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Biosensor-Guided Atmospheric and Room-Temperature Plasma Mutagenesis and Shuffling for High-Level Production of Shikimic Acid from Sucrose in *Escherichia coli*

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ABSTRACT: Here, we first developed a combined strain improvement strategy of biosensor-guided atmospheric and room-temperature plasma mutagenesis and genome shuffling. Application of this strategy resulted in a 2.7-fold increase in the production of shikimic acid (SA) and a 2.0-fold increase in growth relative to those of the starting strain. Whole-cell resequencing of the shuffled strain and confirmation using CRISPRa/CRISPRi revealed that some membrane protein-related mutant genes are identified as being closely related to the higher SA titer. The engineered shuffling strain produced 18.58 ± 0.56 g/L SA from glucose with a yield of 68% (mol/mol) by fed-batch whole-cell biocatalysis, achieving 79% of the theoretical maximum. Sucrose-utilizing *Escherichia coli* was engineered for SA production by introducing *Mannheimia succiniciproducens* β-fructofuranosidase gene. The resulting sucrose-utilizing *E. coli* strain produced 24.64 ± 0.32 g/L SA from sucrose with a yield of 1.42 mol/mol by fed-batch whole-cell biocatalysis, achieving 83% of the theoretical maximum.

KEYWORDS: *shikimic acid, genome shuffling, biosensor-guided, ARTP, Escherichia coli, sucrose, whole-cell biocatalysis*

INTRODUCTION

Shikimic acid (3,4,5-trihydroxy-1-cyclohexene-1-carboxylic acid, SA) is an important metabolic intermediate in plants and microorganisms. SA has received special attention because it is a key component for synthesizing the antiviral drug oseltamivir (Tamiflu). Oseltamivir, a viral neuraminidase inhibitor, has been found to be effective against influenza A and B, avian influenza H5N1, and human influenza H1N1. Especially if used early, it can also be used for prevention.[1] SA was at first produced from the seeds of species of the Chinese star anise plant *Illicium*, such as *Illicium verum* or *Illicium anisatum*. However, the conventional method of producing SA from *I. verum* is typically low yield and costly. Over the past years, microbial production of SA has been realized by engineering *Escherichia coli*,[2−6] *Bacillus subtilis*,[7] and *Corynebacterium glutamicum*.[8−10] Among the abovementioned studies, gene disruption of the shikimate pathway downstream of SA and rational engineering of metabolic networks in shikimate-accumulating strains have been applied to obtain highly productive strains. These have led to many genetically engineered strains with yields that are relatively high (between 40 and 51% mol/mol) but still far from the theoretical maximum (85.7% mol/mol).[11]

The development of productive bacterial strains using genetic or rational engineering strategies is often restricted by limited knowledge of metabolic regulation. Genome shuffling has become one of the promising approaches used for rapid strain improvement because it does not require knowledge of metabolic networks.[12−14] Screening and selecting the desired phenotypes are the last step in genome shuffling. A powerful high-throughput screening method is essential for the success of genome shuffling. Recently, a number of metabolic biosensors have been developed for use in detecting *in vivo* metabolite concentrations and have emerged as promising tools for screening mutant strains for improved product formation.[15] Li et al. developed a shikimate biosensor for monitoring SA production in *E. coli*.[16] Subsequently, another biosensor was constructed for monitoring SA synthesis in *C. glutamicum* by another group.[9]

Sucrose is an attractive raw material for the production of bio-based products. Sucrose is one of the cheapest carbon sources that can be used as a raw material.[17] However, *E. coli* K-12 strain, which has been used for several decades as a host strain for the production of chemicals, unfortunately cannot catabolize sucrose as a sole carbon source. In all of the abovementioned engineered microorganisms, SA was produced from glucose or glycerol as a carbon source. Thus, the development of sucrose-utilizing *E. coli* is desirable for the production of SA.

Thus, in this study, we first applied the strategy of biosensor-based genome shuffling to improve the production of SA. The relationship between the shuffled strain genotype and the phenotype of high SA titer was then revealed by using the combinatorial strategy of comparative genomics analysis with CRISPR activation (CRISPRa) and interference (CRISPRi).

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Table 1. Strains and Plasmids Used in This Study

name	description	source/ purpose
Strain		
E. coli DH5α	*supE44* Δ(*lacZYA-argF*) *U169* (Φ80*lacZ* Δ*M15*) *hsdR17 recA endA1 gyrA96 thi-1 relA1*	Invitrogen
E. coli BW 25113	$lacI^q$ $rrnB_{T14}$ Δ$lacZ_{WJ16}$ hsdR514 Δ$araBAD_{AH33}$ Δ$rhaBAD_{LD78}$	18
E. coli SA30	shikimic acid overproducer, *E. coli* BW 25113 ΔPTS, P_{glk}::P5TG, P_{galP}::P5TG, $attP_{HK}$::P37-tktA-$aroG^{fbr}$-aroB-aroE, $attP_{21}$::P37-csrB-ppsA-P37-nadK, 186::P37-nadK, P21::P37-ppsA-csrB	lab storage
E. coli SA447	shikimic acid overproducer, obtained after ARTP mutant	this study
E. coli SA-GS-1-414	shikimic acid overproducer, obtained after ARTP mutant and genome shuffling	this study
E. coli SA-GS-2-7	shikimic acid overproducer, obtained after ARTP mutant, genome shuffling, and ep-WGS	this study
Plasmid		
pSenSA	shikimic acid biosensor, pBR322 *ori*, Amp^r	this study
pEC-sacC	pEC-XK99E derivate containing *M. succiniciproducens* β-fructofuranosidase gene (*sacC*)	19
pBbB2k-GFP	expressing plasmid, BglBrick vectors, tet promoter, pBBR1 *ori*, kan^r, Addgene plasmid #35345	20
pBbB2k-sacC	pBbB2k derivatives harboring *M. succiniciproducens* β-fructofuranosidase gene (*sacC*)	this study
pBbB2K-dCas9*-MCPSoxS	pBbB2K-dCas9* containing the MCPSoxS sequences	21
pTargetA	*E. coli* scRNA expression vector, BglBrick vector, Ptet promoter, Spe^r, pMB1 *ori*	21

Finally, sucrose-utilizing *E. coli* was constructed for the production of SA.

METHODS

Strains, Plasmids, and Primers. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* DH5α was used for gene cloning in this study. Primers used in this study are presented in Supplementary Table 1.

Construction of Plasmids. Li et al. previously developed a shikimate biosensor for monitoring SA production in *E. coli*.[16] For easy screening, mCherry, which is visible to the naked eye, was used as a reporter for the biosensor. The monitoring device for the shikimate biosensor P_{CP6}-$HucR^{SH4}$-P_{hucR} was synthesized by GENEWIZ (Suzhou, China) and ligated into pUC57 to form pUC57-SA. The P_{CP6}-$HucR^{SH4}$-P_{hucR} fragment was excised from pUC57-SA using *Bgl*II and *Nhe*I and then cloned into pZEA-mCherry to obtain pSenSA.

The *M. succiniciproducens* β-fructofuranosidase gene (*sacC*) was amplified from pEC-sacC and cloned into the *Eco*RI/*Bam*HI sites of pBbB2k-GFP to obtain pBbB2k-sacC.

ARTP Mutagenesis. A 5 mL cell broth cultured for about 5 h was harvested by centrifugation, washed twice with PBS (pH 7.0), and resuspended in 1 mL PBS (pH 7.0). Then, atmospheric and room-temperature plasma (ARTP) mutagenesis was carried out using an ARTP mutation system (ARTP-IIS, Tmaxtree Biotechnology Co, Ltd., Wuxi, China) as described by Niu et al.[22] The parameters were set as follows: (1) the radio frequency power input was set at 120 w, the flow of helium was set at 10 SLM, and the distance between the plasma torch nozzle exit and the slide was set at 2 mm; (2) the different treatment times were selected as 20, 30, 40, 50, 60, 70, 80, and 90 s. After treatment, slides were washed with PBS (pH 7.0) to generate the ARTP mutant library. The mutant strains were transformed by the SA sensor plasmid pSenSA and plated on LB agar with 100 μg/mL ampicillin and 1% glucose. The plates were incubated at 37 °C for about 12 h. A single colony was inoculated into a single well of 48 deep-well microplates containing 1 mL of the fermentation medium and cultured at 37 °C and 1000 rpm for 48 h on an MBR-420FL shaker (TAITEC, Japan). The cells were collected by centrifugation at 14,000*g* for 2 min, washed twice with PBS (pH 7.0), and resuspended in the same volume of PBS (pH 7.0). Then, the bacterial culture (200 μL) was transferred into a 96-well plate, and the fluorescence and OD_{600} were measured using a SynergyNeo2 multi-mode reader (SynergyNeo2, BioTek, USA) with an excitation wavelength of 485 nm and an emission wavelength of 528 nm.

Genome Shuffling. Protoplasts of *E. coli* were generated using a modified version of the protocol of Dai et al.[23] Single colonies were inoculated in 50 mL of LB medium (with 1% glucose) until an OD_{600} of about 1.0 was reached. The cells were harvested by centrifugation at 2795*g* and 4 °C for 15 min, washed three times using ice-cold 0.01 mol/L Tris-HCl buffer (pH 8.0), and resuspended in 0.01 mol/L Tris-HCl buffer (pH 8.0) until an OD_{600} of about 1. EDTA (0.1 M, pH 8.0) was added slowly over 20 min to a final concentration of 0.01 M. The cells were incubated at 100 rpm and 37 °C for an additional 20 min to remove the outer membrane. The cells were harvested by centrifugation, washed twice using SMM buffer (0.5 M sucrose, 20 mM sodium maleate monohydrate, 20 mM $MgCl_2$, pH 6.5), and resuspended in SMM buffer containing 1 mg/mL lysozyme and 5 U/mL DNase I. The cells were incubated at 100 rpm and 37 °C for 1 h to digest the peptidoglycan layer and to digest the DNA released from lysed cells. The protoplasts were harvested by centrifugation and resuspended in a small amount of ice-cold SMM buffer.

Samples of protoplasts were inactivated with UV for 20 min or by heating at 60 °C for 90 min. The two samples of inactivated protoplasts were mixed in a ratio of 1:1, resuspended in a 9-fold volume of PEG buffer (SMM containing 40% PEG6000 and 1.11% $CaCl_2$, pH 7.0), and incubated at 25 °C for 20 min to allow the fusion of the protoplasts. The protoplasts were harvested again and resuspended in a small amount of SMM buffer. This resuspension was inoculated into 10 mL of R-LB medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 1.9 g/L $MgCl_2$, and 1% glucose) and incubated at 100 rpm and 37 °C for 24 h. The cells were then inoculated into 10 mL of R-LB medium and incubated at 200 rpm and 37 °C for 24 h. The cells were established as the library for genome shuffling and stored at −80 °C.

The cells from the library were diluted and then spread on LB plates containing 100 μg/mL ampicillin and 1% glucose. Single colonies with a red color were selected for deep-well microplate analysis to measure OD_{600} and fluorescence intensity as described above.

Error-Prone Whole-Genome Shuffling (ep-WGS). Error-prone whole-genome shuffling (ep-WGS) was carried out according to the protocols reported by Ye et al.[24] with some modifications. Genomic DNA of *E. coli* SA-GS-1-414 was used for the epPCR templates. The error-prone whole-genome amplification was carried out in a total volume of 50 μL, containing 100 ng of genomic DNA, 33.3 μM 15-mer random primers, 0.2 mM dATP, 0.2 mM dGTP, 1 mM dCTP, 1 mM dTTP, 5 mM $MgCl_2$, 0.3 mM $MnCl_2$, and 2 U Taq DNA polymerase. The PCR reaction conditions were as follows: 92 °C for 1 min, 50 cycles at 37 °C for 2 min, a programmed ramping of 0.1 °C per s to 55 °C, and 4 min extension at 55 °C. The amplified PCR products were ethanol-precipitated and stored at −80 °C.

E. coli SA-GS-1-414 (pSenSA, pKD46) were incubated in LB media with 100 μg/mL ampicillin and 50 μg/mL kanamycin at 30 °C to an OD_{600} value of ca. 0.3−0.35, and then 20% L-arabinose was added to a final concentration of 20 mM to induce expression of the Red

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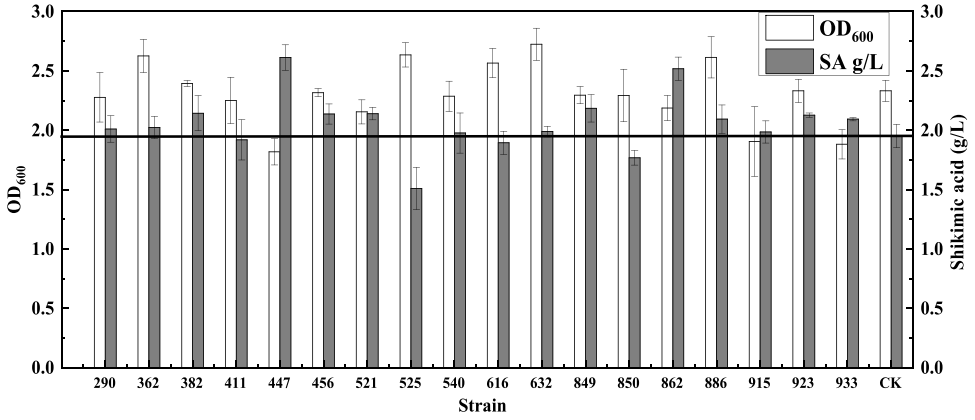

Figure 1. Shikimic acid production by the selected ARTP mutant strains harboring pSenSA. *E. coli* SA30 (pSenSA) was used as the control. The data represent the means of three replicates, and error bars indicate standard deviations.

recombinase. When the cultures reached an OD_{600} of 0.5−0.55, electro-competent cells were prepared, and 100 μL of competent cells was mixed with 200 ng of the above epPCR products and incubated in an ice bath for 5 min. Then, the cells were electroporated with a pulse of 2500 V at 5−6 ms using an Electroporator MicroPulser 6600 (Bio-Rad), and suspensions were immediately mixed with 600 μL of SOC (2% tryptone, 0.5% yeast extract, 0.05% NaCl, 0.0186% KCl, 0.095% $MgCl_2$, and 0.036% glucose) and transferred to a micro-centrifuge tube for culture at 37 °C with 200 rpm for 3 h. All the culture medium was plated on LB agar with 100 μg/mL ampicillin and 1% glucose at 37 °C overnight. The red colonies were first screened by the naked eye, and then the colonies with deeper red color were selected for 48 deep-well microplates containing 1 mL of the fermentation medium and cultured at 1000 rpm and 37 °C for 48 h on an MBR-420FL shaker (TAITEC, Japan). Finally, the SA concentrations were determined using the periodic acid reagent method described by Cromartie and Polge.[25] An SA sample of 250 μL was mixed with 250 μL of the periodic acid reaction solution (5 g/L $HIO_4{\cdot}2H_2O$ and 5 g/L $NaIO_4$) and incubated at 37 °C for 30 min. Next, 500 μL of the combined solution (0.3 M NaOH and 0.0112 M $NaSO_3$) was added to terminate the reaction. Absorbance of the solution at 382 nm was immediately determined. The standard curve was also constructed.

Quantitative Real-Time PCR (qRT-PCR). Total RNA was extracted from *E. coli* cells cultured for 36 h in shake flasks using a HiPure Bacterial RNA kit (Magen, Guangzhou, China) following the manufacturer's instructions. After measuring RNA concentration using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific), only the RNA samples with 260/280 ratio (an indication of protein contamination) greater than 1.8 and 260/230 ratio (an indication of reagent contamination) greater than 2.0 were used for qRT-PCR analysis. The first-strand cDNA was obtained using an All-in-OneTM First-Strand cDNA Synthesis kit (GeneCopoeia, Guangzhou, China). qRT-PCR was carried out on an iCycler iQ5 Real-Time PCR system (Bio-Rad Laboratories, California, USA) using the All-in-OneTM qPCR Mix kit (GeneCopoeia). The template was 100 ng of cDNA. The PCR amplification was performed according to the following conditions: 95 °C for 10 min followed by 45 cycles of denaturation at 95 °C for 10 s, annealing at 55 °C for 20 s, and extension at 72 °C for 15 s. The primers for qRT-PCR are presented in Supplementary Table 1. The fold change of the target gene relative to the reference gene (*cysG*) was determined according to the $2^{-\triangle\triangle Ct}$ method described by Livak and Schmittgen.[26] Each qRT-PCR analysis included at least three biological replicates, and three technical replicates were analyzed for each biological replicate.

Whole-Genome Resequencing and Data Analysis. The total genomic DNA of *E. coli* SA-GS-2-7 was extracted from mid-log-phase bacterial cultures according to the manufacturer's protocol using a TIANamp Bacterial DNA Kit (Tiangen Biotech Co., Beijing, China). Genomic library construction and whole-genome resequencing were performed on an Illumina NOVAseq platform by Sangon Biotech (Shanghai, China). The paired-end reads from *E. coli* SA-GS-2-7 were aligned to the reference genome of *E. coli* MG1655 using BWA software (Burrows-Wheeler Aligner, version 0.7.17). Mutations, including base substitutions, deletions, and insertions, were detected by SAMtools (version 1.9), MarkDuplicates (version 4.1.1.0), and BEDTools (version 2.28.0). DNA frameshift mutations were further validated by PCR and sequencing.

CRISPRa and CRISPRi of the Mutant Genes. Mutant genes with CRISPR activation and interference were performed as described by Niu et al.[21] The pTargetA-X series used in the targeted single-gene activation or repression, with N20 sequences targeting gene loci of interest, was obtained by inverse PCR from pTargetA using primers targeting N20F/N20 genes, then cut with *Kpn*I, and self-ligated. For activation, the N20 sequence was designed to target the non-template strand upstream of the promoter. For repression, the N20 sequence was designed to target the 5′ end (about 100 bp downstream of ATG) of the gene on the non-template DNA strand. As the scRNA fragment was flanked by *Bam*HI and *Bgl*II, the scRNA expressing vector pTargetA-XY could be reassembled from pTargetA-X and pTargetA-Y using the standard BglBrick assembly approach.

To investigate the effects of activation and repression on growth and SA production, pBbB2K-dCas9*-MCPSoxS and pTargetA-X were co-transferred into *E. coli* SA30. A single colony was grown in 5 mL of LB (with 1% glucose) in a falcon tube at 37 °C overnight. The overnight cultures were inoculated into 10 mL of the fermentation medium at 37 °C until the OD_{600} reached 0.8; anhydrous tetracycline was added to the media at a final concentration of 200 nM to induce the expression of dcas9* and then further cultured for 72 h.

Shake Flask Culture for SA Production. A single colony was inoculated into 5 mL of LB (with 1% glucose) in a falcon tube and then cultured overnight at 37 °C and 200 rpm. The overnight seed culture was then inoculated into 10 mL of fermentation medium with an initial OD_{600} of 0.2. The fermentation medium contained (per liter) KH_2PO_4 (3 g), $Na_2HPO_4{\cdot}7H_2O$ (13 g), NaCl (0.5 g), $MgSO_4{\cdot}7H_2O$ (0. 24 g), NH_4Cl (1 g), $CaCl_2$ (0.1 g), proline (1 g), acid-hydrolyzed casein (1 g), glucose (10 g), and glycerol (10 g). The cultures were incubated at 37 °C and 200 rpm for 72 h.

Whole-Cell Biocatalysis for SA Production. The overnight seed culture was inoculated into 50 mL of SBMSN (10 g/L glucose, 12 g/L peptone, 24 g/L yeast extract, 1.7 g/L KH_2PO_4, 11.42 g/L K_2HPO_4, 1 g/L $MgCl_2{\cdot}6H_2O$, 1.42 g/L ammonium oxalate, and 2 g/L Tween-80) medium with an initial OD_{600} of 0.2. The cultures were then incubated at 37 °C and 200 rpm until an OD_{600} of about 0.8. Then, the cultures were incubated at 30 °C and 150 rpm for 6 h and finally at 20 °C and 150 rpm for 12 h. The cells were collected by centrifugation at 6000*g* and 4 °C for 15 min and washed with ice-cold 0.1 M phosphate buffer (pH 7.0) twice.

For biocatalysis, cells were resuspended in a reaction mixture (2.5 mL) containing 10 g/L glucose or sucrose, 1 mM $MgSO_4$, 1 mM

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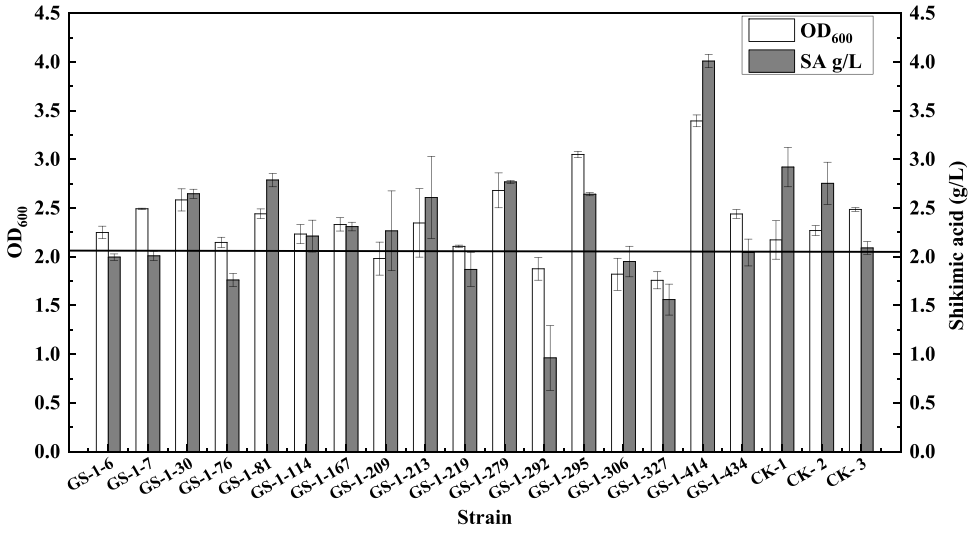

Figure 2. Shikimic acid production by the selected genome shuffling strains harboring pSenSA. *E. coli* SA-447 (pSenSA) was used as control strain 1 (CK1); *E. coli* SA-862 (pSenSA) was used as control strain 2 (CK2); *E. coli* SA30 (pSenSA) was used as control strain 3 (CK3). The data represent the means of three replicates, and error bars indicate standard deviations.

$CaCl_2$, and 0.1 M phosphate buffer (pH 7.0) to form cell suspensions (OD_{600} of 75 for using glucose or 30 for using sucrose). When using sucrose as a carbon source, 200 nM anhydrous tetracycline was added. The biocatalysis was performed at 37 °C and 200 rpm for 48 h.

Analysis. Growth was monitored by measuring the optical density at 600 nm using a spectrophotometer (UV2400, Shimadzu, Japan). SA concentration was analyzed using a Shimadzu HPLC system (LC-20A, Shimadzu, Japan) equipped with an Aminex HPX-87H column (9 μm, 7.8 × 300 mm, Bio-Rad, USA). The mobile phase was 5 mmol/L H_2SO_4 with a flow rate of 0.5 mL/min. SA was detected using a photodiode array detector (SPD-M20A) operating at 210 nm and quantified by comparison with the standard curve. Glucose concentration was determined by glucose oxidase using a glucose assay kit (Shanghai Rongsheng Biotech Corporation, China) as described by the manufacturer. The sucrose concentration was determined by analyzing the glucose concentration of the HCl hydrolysate. A total of 100 μL of 6 mol/L HCl was added to 1 mL of cell-free culture medium. The mixture was hydrolyzed at 70 °C for 15 min, and subsequently, glucose analysis was performed using a glucose assay kit. Sucrose concentration was calculated according to the difference in the glucose concentration between the acid-treated sample and the untreated sample.

RESULTS AND DISCUSSION

Shuffling Improved the Production of Shikimic Acid. ARTP is a rapid, effective, and environmentally friendly mutagenesis tool capable of inducing high mutation rates for generating a microbial mutant library with high diversity.[27] After *E. coli* SA30 cells were treated with ARTP, the SA biosensor pSenSA was transformed into the mutant strains for screening. Nine hundred and forty-five red colonies were selected for deep-well microplate analysis (Supplementary Figure 1). After measuring the fluorescence strength of each deep-well culture, we selected 18 strains with higher fluorescence strength for further shake flask analysis. From Figure 1, we can find that strains 447, 862, 849, 886, 456, 521, 382, 923, and 933 produced higher levels of SA than the parent strain. Some mutants exhibited higher growth density than the parent strain. Of these 18 strains, 9 strains showed higher SA titer, 16 strains showed higher growth, and 7 strains showed both higher SA titer and growth. These results also indicate that ARTP can form a mutant library with high diversity.

Genome shuffling can integrate the good traits of different strains into one strain and is a powerful technology for strain improvement that does not require knowledge of the genetic background of the microbe. To improve SA production and growth, four strains with higher SA production (strains 447, 862, 849, and 886) and four strains with higher growth (strains 616, 362, 525, and 632) obtained after the above-described ARTP mutagenesis were selected as starting strains for genome shuffling. The genome shuffling strains were named as SA-GS-*n*-*x*, where "*n*" denotes the round number for genome shuffling and "*x*" denotes the ordinal number of the genome shuffling strain. Of 450 colonies with the reddest coloration, 17 genome shuffling strains with higher fluorescence strengths (Supplementary Figure 2) were selected for assays of SA production in shake flasks. As shown in Figure 2, only strain SA-GS-1-414 harboring pSenSA produced more SA than the starting strains.

Because only one shuffling strain with a higher level of SA was obtained, it was not possible to complete the second-round genome shuffling. To solve this problem, ep-WGS first developed by Luhe et al.[28] was employed. This technique does not require multiple parental strains, as does conventional genome shuffling. Thus, the strain SA-GS-1-414 harboring pSenSA was used for ep-WGS. From the above data, we find that only one strain with higher SA titer in the shake flask analysis was obtained from the 17 shuffling strains with higher fluorescence strength in deep-well microplate analysis. It indicates that some strains with higher fluorescence strength did not produce higher levels of SA. This may be caused by the specificity of the biosensor. Besides SA, the biosensor can also monitor SA analogs, such as quinic acid, gallic acid, caffeic acid, uric acid, and cinnamic acid.[16] It indicates that the fluorescence strength of the biosensor reflects the concentration of all SA analogs in the SA biosynthetic pathway, not only the SA concentration. Thus, to improve the efficiency of screening, the periodic acid reagent method[25] was used to measure SA concentrations of the deep-well microplate cultures. A total of 190 colonies with the reddest coloration were selected for analysis of SA concentration in deep-well microplate cultures using the periodic acid reagent method (Supplementary Figure 3). Then, 12 strains with higher SA concentration in the deep-

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well microplate analysis were selected for further shake flask analysis. As shown in Figure 3, 10 strains indeed produced higher levels of SA than the control strain. Strain SA-GS-2-7 harboring pSenSA produced 5.95 ± 0.04 g/L SA.

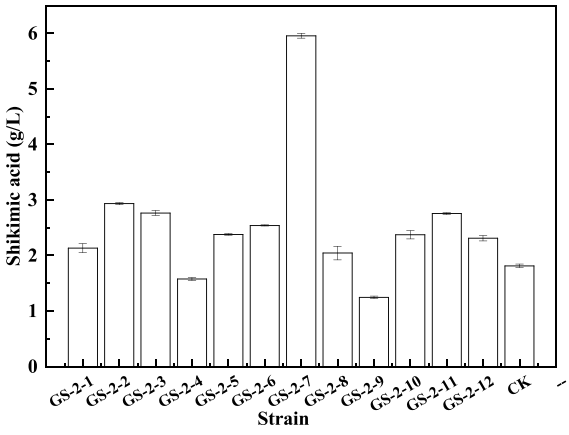

Figure 3. Shikimic acid production by the selected ep-WGS strains harboring pSenSA. *E. coli* SA-GS-1-414 (pSenSA) was set as the control strain. The data represent the means of three replicates, and error bars indicate standard deviations.

After removing the biosensor pSenSA, all mutant strains were cultured for shake flask analysis again. As shown in Figure 4, applying the combined strategy of biosensor-based ARTP

Strain	OD_{600}	Shikimic acid (g/L)
E. coli SA30	2.03 ± 0.23 (100.0%)	2.23 ± 0.04 g/L (100.0%)
↓ ARTP		
E. coli SA447	2.23 ± 0.21 (109.9%)	3.54 ± 0.05 g/L (158.7%)
↓ Genome shuffling		
E. coli SA-GS-1-414	3.04 ± 0.07 (149.7%)	3.84 ± 0.07 g/L (172.2%)
↓ ep-WGS		
E. coli SA-GS-2-7	4.08 ± 0.13 (201.0%)	6.10 ± 0.06 g/L (273.5%)

Figure 4. Diagram summarizing shikimic acid production and growth obtained by using the combined strategy of biosensor-based ARTP mutagenesis and genome shuffling.

mutagenesis and genome shuffling resulted in a 1.7-fold increase in the production of SA and a 1.0-fold increase in growth. However, the specific titer of *E. coli* SA-GS-1-414 (1.26 g/L OD_{600}) was lower than that of *E. coli* SA 447 (1.59 g/L OD_{600}). This may be because we selected four strains with higher growth (strains 616, 362, 525, and 632) for the conventional genome shuffling.

We also investigated the genetical stability of the final strain *E. coli* SA-GS-2-7. The level of SA production in *E. coli* SA-GS-2-7 did not show a significant difference after 25 rounds of subculturing (Supplementary Figure 5). In this study, a novel breeding approach, termed biosensor-based ARTP mutagenesis and genome shuffling, was developed for improving SA production.

Relationship between the Genotype and the Phenotype. To reveal the mechanism underlying the higher SA production of *E. coli* SA-GS-2-7, the transcription levels of the related genes were analyzed. As shown in Figure 5, the transcriptional levels of genes involved in the SA biosynthetic pathway, such as *tktA*, *ppsA*, *pgi*, *aroF*, *aroG*, *aroH*, and *aroB*,

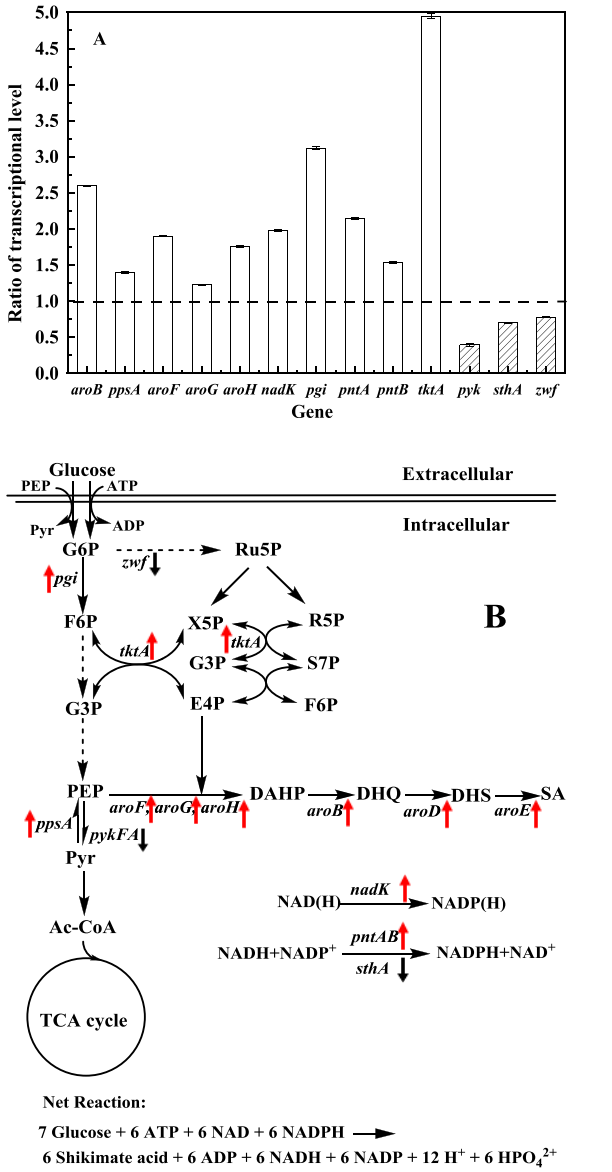

Figure 5. (A) Relative transcription levels of the genes involved in shikimic acid biosynthesis in *E. coli* SA-GS-2-7 compared with those of *E. coli* SA30. (B) Differentially expressed genes involved in shikimate acid biosynthesis. The downward direction represents the downregulation of the transcription level, and the upward direction represents the upregulation of the transcription level. G6P: glucose-6-phosphate; F6P: fructose-6-phosphate; G3P: glyceraldehde-3-phosphate; PEP: phosphoenolpyruvate; Pyr: pyruvate; Ac-CoA: acetyl-CoA; TCA cycle: tricarboxylic acid cycle; Ru5P: ribulose-5-phosphate; X5P: xylulose-5-phosphate; R5P: ribose-5-phosphate; S7P: sedoheptulose-7-phosphate; E4P: D-erythrose 4-phosphate; DAHP: 3-deoxy-D-arabino-heptulosonate-7-phosphate; DHQ: 3-dehydroquinate; DHS: 3-dehydroshikimate; SA: shikimic acid; *zwf*: glucose-6-phosphate dehydrogenase gene; *tkt*: transketolase 1 gene; *tal*: transaldolase A gene; *ppsA*: phosphoenolpyruvate synthase; *pykFA*: pyruvate kinase I and II genes; *aroF*/*aroG*/*aroH*: 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase genes; *aroB*: 3-dehydroquinate synthase gene; *aroD*: 3-dehydroquinate dehydratase gene; *aroE*: dehydroshikimate reductase gene; *nadK*: NAD kinase; *pntA*/*pntB*: NAD(P) transhydrogenase; *sthA*: NAD(P)(+) transhydrogenase.

were upregulated in *E. coli* SA-GS-2-7. Also, the transcription levels of genes, such as *zwf* and *pykA*, involved in competing

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pathways were downregulated. It has been demonstrated that overexpression of *tktA* and the 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase genes (*aroF*, *aroG*, and *aroH*) improved the production of SA in *E. coli*.[2−6] Patnaik and Liao reported that co-overexpression of phosphoenolpyruvate (PEP) synthase (encoded by *ppsA*) and transketolase (encoded by *tktA*) increased the final concentration and the yield of DAHP by almost twofold, close to the theoretical maximum.[11] They speculated that to achieve the theoretical yield of DAHP, the nonoxidative part of the pentose pathway provided erythrose 4-phosphate (E4P) and the Pps reaction recycled pyruvate to PEP. The downregulation of *zwf* can drive the carbon flux into the nonoxidative part of the pentose pathway. We can also determine that the transcription level of *pykA* in *E. coli* SA-GS-2-7 was downregulated (Figure 5). The downregulation of *pykA* resulted in more carbon flux into the shikimate pathway. In the shikimate pathway, shikimate dehydrogenase (encoded by *aroE*) catalyzes the reduction of 3-dehydroshikimate to SA, which requires NADPH as a cofactor. Our previous research has demonstrated a strong correlation between NADPH availability and SA production.[2] From Figure 5, we can determine that transcription levels of *nadK*, *pntA*, and *pntB* were significantly upregulated. This upregulation led to the enhancement of NADPH availability.

To reveal the relationship between the phenotype and genotype of *E. coli* SA-GS-2-7, the whole genome of *E. coli* SA-GS-2-7 was resequenced. Single nucleotide polymorphism (SNP) and insertion or deletion (InDel) analyses were performed by mapping the sequences of *E. coli* MG1655. A total of 38 mutations in 20 genes were identified in *E. coli* SA-GS-2-7 (Table 2). Of these, there were 30 SNPs and 8 InDel

Table 2. SNPs and InDel Mutations in *E. Coli* SA-GS-2-7

type	amounts of mutant	amounts of gene
synonymous SNP	9	3
non-synonymous SNP	11	11
stop-gain mutation	4	4
frameshift mutation	6	2
intergenic mutation	8	
total	38	20

mutations. Of the SNPs, 9 SNPs were synonymous mutations clustered in 3 genes (*icd*, *hycG*, and *selA*), 11 SNPs were non-synonymous mutations in 11 genes (*lacI*, *nagE*, *hlyE*, *acnA*, *ygiC*, *yrfF*, *rhaD*, *fabR*, *btuB*, *yjjI*, and *intQ*), 3 SNPs were stop-gain mutations in 3 genes (*fhuA*, *hsdR*, and *yjjP*), 6 SNPs were intergenic region mutations, and 1 SNP was a non-synonymous, stop-gain, and InDel mutation (*araD*) (Supplementary Table 2).

In our previous study,[21] we reported a CRISPRa/CRISPRi approach for investigating gene function. Thus, we applied the CRISPRa/CRISPRi approach for activating and repressing target genes in *E. coli* SA30 to confirm the functions of all mutant genes. We first constructed the scRNA plasmid (pTargetA-Xa and pTargetA-Xi) for CRISPRi of all mutant genes. pBbB2K-dCas9*-MCPSoxS and pTargetA-Xi were co-transferred into *E. coli* SA30 for repression. Growth and SA production were analyzed and compared with the control strain with empty vector pTargetA. The results are shown in Table 3 and Supplementary Table 3). As shown in Table 3, the repression of some membrane protein genes (*ybaL*, *nagE*, and *gatC*) and genetic information-related genes (*rrfD*, *ygiC*, *fabR*, *hsdR*, and *pheV*) significantly improved SA production. The repression of these genes may affect the transport of mass, enhancing SA production. The *ybaL* gene encodes the transporter protein YbaL. The *fabR* gene encodes the fatty acid biosynthesis transcription repressor FabR, which inhibits the expression of *fabA* and *fabB*. The repression of *fabR* can affect membrane biosynthesis, enhancing the transport of mass. Many studies have demonstrated that membrane-modified transport engineering is a powerful tool for metabolically engineering microorganisms.[29−31] We also found that the repression of most genes did not show significant effects or had slight inhibitory effects on growth. We also investigated the simultaneous effect of the three genes with higher SA titer after repression. The simultaneous repression of *ybaL*, *rrfD*, and *ygiC* further improved SA production. These results further demonstrate that the phenotype of *E. coli* SA-GS-2-7 results from the mutant genes.

Those genes that did not show a significant effect on SA production after repression were selected for activation to confirm their functioning. pBbB2K-dCas9*-MCPSoxS and pTargetA-Xa were co-transferred into *E. coli* SA30 for activation. As shown in Table 4 and Supplementary Table 4, activation of *nagE*, *lacI*, and *hycG* increased growth by 5%, 11%, and 26%, respectively. Activation of *acnA*, *btuB*, *selA*, and *intQ* enhanced the production of SA by 5%, 7%, 8%, and 11%, respectively.

The above results demonstrate that the higher growth and SA titer of *E. coli* SA-GS-2-7 were caused by mutations in certain genes, inhibiting or activating their expression. These mutant genes mainly included membrane protein-related genes. Mutations in these genes improved metabolite transport, enhancing SA production.

Whole-Cell Biocatalysis Using Glucose and Sucrose as Carbon Sources. Compared with fermentation, whole-cell biocatalysis is an attractive method because of its high efficiency and relatively simple downstream processing.[32] The whole-cell biocatalytic process consists of two stages: substrate growth and transformation. After culturing, the cells were collected, washed with buffer solution, and resuspended in a buffer for biocatalysis. Thus, whole-cell biocatalysis was used to produce SA. We first optimized the conditions for whole-cell biocatalysis using glucose as a sole carbon source. To examine the effects of cell concentration on SA production, whole-cell biocatalysis was conducted at OD_{600} values of 15, 30, 45, 60, 75, and 90. As shown in Figure 6A, the maximal SA production was obtained at an OD_{600} of 75. Mass transport is another important parameter of whole-cell biocatalysis that needs to be considered. Working volume affects mass transport. Thus, we also investigated the effect of working volume on SA production. It was found that the optimum working volume was 2.50 mL (Figure 6B). To increase SA productivity, we attempted to increase the initial glucose concentration. However, increasing the initial glucose concentration did not improve the production of SA (data not shown). Fed-batch fermentation is another approach for improving the productivity of a bioprocess. Thus, we investigated the effect of the fed-batch process. When the concentration of glucose was lower than 5 g/L, additional glucose was added until a concentration of 10 g/L. As shown in Figure 7A, *E. coli* SA-GS-2-7 produced 18.58 ± 0.56 g/L SA from glucose after 48 h with a yield of 68% (mol/mol). The productivity was 0.39 g/L/h. This yield is the highest value attained so far, higher than that (51%, mol/mol) reported by

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Table 3. Effect of CRISPR Repression of the Selected Mutant Genes on the Growth and Production of SA in *E. coli* SA30

gene	protein	ratio of growth[a]	ratio of SA concentration[b]
Metabolic pathway-related genes			
rhaD	rhamnulose-1-phosphate aldolase. This protein is involved in the subpathway that synthesizes glycerone phosphate from L-rhamnose.	0.85 ± 0.05	1.07 ± 0.04
Membrane protein transport-related genes			
fhuA	ferrichrome outer membrane transporter	1.07 ± 0.05	0.87 ± 0.02
nagE	*N*-acetyl glucosamine-specific PTS enzyme IIC, IIB, and IIA components; play a significant role in the recycling of peptidoglycan	0.89 ± 0.04	1.08 ± 0.04
gatC	PTS system galactitol-specific EIIC component; involved in galactitol transport	0.76 ± 0.04	1.05 ± 0.04
ybaL	putative cation/proton transporter YbaL	0.75 ± 0.03	1.26 ± 0.03
hlyE	hemolysin E	1.04 ± 0.05	1.05 ± 0.03
Genetic information-related genes			
pheV	tRNA	0.84 ± 0.04	1.05 ± 0.04
rrfD	5S ribosomal RNA	0.87 ± 0.05	1.25 ± 0.05
ygiC	ATP-Grasp family ATPase	0.96 ± 0.05	1.21 ± 0.04
hsdR	type-I restriction enzyme EcoKI R protein	0.81 ± 0.04	1.12 ± 0.04
Transcriptional factor			
fabR	HTH-type transcriptional repressor; represses the transcription of fabA and fabB; involved in unsaturated fatty acid (UFA) biosynthesis; by controlling UFA production, FabR directly influences the physical properties of the membrane bilayer.	0.96 ± 0.05	1.14 ± 0.04
ybaL-rrfD-ygiC		0.84 ± 0.05	1.34 ± 0.05

[a]The ratio of $OD_{600}$with and without CRISPRi. [b]The ratio of SA production with and without CRISPRi.

Table 4. Effect of CRISPR Activation of the Selected Mutant Genes on the Growth and Production of SA in *E. coli* SA30

gene	protein	ratio of growth[a]	ratio of SA concentration[b]
Membrane protein transport-related genes			
btuB	cobalamin outer membrane transporter	1.02 ± 0.04	1.07 ± 0.05
Stress response-related genes			
acnA	aconitate hydratase 1; catalyzes the reversible isomerization of citrate to isocitrate via cis-aconitate. AcnA is resistant to oxidation *in vivo*.	0.94 ± 0.05	1.05 ± 0.04
Genetic information-related genes			
lacI	lactose-inducible lac operon transcriptional repressor	1.11 ± 0.05	0.98 ± 0.04
intQ	putative defective protein IntQ	1.04 ± 0.05	1.11 ± 0.04
Amino acid and cofactor-related genes			
hycG	formate hydrogenlyase subunit HycG	1.26 ± 0.08	0.87 ± 0.03
selA	selenocysteine synthase	1.02 ± 0.05	1.08 ± 0.05

[a]The ratio of OD_{600} with and without CRISPRa. [b]The ratio of SA production with and without CRISPRa.

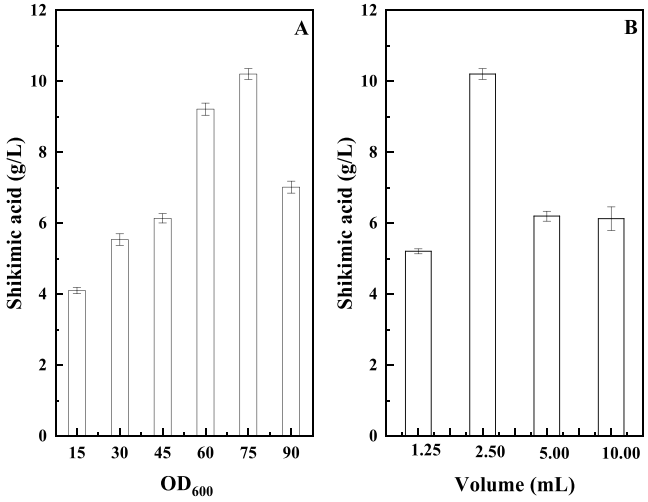

Figure 6. Shikimic acid production by whole-cell biocatalysis using glucose as a sole carbon source. (A) Effect of cell density. (B) Effect of working volume. The data represent the means of three replicates, and error bars indicate standard deviations.

Kogure et al.[8] They have reported the highest SA titer so far. The yield obtained in this study using glucose as a sole carbon source achieved 79% of the theoretical maximum (85.7%).

Lee et al.[33] constructed a sucrose-utilizing *E. coli* K-12 strain for L-threonine production by overexpressing *M. succiniciproducens sacC*. Thus, *sacC* was introduced into *E. coli* SA-GS-2-7 for SA production from sucrose. As shown in Figure 8, the maximal SA production was obtained at an OD_{600} of 30 and a working volume of 2.50 mL. We also investigated the effect of the fed-batch process. As shown in Figure 7B, *E. coli* SA-GS-2-7 (pBbB2k-sacC) produced 24.64 ± 0.32 g/L SA from sucrose after 60 h with a yield of 142% (mol/mol), achieving 83% of the theoretical maximum (171.4%). These results demonstrate that using sucrose resulted in higher yield than using glucose, indicating that sucrose is a more beneficial carbon source for SA production. It may be caused by the simultaneous hydrolysis and biocatalysis.

In this study, the novel breeding approach of biosensor-based ARTP mutagenesis and genome shuffling was developed. Application of this strategy resulted in a 2.7-fold increase in the production of SA and a 2.0-fold increase in growth from those of the starting strain. Through whole-cell resequencing of the shuffling strain and confirmation using CRISPRa/CRISPRi, certain mutant genes were identified as being closely involved with the higher SA titer. A sucrose-utilizing *E. coli* strain was obtained by introducing *M. succiniciproducens sacC*. The resulting strain produced 24.64 ± 0.32 g/L SA from sucrose with a yield of 1.42 mol/mol, achieving 83% of the theoretical maximum.

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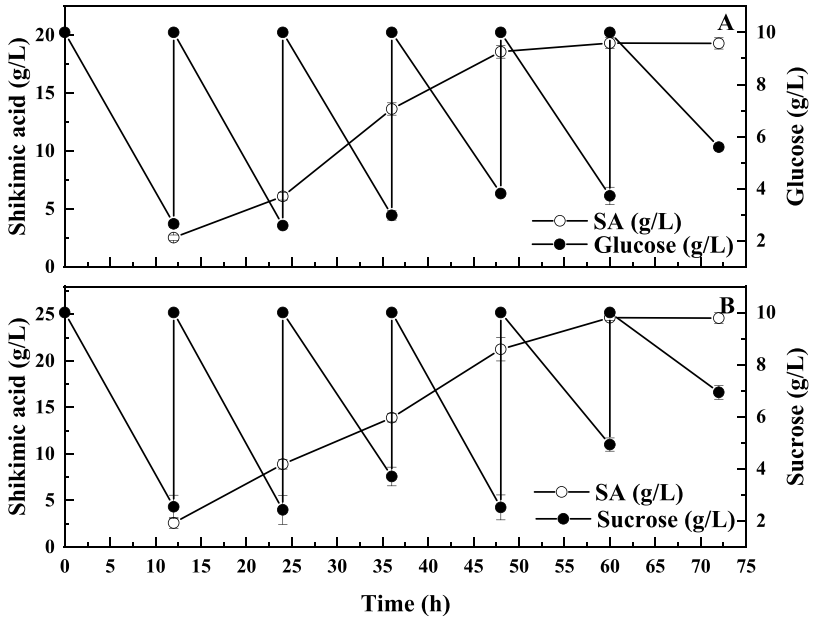

Figure 7. Shikimic acid production by fed-batch whole-cell biocatalysis. (A) Glucose as a sole carbon source. (B) Sucrose as a sole carbon source. The data represent the means of three replicates, and error bars indicate standard deviations.

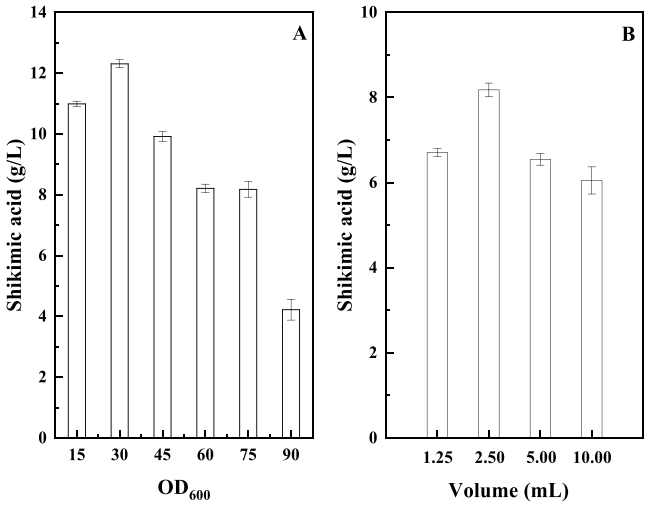

Figure 8. Shikimic acid production by whole-cell biocatalysis using sucrose as a sole carbon source. (A) Effect of cell density. (B) Effect of working volume. The data represent the means of three replicates, and error bars indicate standard deviations.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.0c05253.

Primers used in this study (Supplementary Table 1); mutations in *E. coli* SA-GS-2-7 (Supplementary Table 2); effect of CRISPRi of the mutant genes on growth and SA production in *E. coli* SA30 (Supplementary Table 3); effect of CRISPRa of the mutant genes on growth and SA production in *E. coli* SA30 (Supplementary Table 4); fluorescence strength of the ARTP mutant strains (Supplementary Figure 1); fluorescence strength of the genome shuffling strains (Supplementary Figure 2); SA production of the ep-WGS strains harboring pSenSA in the deep-well microplate culture (Supplementary Figure 3); standard curve for detecting shikimic acid with the periodic acid reagent method (Supplementary Figure 4); and genetical stability of *E. coli* SA-GS-2-7 (Supplementary Figure 5) (PDF)

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Notes

The authors declare no competing financial interest.

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