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BIOENERGY/BIOFUELS/BIOCHEMICALS

Enhanced succinic acid production by *Actinobacillus succinogenes* after genome shuffling

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Abstract Succinic acid is an important platform chemical for synthesis of C4 compounds. We applied genome shuffling to improve fermentative production of succinic acid by *A. succinogenes*. Using a screening strategy composed of selection in fermentation broth, cultured in 96-deep-well plates, and condensed HPLC screening, a starting population of 11 mutants producing a higher succinic acid concentration was selected and subjected to recursive protoplasts fusion. After three rounds of genome shuffling, strain F3-II-3-F was obtained, producing succinic acid at 1.99 g/l/h with a yield of 95.6 g/l. The genome shuffled strain had about a 73 % improvement in succinic acid production compared to the parent strain after 48 h in fed-batch fermentation. The genomic variability of F3-II-3-F was confirmed by amplified fragment-length polymorphism. The activity levels of key enzymes involved in end-product formation from glucose and metabolic flux distribution during succinic acid production were compared between *A. succinogenes* CGMCC 1593 and F3-II-3-F. Increased activity of glucokinase, fructose-1,6-bisphosphate aldolase, PEP carboxykinase and fumarase, as well as decreased activity of pyruvate kinase, pyruvate formate-lyase, and acetate kinase explained the enhanced succinic acid production and decreased acetic acid formation. Metabolic flux analysis suggested that increased flux to NADH was the main reason for increased activity of the C4 pathway resulting in increased yields of succinic acid. The present work will be propitious to the development of a bio-succinic acid fermentation industry.

Keywords Succinic acid · Genome shuffling · High-throughput screening

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Introduction

Increased oil prices, declining oil reserves, and environmental impacts of many petrochemical processes have propelled the search for alternative renewable chemicals that can be produced by microbial or enzymatic routes [19]. Succinate, currently produced petro-chemically to satisfy a specialty chemical market in food, agriculture, and pharmaceutical industries, is an optional starting material for producing bulk chemicals. As such, succinate could transform a multi-billion dollar petrochemical market into one based on renewable resources if succinate could be produced by fermentation at a price competitive with that of maleic anhydride, the current precursor of many bulk chemicals. Additionally, fermentative production of succinate uses CO_2 as a substrate. Therefore, both economic and environmental benefits impel the development of a bio-based succinate industry [15, 25, 35].

Several microbial fermentative processes for production of succinic acid have shown promise in recent years. The rumen organisms, *A. succinogenes* and *M. succiniciproducens,* have been a focus of research on succinate production. The mutant FZ53 derived from *A. succinogenes* 130 was reported to produce the highest concentration

of succinate at 105.8 g/l with a yield of 0.82 g/g glucose [4]. Besides natural succinate producers, *E. coli* or *C. glutamicum* utilize multiple pathways to form succinic acid. Some promising strains have been developed by engineering central metabolism. For example, *E. coli* AFP111/pTrc99A-pyc was reported to produce succinate fermentatively at a concentration of 99.2 g/l with an overall yield of 110 % using dual-phase fermentation [26]. *E. coli* HL27659 k (pKK313) produced 58.3 g/l of succinate in 59 h under aerobic conditions using fed-batch culture [9]. A recombinant *E. coli* SBS550MG produced succinate at about 40 g/l, and the yield from glucose was about 1.5 mol/mol with an average anaerobic productivity rate of 10 mM/h [24]. Nonrecombinant strains *E. coli* KJ060 and KJ122 produced 87 and 83 g/l of succinate, respectively, with molar yields of about 1.2 per mole of metabolized glucose [6, 7]. *C. glutamicum*△*ldhA*-pCRA717 produced succinate at 146 g/l with a yield of 0.92 g/g glucose at high cell density under oxygen deprivation with intermittent addition of sodium bicarbonate and glucose [21]. However large-scale production of succinic acid by these approaches is still likely to be too expensive. Requirements for auxotrophic complementation or the production of a large number of by-products impede the application, even though some approaches have nearly reached the maximal theoretical yield [1]. Given that fermentation-based bioprocesses rely extensively on strain improvement for commercialization [22], strain improvement is still very important to make industrial production of succinic acid economically viable.

A. succinogenes is one of most promising succinic acid producers examined to date. *A. succinogenes* CGMCC1593 has been used to produce succinic acid from cane molasses and corn stover [13, 32]. Using pH control and glucose fed-batch fermentation, succinic acid production by strain CGMCC1593 has achieved a concentration, productivity, and yield of 60.2 g/l, 1.3 g/l/h, and 0.75 g/g, respectively [14]. While classical strain improvement techniques have significantly increased succinic acid production by *A. succinogenes*, classical approaches are both laborious and time-consuming. Rational metabolic engineering is constrained, despite the availability of an *A. succinogenes* genome sequence [16] because of the lack of tools for genetic manipulation.

Genome shuffling offers an alternative for rapid production of improved organisms. Genome shuffling can produce genetic changes simultaneously at different positions throughout a genome and does not require information about the gene regulatory network [29]. This engineering approach has been proven effective in the rapid improvement of industrially important microbial phenotypes, such as improved acid tolerance of *Lactobacillus* [22], increased ethanol production by *Saccharomyces cerevisiae* [12], and improved 1,3-propanediol production by *Clostridium diolis* [20]. We combined genome shuffling with an effective, high-throughput screening method to obtain an *A. succinogenes* CGMCC1593 mutant with more efficient succinate production.

Materials and methods

Chemicals and gases

All the chemicals used were of reagent grade and were obtained from either Sinochem Shanghai (Shanghai, PRC) or Fluka Chemical Co. (Buchs, Switzerland). Enzymes and coenzymes were purchased from Sigma-Aldrich (Shanghai, Trading Co., Ltd). Gas was obtained from Xinnan Gases Co. (Wuxi, PRC).

Microbial strain and media

A. succinogenes CGMCC1593, isolated from bovine rumen and stored at China General Microbiological Culture Collection Center (CGMCC), was used as the parent strain [34]. A mutant library of CGMCC1593 was generated using nitrosoguanidine (NTG) as the mutagen [18] and treated with UV (15 W-UV lamp) irradiation at a distance of 30 cm for 30 s. Stain F3-II-3-F, the final mutant strain obtained from genome shuffling, was deposited at the China Center for Type Culture Collection (CCTCC) with the designation CCTCC M2012036.

All frozen stocks of strains and cultures were propagated on TSB medium. Selective plates were based on TSB medium containing either 60 g/l sodium succinate (S-TSB), or 12 g/l fluoroacetate (F-TSB), or diluted fermentation broth (DF-TSB) and 20 g/l agar. Regeneration medium (PM) was TSB agar medium supplemented with 2.0 g/l magnesium chloride and 103 g/l sucrose. Seed and fermentation medium were the same as described in [14]. All cultures were incubated at 37 °C with CO_2 as the gas phase.

High-throughput screening method

Mutants were spread on selective plates and incubated at 37 °C in a CO_2 atmosphere for 72 h. Colonies were inoculated into 96-deep-well plates containing 1 ml of seed medium. After 10-h incubation, 90 % of the culture in each well was removed, replaced with fresh fermentation medium, and the cultures were incubated in 100 % CO_2 environment for 72 h. A condensed HPLC screening (CAS) was used to identify superior succinic acid producers in the 96-deep-well plates. The procedure consisted of four steps. First, the average succinic acid concentration of each

column (i.e., each set of eight wells) of each 96-deep-well plate was measured. Second, the column whose average value was highest in every 96-deep-well plate was selected. Third, each well of the selected column was measured. Fourth, the well with the highest succinate concentration was picked for subsequent screening in anaerobic fermentation bottles.

Genome shuffling

The initial population for genome shuffling was composed of 11 strains with higher succinic acid production from the mutant library of CGMCC1593. Genome shuffling was carried out as described previously [22] with some modification. Ten milliliters of each of the 11 strains cultured in TSB medium were harvested during the logarithmic growth phase, centrifuged (8,000 rpm, 10 min), washed, and resuspended in protoplasting buffer (P buffer) [8]. Preparation of protoplasts was as described by Liu et al. [11], with an enzyme treatment of 10 mg/ml of lysozyme (>20,000 U/mg, Shanghai Sangon Biological Engineering Technology & Services Co., Ltd) in P buffer for about 30 min. The appearance of spherical cells as judged by light microscopy was used as an indicator of protoplast formation. The prepared protoplasts were mixed and divided into two parts, one of which was inactivated with UV irradiation for 5 min and the other inactivated by heating at 55 °C for 25 min. Each of the inactivated protoplast solutions of 0.5 ml were mixed, centrifuged (6,000 rpm, 10 min), re-suspended in 0.1 ml P buffer, mixed with 0.9 ml of 40 % PEG 6000, and incubated at 37 °C for 5 min for fusion to occur. The fused protoplasts were centrifuged and suspended in 1 ml of P buffer. Serial dilutions were anaerobically regenerated on PM at 37 °C for 3-5 days. The pooled regenerated colonies were copied to selective plates for high-throughput screening. Based on high-throughput screening, regenerated colonies with higher succinate yields were used in the subsequent round of genome shuffling. The pooled fusion libraries were designated sequentially as F1, F2, and F3.

Succinic acid production in anaerobic bottles and stirred bioreactors

Fermentation in 150-ml anaerobic bottles and fed-batch fermentation in a 5-l stirred fermenter (BioFlo 110, New Brunswick Scientific, Enfield, CT, USA) were carried out at 37 °C as described by Liu et al. [14]. The initial glucose concentration in the fermentation medium was 50 g/l. When fed-batch fermentation was performed, a concentrated solution containing 600 g/l glucose was fed into the stirred bioreactors to maintain the glucose concentration within 10 ~ 40 g/l. The pH was adjusted to pH 6.5 with using magnesium carbonate. The cultures were sparged with CO_2 gas at 0.10 vvm to maintain an anaerobic environment, and the agitation speed was maintained at 200 rpm. Samples were periodically drawn from the reactors for analysis.

Metabolic flux analysis of succinic acid production

The metabolic network for succinic acid production comprises glycolysis, the pentose phosphate pathway (PPP), the C4 pathway defined as PEP → OAA → -Mal → Fum → Suc, and the C3 pathway defined as PEP → Pyr → AcCoA → Ace + EtOH (Fig. 5). Metabolic flux analysis was based on the pseudo steady-state assumption and calculated by MATLAB7.0 software, in which the biochemical reactions and considered metabolites form a set of linear equations and can be expressed in a matrix [27]. The metabolic flux distribution was represented as the volumetric rates (mmol/l/h) of intracellular metabolite formation at the stationary period in 20 h (no consideration of cell growth). The flux of glucose and carbon dioxide uptake during the production phase were set to 100 and 200 % respectively, and other fluxes in the network were expressed as the relative molar flux normalized to the flux of glucose.

Fermentation broth assays

The fermentation samples were diluted tenfold with 0.2 M HCl, and the optical density was monitored by measuring the absorbance at 660 nm (OD660) using a spectrophotometer (U-3000, Hitachi, Japan). Dry cell weight (DCW) was measured by drying the harvested cells (washed three times with distilled water) to a constant weight at 105 °C, an OD660 of 1.0 was equivalent to 505 ± 18 mg/DCW/l. The concentrations of glucose, succinic acid, acetic acid, formic acid, and lactic acid in the diluent were quantified using an HPLC system (Waters, Milford, MA, USA) equipped with a cation-exclusion column (Aminex HPX-87H; 300 × 7.8 mm, 9 μm; Bio-Rad Chemical Division, Richmond, CA) and a refractive index detector (Waters). The mobile phase was 10 mM H_2SO_4 solution at a flow rate of 0.5 ml/min, and the column was operated at 55 °C as described previously [14].

Enzyme assays

Enzyme activities were measured spectrophotometrically in a temperature-controlled spectrophotometer (PowerWave XS2, Gene Company Limited) as described in [28]. The following enzymes were assayed: glucokinase (EC2.7.1.1), fructose-1,6-bisphosphate aldolase (EC 4.1.2.13), phosphogluconate dehydratase (EC1.2.1.12),

enolase (EC4.2.1.11), phosphoenolpyruvate (PEP) carboxykinase (EC 4.1.1.49), L-malate dehydrogenase (EC 1.1.1.37), fumarase (EC 4.2.1.2), fumarate reductase (EC 4.2.1.2), pyruvate kinase (EC 2.7.1.40), pyruvate formate-lyase (EC 2.3.1.54), phosphotransacetylase (EC 2.3.1.8), acetate kinase (EC 2.7.2.1), alcohol dehydrogenase (EC 1.1.1.1). All the assays were performed at 37 °C using cell extracts prepared from cells grown in seed cultures. Reactions were started by the addition of the substrate, and the rates were monitored by the production or extinction of NAD(P)H at 340 nm, or by the formation of PEP or fumarate at 240 nm, where their extinction coefficients were 6.23, 1.50, and 2.53 cm^{-1} mM^{-1}, respectively. One unit (U) of enzyme activity was defined as the amount of enzyme necessary to catalyze the conversion of 1 μmol of substrate per min into specific products. Protein contents were determined using the modified Bradford method.

Amplified fragment length polymorphism

The method of amplified fragment length polymorphism (AFLP) was performed as described by Zheng et al. [33]. Genomic DNA samples of three strains (CGMCC 1593, IV-10-C, and F3-II-3-F) were obtained using a Genomic DNA Extraction Kit (Generay Biotech Co., Ltd., Shanghai, China), and were restricted with a rare-cutting endonuclease *Apa*I and a frequent-cutting endonuclease *Taq*I. An *Apa*I-adapter and a *Taq* I-adapter were ligated to the restriction sites of the digested DNA by addition of T4 DNA ligase. The linkage products were used to perform pre-amplification reactions by the addition of *Taq* polymerase, *Apa*I, and *Taq*I pre-amplification primers. The pre-amplification products were used as templates for selective amplification. Each selective primer contained one selective nucleotide, resulting in 16 unique primer pairs for each restriction enzyme combination (Table 1). The selective amplification reactions were performed by addition of *Taq* polymerase, and *Apa*I and *Taq*I selective primers using a touch down (TD) PCR procedure. The amplified fragments were resolved by electrophoresis on denaturing polyacrylamide gels and stained in $AgNO_3$ after electrophoresis.

Table 1 Sequence of adaptor and primer used in AFLP

Oligonucleotide	Sequence (5′-3′)
Adaptor	
*Apa*I	TCGTAGACTGCGTACAGGCC
	TGTACGCAGTCTAC
*Taq*I	GACGATGAGTCCTGAC
	CGGTCAGGACTCAT
Per-amplification primer	
*Apa*I	GACTGCGTACAGGCCC
*Taq*I	GATGAGTCCTGACCGA
Selective amplification primers	
*Apa*I-A (T, C, G)	GACTGCGTACAGGCCCA(T, C, G)
*Taq*I-A (T, C, G)	GATGAGTCCTGACCGA A(T, C, G)

Results

High-throughput screening for overproducing mutants

A. succinogenes CGMCC1593 was mutagenized by exposure to NTG and ultraviolet irradiation to obtain diverse strains with superior succinic acid yields as the starting population for genome shuffling. Following mutagenesis, nearly 1,056 colonies were isolated and cultured in twelve 96-deep-well plates. The average succinic acid concentration of each of the 144 columns (i.e., each set of eight wells) among the 96-deep-well plates varied up and down to the control, representing a distribution that included succinate yields lower and higher than the control (Fig. 1a). Subsequent measurements of each well in the "best" column of each 96-deep-well plate (i.e., the column with the highest succinate yield) suggested the rate of positive mutants was about 75 % in the total detected samples (Fig. 1b). Of these, 11 strains (III-9-H, IV-7-A, IV-7-C, V-12-B, VI-10-C, VII-11-H, VIII-10-H, IX-2-C, X-8-E, XI-8-B, and XII-7-B) with superior succinic acid concentrations were selected for further testing in anaerobic fermentation bottles.

The average succinic acid concentration produced by the eleven mutants was 29.9 g/l (Table 2), representing a 28 % increase in concentration compared with the parental strain, CGMCC1593. Production of succinic acid appeared slightly higher for mutants grown in 96-well plates with diluted fermentation broth (DF-TSB) instead of sodium succinate (S-TSB) and fluoroacetate (F-TSB) media. Therefore, DF-TSB was used to screen for superior succinate producers from recursive protoplast fusion experiments.

Genome shuffling for improvement of succinic acid production

Above 11 mutants were used as the initial genetic diversity in the first round of genome shuffling. A total of 360 colonies obtained from the genome shuffling procedure were subjected to high-throughput screening, and four strains labeled F1-I-3-F, F1-II-7-B, F1-III-2-E, and F1-IV-9-D were identified with high succinate yields in plates containing diluted fermentation broth (derived from NTG mutant strain VI-10-C). These four F1 strains were subjected to a second round of genome shuffling. Another four

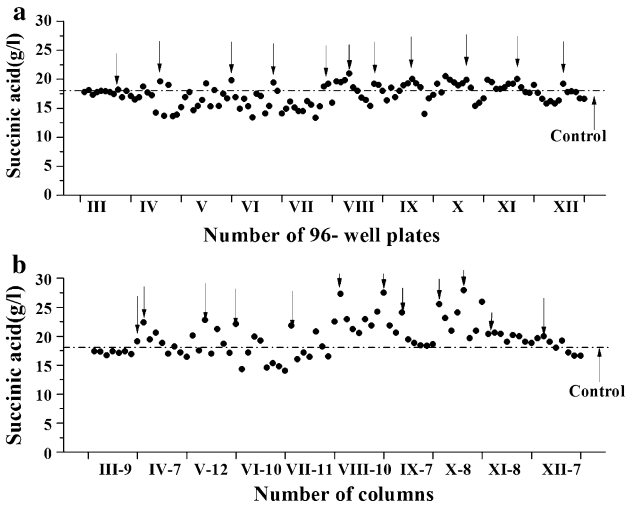

Fig. 1 Condensed HPLC screening. **a** Distribution of the average succinic acid concentration of each column in various 96-well culture plates. **b** Distribution of the succinic acid concentration of each well in the column whose average value was highest in each 96-cell culture plate. *Arrows* represent the high-yielding group or producing strains

strains (F2-5-B, F2-II-5-H, F2-III-6-D, and F2-III-10-F) capable of higher succinic acid production than the F1 strains were chosen from a population of 300 colonies grown on DF-TSB (the diluted fermentation broth from F1-IV-9-D plates) and used as F2 strains. After the third round of fusion within the protoplasts of the F2 strains, three strains (F3-I-9-C, F3-II-3-F, and F3-II-10-D) were isolated from the F3 population of 200 colonies grown on DF-TSB. Succinic acid production by the shuffled strains showed iterative improvements (Fig. 2). The average concentration of succinate was about 5 % higher with the F1 strains than the NTG mutants, about 10 % higher with the F2 strains than the F1, and about 9 % higher with the F3 than the F2. The highest producers in each round of screening were VI-10-C, F1-IV-9-D, F2-III-6-D, and F3-II-3-F. These strains produced succinate at 31.5, 32.8, 35.5, and 38.9 g/l, respectively. The shuffled strain F3-II-3-F had a 1.6-fold increase in succinate production compared with the parental strain CGMCC1593.

To test the performance of the above four in fermentative production of succinate, fed-batch fermentations were conducted in a 5-l bioreactor (Fig. 3; Table 3). After fermentation for 48 h, a clear progression in the concentration of succinate was evident among bioreactor samples from the parental strain (CGMCC 1593), the NTG mutant strain (VI-10-C), and the genome-shuffled strains (F1-IV-9-D, F2-III-6-D, and F3-II-3-F) with values of 55.2, 77.9, and 95.6 g/l, respectively. The residual glucose was reduced to less than 10 g/l. Compared to the parent strain, the maximum biomass concentration of the shuffled strains increased over 1.3 times. Although the yield of the shuffled strains relative to the parent increased only slightly (from 0.71 to 0.79 g/g), the productivity increased more than 1.4 times. The productivity of the F3 shuffled strain F3-II-3-F (CCTCC M2012036) reached 1.99 g/l/h, more than 1.5 times that of the parent strain. The mass ratio of succinic acid to acetic acid (S/A) sequentially increased among strains VI-10-C, F1-IV-9-D, F2-III-6-D, and F3-II-3-F representing the iterations of mutagenesis (Table 3), and the values progressed from 9.3 to 10.7, to 11.8, to 14.3, and to 15.4. The specific rates of glucose consumption and of succinic acid production (at 4 h) by the shuffled strains were also improved at a certain extent relative to the parent strain.

Comparative AFLP fingerprints, enzyme activities, and metabolic flux

A sensitive and stable genome-wide fingerprinting technique, AFLP, was applied to explore the genomic variability between the parent strain CGMCC 1593, the NTG mutant VI-10-C, and the genome-shuffled strain F3-II-3-F. Among the AFLP banding patterns produced with 16 different primer pairs, numerous AFLP bands were observed that were polymorphic among the strains (Fig. 4). The polymorphisms confirmed that genome changes had occurred in the mutant VI-10-C and the shuffled F3-II-3-F strains.

The activity levels of enzymes involved in succinic acid formation were determined in cell crude extracts from F3-II-3-F and CGMCC 1593 to provide insight into the superior succinate yield from F3-II-3-F. As shown in Table 4, the levels of glucokinase, fructose-1, 6-bisphosphate aldolase, PEP carboxykinase, and fumarase were increased more than 20 % in strain F3-II-3-F. PEP carboxykinase activity was 67 % higher in strain F3-II-3-F, but key enzymes of the C3 pathway (the formate-, acetate-, and ethanol-producing pathway), such as pyruvate kinase, pyruvate formate-lyase, and acetate kinase, were reduced about 30 % in strain F3-II-3-F relative to strain CGMCC 1593. These data suggested the genome-shuffled strain, F3-II-3-F, had an increased capacity to form succinic acid through CO_2 fixation with less formation of acetic acid through the C3 pathway, resulting in greater production of succinic acid.

The intracellular metabolic flux distribution in the steady-state growth period during fermentation was examined using a mass balance based on a previous metabolic flux analysis [27]. Compared to the parent strain CGMCC 1593, the metabolic flux to PPP, NADH, and succinic acid in the shuffled strain F3-II-3-F was remarkably enhanced. These fluxes were increased about 77, 70, and 31 %, respectively. In contrast, the flux to acetic acid and formic acid in F3-II-3-F was decreased about 67 and 45 %, respectively (Fig. 5). The major role of the PPP

Table 2 Succinic acid production in anaerobic bottles of mutants from different selective plates

Selective plate	Strain	Succinic acid (g/l)	Acetic acid (g/l)	Formic acid (g/l)
DF-TSB	VI-10-C	31.47 ± 0.86	5.03 ± 0.17	1.96 ± 0.22
	VII-11-H	30.58 ± 0.43	5.20 ± 0.07	1.91 ± 0.09
	XI-8-B	30.99 ± 0.52	5.12 ± 0.09	1.90 ± 0.11
	XII-7-B	29.66 ± 0.59	4.90 ± 0.10	1.62 ± 0.12
S-TSB	III-9-H	29.76 ± 0.79	5.17 ± 0.13	1.96 ± 0.17
	IV-7-A	30.78 ± 1.06	5.28 ± 0.23	2.10 ± 0.28
	IV-7-C	30.27 ± 1.10	5.25 ± 0.18	2.06 ± 0.23
	V-12-B	29.31 ± 0.28	5.09 ± 0.05	1.86 ± 0.06
F-TSB	X-8-E	29.73 ± 1.27	4.60 ± 0.10	1.95 ± 0.26
	VIII-10-H	28.47 ± 0.49	4.86 ± 0.21	1.69 ± 0.10
	IX-2-C	29.16 ± 0.33	4.82 ± 0.09	1.83 ± 0.07
Control	CGMCC1593	23.35 ± 1.02	6.15 ± 0.17	1.72 ± 0.34

Standard deviations were calculated from the results of three independent experiments

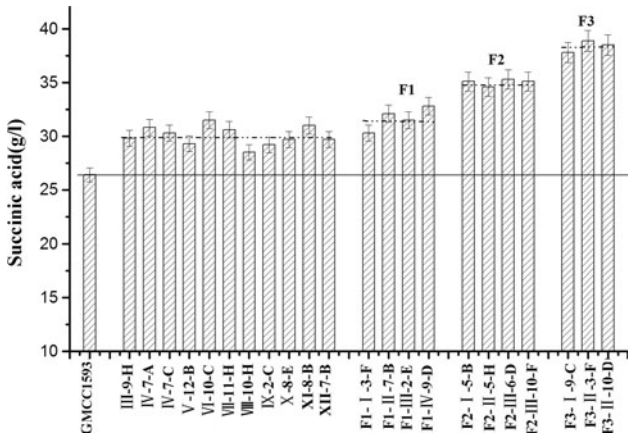

Fig. 2 Comparison of succinic acid production by the parental CGMCC 1593 strain, the NTG mutant strains, and the shuffled strains in anaerobic bottles

pathway is to regenerate NADPH. Increased NADH regeneration in F3-II-3-F would enhance the activity of the C4 pathway in an environment rich in carbon dioxide. Although the metabolic flux to PEP in the two strains was not dramatically different, the reduced flux to the C3 pathway in F3-II-3-F increased the flux to the C4 pathway. These data reinforce the notion that increased succinic acid production by F3-II-3-F was mainly due to increased activity of the C4 pathway, enabled by an increase flux to NADH.

Discussion

Genome shuffling is a relatively new tool to enable improvements in the microbial production of biochemicals through complex metabolic pathways. The success of genome shuffling depends on the initial selection of variants, the efficiency of the genetic recombination process, and the power of the selection method [3]. In the present study, we aimed to determine if genome shuffling could improve succinic acid production by *A. succinogenes*. This required development of an efficient screening method.

In generating novel succinic acid-producing strains, the succinic acid concentration must be evaluated by HPLC. This involves many time-consuming and costly steps. A sample usually requires about 20–30 min. Genome shuffling begin with genetic diversity generated using classical mutagenesis techniques such as NTG, UV, and/or chemostat enrichments, followed by recursive protoplast fusion of mutant populations. These two steps generate a huge number of mutants that are difficult to evaluate in screens in the absence of selections [23]. We demonstrated a novel selective screening strategy, wherein mutant colonies grown on diluted fermentation broth were picked, cultured in 96-deep-well plates, and superior succinic acid producers were identified by a condensed HPLC screening approach. Instead of performing HPLC analysis of all 96 wells in each 96-well plate, our approach condensed the analysis to 20 assays per plate (12 mean values of every column plus eight samples in the highest-value column). This approach increased the efficiency by approximately 80 %. The rate of positives (i.e., detection of strains with higher succinate production) in the second round of HPLC assays reached 75 %, allowing us to rapidly obtain strains with desirable properties.

We assessed three media for selection of mutants and found that diluted fermentation medium was best. Substrate and product inhibition can reduce the growth of *A. succinogenes* [2, 10], For example, strains resistant to fluoroacetate were reported to produce more succinic acid and less formic and acetic acid [5]. We found that mutants selected on DF-TSB generally produced more succinic acid

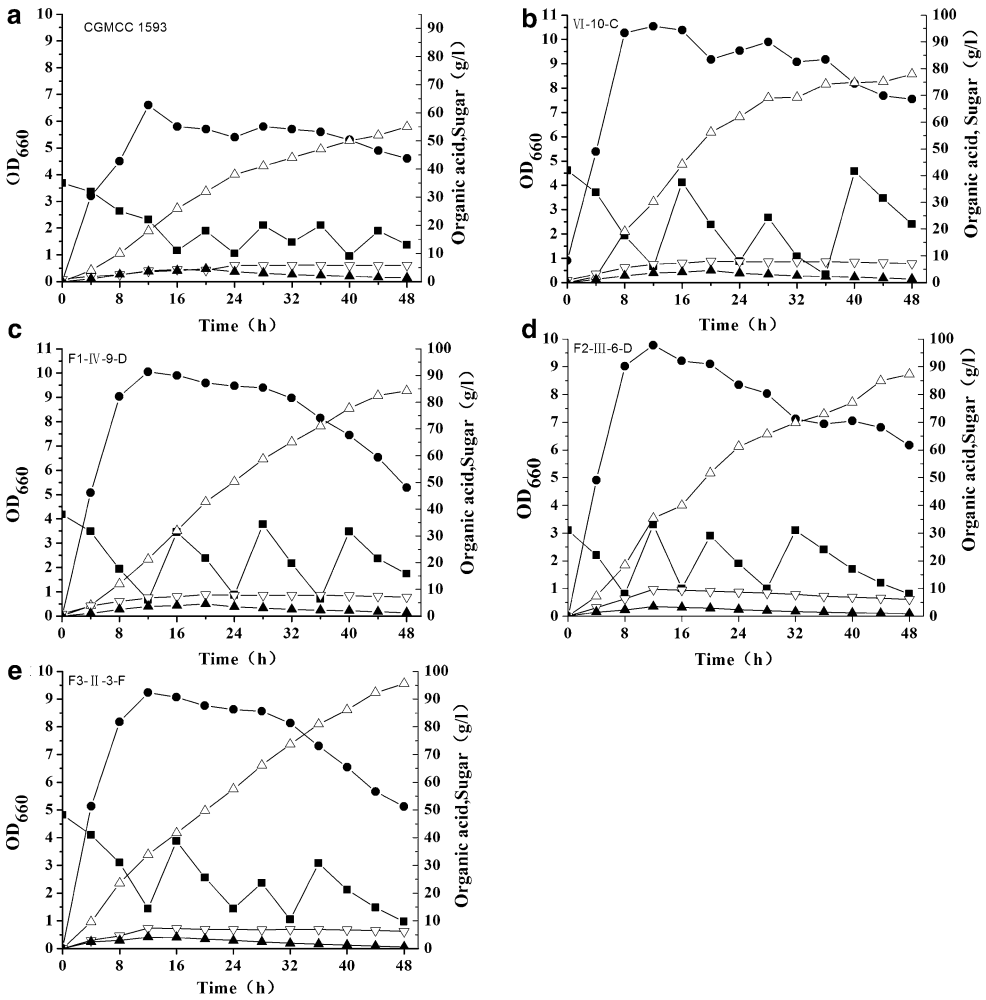

Fig. 3 Time courses of cell growth and production of organic acids in fed-batch fermentation in 5–l stirred bioreactors. **a** Strain CGMCC 1593, **b** strain VI-10-C, **c** strain F1-IV-9-D, **d** strain F2-III-6-D, and **e** strain F3-II-3-F. OD660 (*filled circle*), glucose (*filled square*), succinic acid (*triangle*), acetic acid (*inverted triangle*), formic acid (*filled triangle*)

Table 3 Characterization of succinic acid production of the shuffled and initial strains

	CGMCC 1593	VI-10-C	F1-IV-9-D	F2-III-6-D	F3-II-3-F
Total glucose (g/l)	90.2	122.5	121.8	118.7	130.8
Maximum optical density (OD660)	6.66	10.45	9.95	9.78	9.23
Succinic acid titer (g/l)	55.2	77.9	84.2	87.3	95.6
Residual glucose (g/l)	13.1	15.8	15.8	8.0	9.7
Acetic acid (g/l)	5.8	7.1	7.1	6.1	6.2
Yield g/g (the consumed)	0.71	0.77	0.79	0.79	0.79
Productivity (g/l/h)	1.25	1.62	1.75	1.82	1.99
S/A mass ratio (g/g)	9.38	10.97	11.86	14.31	15.42
Specific growth rate (/h) (4 h)	0.176	0.218	0.222	0.230	0.199
Specific glucose consumed rate (g/g DCW/h) (4 h)	2.34	3.39	3.01	3.51	2.51
Specific succinic acid production rate (g/g DCW/h) (4 h)	2.32	2.62	1.54	2.78	3.41

than mutants selected on S-TSB and FD-TSB media (Table 2). Strain VI-10-C selected from DF-TSB produced 77.9 g/l succinic acid in a 48-h fed-batch fermentation in a 5-l bioreactor (Fig. 3b), and the biomass concentration of VI-10-C was almost 1.4 times higher than that of the parent strain. The improvement in succinic acid production by this strain was due to its higher biomass to a great extent. Growth on DF-TSB resulted in strains with better adaptation (e.g., resistance to metabolites such as succinic, acetic, and formic acids) to the fermentation environment.

Genome shuffling enables the integration of different adaptations that occur in multiple parents. After three

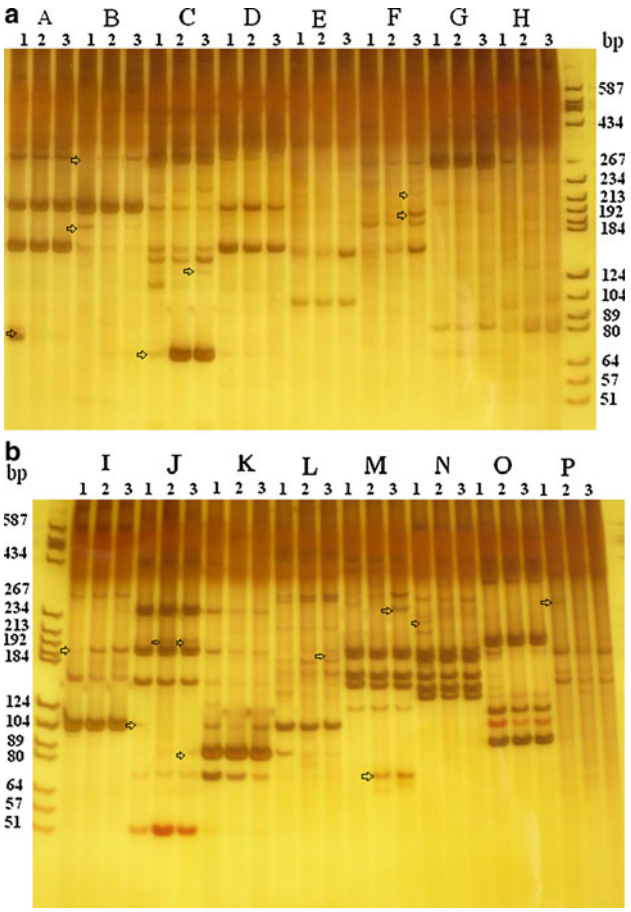

Fig. 4 AFLP banding patterns. The DNA marker was pBR322/BsuRI. *Letters A to P* represent different AFLPs from different primer sets as follows: A, Apa I-A/Taq I-A; B, Apa I-A/Taq I-T; C, Apa I-A/Taq I–C; D, Apa I-A/Taq I-G; E, Apa I-T/Taq I-A; F, Apa I-T/Taq I-T; G, Apa I-T/Taq I–C; H, Apa I-T/Taq I-G; I, Apa I–C/Taq I-A; J, Apa I–C/Taq I-T; K, Apa I–C/Taq I–C; L, Apa I–C/Taq I-G; M, Apa I-G/Taq I-A; N, Apa I-G/Taq I-T; O, Apa I-G/Taq I–C; P, Apa I-G/Taq I-G. *Numbers 1 to 3* above the lanes represent different strains as follows: *1* strain CGMCC 1593; *2* strain VI-10-C; *3* strain F3-II-3-F

rounds of genome shuffling, we obtained strain F3-II-3-F, which could produce 95.6 g/l succinic acid—a 73 % improvement in yield compared to the parent strain. The productivity of strain F3-II-3-F was enhanced to 1.99 g/L/h, which is the highest level in *A. succinogenes* batch or fed-batch fermentation to the best of our knowledge. Strain F3-II-3-F had a higher specific succinic acid production rate and a higher specific growth rate than the parent strain. It was also superior to the mutant VI-10-C in succinic acid production (Table 2), suggesting that several traits related to succinate overproduction were improved in F3-II-3-F. The presence of multiple genetic changes in strain F3-II-3-F relative to the parent strain and the NTG mutant VI-10-C was confirmed by AFLP (Fig. 4).

Few studies have explored the mechanisms by which strains have been improved through genome shuffling. Zhang et al. carried out a comparative proteome analysis of

Table 4 Comparison of key enzyme activity in glucose metabolic of F3-II-3-F and CGMCC 1593

Enzyme	Specific activity (U/mg)	
	F3-II-3-F	CGMCC 1593
Glucokinase	105 ± 5.3	87 ± 4.9
Fructose-1, 6-bisphosphate aldolase	722 ± 23	560 ± 25
Phosphogluconate dehydratase	408 ± 20	401 ± 21
Enolase	2,735 ± 97	2,594 ± 92
PEP carboxykinase	1,856 ± 87	1,108 ± 42
L-Malate dehydrogenase	926 ± 36	836 ± 41
Fumarase	3,501 ± 102	2,867 ± 99
Fumarte reductase	522 ± 26	506 ± 25
Pyruvate kinase	403 ± 20	537 ± 26
Pyruvate formate-lyase	251 ± 12	325 ± 16
Phosphotransacetylase	320 ± 16	338 ± 17
Acetate kinase	1,218 ± 59	1,600 ± 66
Alcohol dehydrogenase	<5	10 ± 1.5

Each value is the mean of two parallel replicates and is represented as mean ± standard deviation

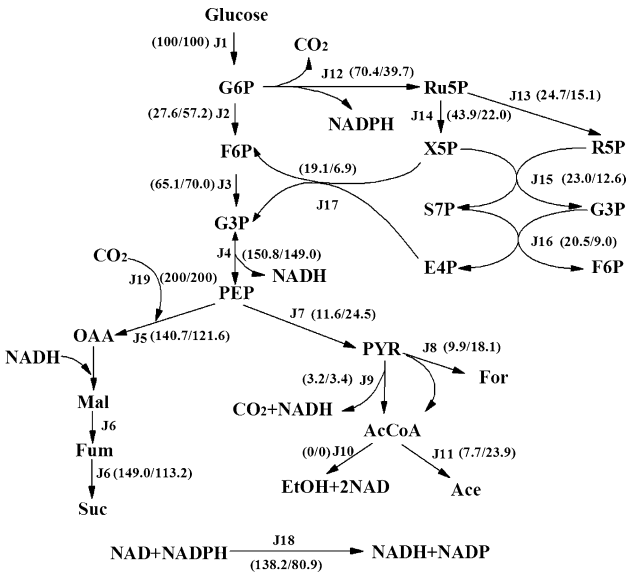

Fig. 5 Simplified map of succinate metabolism in *A. succinogenes*. Values from *left* to *right* were from *A. succinogenes* F3-II-3-F and CGMCC 1593, respectively

an improved vitamin B12 producing strain of *Propionibacterium shermanii* [30]. Among 38 protein spots in 2-DE electrophoresis that consistently exhibited significant changes in abundance, six were potentially key enzymes involved in vitamin B12 biosynthesis [30]. Zhao et al. conducted comparative analysis of synthetase gene expression between the initial and shuffled strains of *B. amyloliquefaciens* and showed that changes in mRNA transcriptional levels explained the enhanced flux to lipopeptide production in the shuffled strain [31]. Unlike

E. coli, which uses multiple pathways to form succinic acid, *A. succinogenes* exclusively forms succinate via the PEP carboxykinase pathway [28]. Therefore, we compared the levels of critical enzymes in glucose metabolism and the metabolic flux distributions between the shuffled strain F3-II-3-F and the parent strain. Some distinct differences were apparent (Table 4; Fig. 5). The increased activity of glucokinase and fructose-1, 6-bisphosphate aldolase in F3-II-3-F explained the rapid growth, and high activities of PEP carboxykinase and fumarase as well as the enhanced metabolic flux to NADH increased succinic acid formation through the C4 pathway. In parallel, lower activity of other key enzymes and reduced metabolic fluxes in the C3 pathway decreased the formation of acetic acid during fermentation. These observations demonstrated that the improvement productivity of F3-II-3-F was linked to a combination of factors.

Guettler et al. first used *A. succinogenes* as a succinic-acid-producing strain [5]. They reported the production of succinate and acetate in a ratio of about 4:1 in glucose-containing complete medium. They also described a fluoroacetate-resistant variant (FZ53) that produced some of the highest succinate concentrations ever reported and simultaneously produced some formate and acetate (the ratio of S/A was about 6 ~ 9:1). Genetic engineering of *A. succinogenes* to produce succinate as the sole fermentation end product has been a goal [16, 17]. Our genome shuffled strain, F3-II-3-F, had an S/A ratio of 15:1, which had increased from 9:1 as a result of three rounds of genome shuffling. This indicates that recursive genome shuffling and selection not only increased succinate concentrations but also reduced acetic acid formation.

In summary, we demonstrated the first use of genome shuffling as a means of improving succinate production by *A. succinogenes*. Our novel screening approach was an essential feature in success of the effort. After three rounds of genome shuffling from a staring population of 11 mutants, significant improvements in succinate production were observed. The shuffled strain F3-II-3-F produced succinic acid at 95.6 g/l with a productivity of 1.99 g/l/h in fed-batch fermentation. These results will be helpful in the development of strains for industrial production of succinic acid.

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