors used for generating CRISPR/Cpf1 system and for homologous recombination: a) the pJYS3_Am_MCS vector was derived from the pJYS3_ΔcrtYF plasmid, which was obtained from Addgene (www.addgene.org), b) the pCold vector (TaKaRa) was used as an intermediate vector to construct homologous arms and the expression gene set for the pJYS3Am_ldhA-dT [$ldhA_{pro}$-Kn^r-$ldhA_{gen}$] and pJYS3Am_Kn^r-sT [$ldhA_{pro}$-Psod:SucE-rspLm-$ldhA_{gen}$] vectors, and c) the lactate dehydrogenase A (ldhA) gene and its flanking regions were used as homologous arms and two target DNA sequences for CRISPR/Cpf1 genome editing in *Corynebacterium glutamicum*. Lowercase letters indicate the primer annealing sites for PCR amplification of the promoter region (-1 to -998) and the region of the ldhA gene (95 to 945) along with a partial terminator region (+1 to +57). Uppercase letters denote the ldhA gene. Red letters represent PAM sites, and green arrows indicate the target DNA sequences

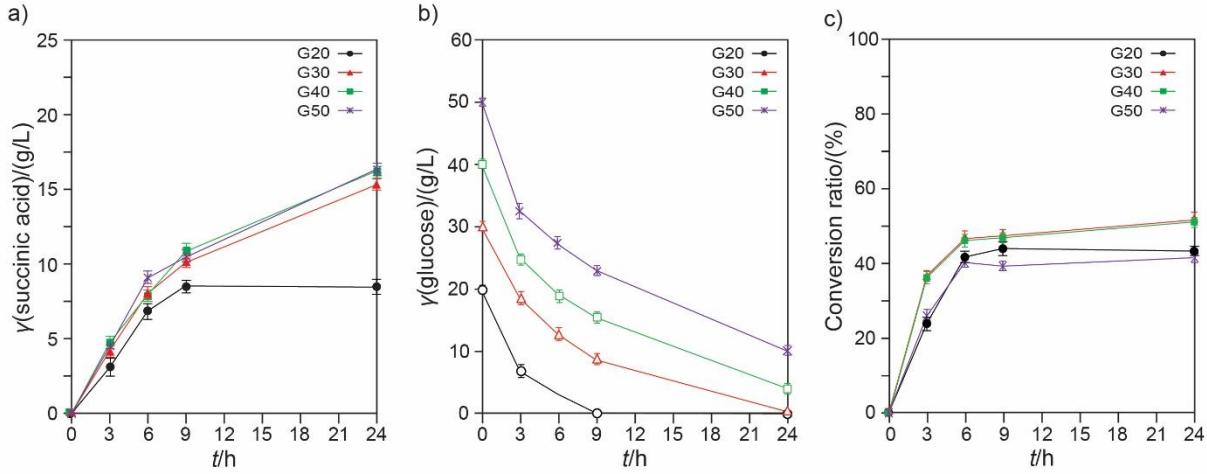

Fig. S2. Succinic acid production depends on glucose concentration: a) succinic acid production, b) glucose consumption, and c) the conversion ratio