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Succinic acid production from corn stalk hydrolysate in an *E. coli* mutant generated by atmospheric and room-temperature plasmas and metabolic evolution strategies

Min Jiang · Qing Wan · Rongming Liu · Liya Liang · Xu Chen · Mingke Wu · Hanwen Zhang · Kequan Chen · Jiangfeng Ma · Ping Wei · Pingkai Ouyang

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Abstract AFP111 is a spontaneous mutant of *Escherichia coli* with mutations in the glucose-specific phosphotransferase system, pyruvate formate lyase system, and fermentative lactate dehydrogenase system, created to reduce byproduct formation and increase succinic acid accumulation. In AFP111, conversion of xylose to succinic acid only generates 1.67 ATP per xylose, but requires 2.67 ATP for xylose metabolism. Therefore, the ATP produced is not adequate to accomplish the conversion of xylose to succinic acid in chemically defined medium. An *E. coli* mutant was obtained by atmospheric and room-temperature plasmas and metabolic evolution strategies, which had the ability to use xylose and improve the capacity of cell growth. The concentration of ATP in the mutant was 1.33-fold higher than that in AFP111 during xylose fermentation. In addition, under anaerobic fermentation with almost 80 % xylose from corn stalk hydrolysate, a succinic acid concentration of 21.1 g l^{-1} was obtained, with a corresponding yield of 76 %.

Keywords Xylose · Atmospheric and room-temperature plasmas · Metabolic evolution · Corn stalk hydrolysate · ATP · Succinic acid

M. Jiang (✉) · Q. Wan · R. Liu · L. Liang · X. Chen · M. Wu · H. Zhang · K. Chen · J. Ma · P. Wei · P. Ouyang
State Key Laboratory of Materials-Oriented Chemical Engineering, College of Biotechnology and Pharmaceutical Engineering, Nanjing University of Technology, Nanjing 211816, People's Republic of China
e-mail: bioengine@njut.edu.cn

Introduction

Succinic acid, a C4 dicarboxylic acid, is used as the precursor of many important chemicals in the food, agricultural, and pharmaceutical industries [45]. Currently, bio-based succinic acid production has the additional environmental benefit of using CO_2, a greenhouse gas, as a substrate. However, bio-based succinic acid production is not yet competitive with petrochemical-based production, mainly due to high production costs [33, 38]. Renewable lignocellulosic biomass, such as agricultural and forestry residues, waste paper, and industrial waste, is an attractive feedstock for succinic acid production. The low cost and high carbohydrate content of these renewable cellulosic materials make them attractive potential substrates for bioconversion to value-added platform chemicals [19].

Corn stalk, a typical cellulosic material, is a major byproduct of the corn milling process, and contains 60–70 % carbohydrates, of which 30 % is hemicellulose. After the pretreatment of corn stalk, glucose and xylose are the major constituents of the hydrolysates, and microorganisms should be required to efficiently ferment these sugars for the successful industrial production of succinic acid. Most studies of succinic acid fermentation by engineered *E. coli* used glucose as the feedstock [2, 27]. Nevertheless, further research is required to accomplish efficient conversion of xylose, which is the main component of corn stalk hydrolysate, to succinic acid.

Genetic manipulations in *E. coli* can increase succinic acid production and reduce byproducts formation. AFP111 is a spontaneous mutant of *E. coli* W1485 with mutations in the glucose-specific phosphotransferase system (*ptsG*), pyruvate formate lyase system (*pflB*), and fermentative lactate dehydrogenase system (*ldhA*) [6], while AFP184

is a metabolically engineered strain obtained by introducing these same three mutations into *E. coli* C600 (ATCC 23724) [8]. In AFP111, xylose is transported by the periplasmic protein that represents a high-affinity system and is driven by ATP [1, 12]. The periplasmic protein consists of two ATP-binding cassette (ABC) domains and two hydrophobic transmembrane domains [28]. Through periplasmic protein, transport systems that were catalyzed by the hydrolysis of ATP concomitantly translocated the substrate [9]. Conversion of xylose to succinic acid in this mutant generates 1.67 ATP per xylose, but requires 2.67 ATP for xylose metabolism [1, 30]. Thus, AFP111 cannot utilize xylose for biomass generation and succinic acid production due to insufficient ATP supply. Conversely, in AFP184, the transport of xylose across the cell membrane is linked to the movement of protons, which needs less ATP to convert xylose to succinic acid [15, 20], compared to AFP111, and has been shown in earlier work to achieve high xylose utilization under anaerobic conditions, with the overexpression of the ATP-forming phosphoenolpyruvate (PEP) carboxykinase (PCK), a PEP carboxylase (*ppc*)-minus *E. coli* strain [29].

The atmospheric and room-temperature plasmas (ARTP) mutation system is becoming increasingly popular in various fields owing to the convenient operation processes, low capital costs, for utilization in plasma-based sterilization and industrial microbe gene mutation breeding [21, 36, 37]. The ARTP can generate diverse breakages of plasmid DNA and oligonucleotides with variations in plasma dosage [26, 40], and has been successfully applied to affect the heterotrophic pathway of *E. coli* and increase the utilization of several substrates [21]. It has also been reported that a hydrogen-producing *Enterobacter aerogenes* strain was obtained by the ARTP mutation system, in which the ATP yield was higher than that of the wild-type strain [31]. These findings suggest that ARTP may be used as a rapid mutation tool to improve ATP generation, and thus succinic acid production in AFP111.

Previous results where the mutants generated by this approach have improved succinic acid profiles have also significantly reduced growth characteristics have been noted under anaerobic conditions [31]. The developments of practical methods for the metabolic evolution (ME) pathways have been proven highly effective in the optimization of production strains. ME has the advantage of letting nonintuitive beneficial mutations occur in many different genes and regulatory regions in parallel [7]. It allows for the metabolic engineering of microorganisms by selecting beneficial mutations [7, 32]. In addition, it can increase the rate of utilization of substrates such as sugars [10, 42], as well as improve the growth rate and product titers [11]. Therefore, ME can be used as a tool to optimize the faster-growing production strains.

Here, we investigated mutation of AFP111 using ARTP, combined with ME, and examined xylose consumption, succinic acid production, and ATP of a typical *E. coli* mutant. Furthermore, anaerobic fermentation of corn stalk hydrolysate for succinic acid production was also performed.

Materials and methods

Bacterial strains and media

E. coli strains used in this study are shown in Table 1. The Luria–Bertani (LB) medium contained: 10 g l^{-1} tryptone (Oxoid, UK), 5 g l^{-1} yeast extract (Oxoid, UK), and 5 g l^{-1} NaCl. The chemically defined (CD) medium contained: citric acid 3 g l^{-1}, $Na_2HPO_4{\cdot}12H_2O$ 4 g l^{-1}, $KH_2PO_4{\cdot}8$ g l^{-1}, $(NH_4)_2HPO_4{\cdot}8$ g l^{-1}, NH_4Cl 0.2 g l^{-1}, $(NH_4)_2SO_4$ 0.75 g l^{-1}, $MgSO_4{\cdot}7H_2O$ 1 g l^{-1}, $CaCl_2{\cdot}2H_2O$ 10.0 mg l^{-1}, $ZnSO_4{\cdot}7H_2O$ 0.5 g l^{-1}, $CuCl_2{\cdot}2H_2O$ 0.25 mg l^{-1}, $MnSO_4{\cdot}H_2O$ 2.5 mg l^{-1}, $CoCl_2{\cdot}6H_2O$ 1.75 mg l^{-1}, H_3BO_3 0.12 mg l^{-1}, $Al_2(SO_4)_3$ 1.77 mg l^{-1}, $Na_2MoO_4{\cdot}2H_2O$ 0.5 mg l^{-1}, ferric citrate 16.1 mg l^{-1}, 20.0 mg thiamine, and 2.0 mg biotin [16]. Solid medium for plates contained 1.5–2 % agar. When needed, the following antibiotics were added: 30 μg ml^{-1} kalamycin and 25 μg ml^{-1} chloramphenicol.

Table 1 Strains used in this work

Strains	Relevant description	Source site
W1485	F + wild type	CGSC 5024
AFP111	F + λ-rpoS396 (Am) rph-1△(pflB::Cam) ldhA::Kan ptsG	Clark
DW111	Derivative of AFP111, selected by ARTP method	This study
DC111	Derivative of AFP111, selected by ARTP method	This study
DX111	Derivative of AFP111, selected by ARTP method	This study
DC112	Derivative of DC111, selected by an anaerobic continuous culture method	This study
DC113	Derivative of DC111, selected by an anaerobic continuous culture method	This study
DC114	Derivative of DC111, selected by an anaerobic continuous culture method	This study
DC115	Derivative of DC111, selected by an anaerobic continuous culture method	This study

Table 2 The major composition of corn stalk hydrolysate after being sterilized at 121 °C for 15 min

Sugar	Concentration (g l^{-1})[a]
Total sugar	45.6 ± 0.52
Xylose	40.0 ± 0.45
Glucose	3.6 ± 0.06
Arabinose	2.0 ± 0.01
TPC	1.8 ± 0.02

Each value is the mean of three parallel replicates ± standard deviation

TPC total soluble phenolic compounds

[a] The total reducing sugar concentration was measured by the 3,5-dinitrosalicyclic acid method. Glucose, xylose, and arabinose were quantified by high-performance liquid chromatography

Corn stalk was obtained from Shandong Zhengde Foods Ltd., China.

Hydrolysis corn stalks

The corn stalk was mixed with 2 % (v v^{-1}) sulfuric acid at a ratio of 1:5 (w v^{-1}), and hydrolyzed in an autoclave at 121 °C for 2.5 h. The raw hydrolysate was adjusted to pH 4.0 with solid $Ca(OH)_2$ at 50 °C and 2 % (w v^{-1}) activated carbon added to remove many inhibitors that affect microorganism culture growth. The mixture was heated to 50 °C for 2 h and then passed through filter paper to remove solids. The resulting corn stalk hydrolysate contained 45.6 g l^{-1} reducing sugar as shown in Table 2.

Protocols for ARTP mutation of AFP111

The ARTP machine was designed at Tsinghua University [26]. For mutation of AFP111, 10 µl of the culture (OD_{600} = 1.0) was dropped onto a sterilized stainless-steel plate (12 mm in diameter) and then dried with sterile air. The metal plate containing cells was placed into the vessel approximately 2 mm away the torch nozzle exit and the vessel. Operating conditions consisted of a helium gas flow rate of Q_{He} = 10 slpm (standard liters per minute) radio-frequency power input of 100 W, and plasma treatment times of 10–60 s. After treatment, the sample plate was put into a test-tube containing sterile saline and shaken for 2 min to resuspend cells. The suspension was spread onto plates of LB medium and the lethality rate of the colonies under different operation time was evaluated.

A cell lethality percentage of 90 % was set as the criterion for mutant generation [14, 22]. After treatment, the suspension was spread onto plates of CD medium supplemented with 0.2 % xylose and colonies were selected.

Metabolic evolution

Seed cultures were prepared by inoculating strain from fresh xylose plates into 25-ml screw-cap tubes containing 10 ml of LB broth with 15 g l^{-1} xylose. After incubating for 12 h (37 °C, 200 rpm), all of the culture was used to inoculate the fermentation vessel containing 100 ml of CD medium broth with 15 g l^{-1} xylose, purged with carbon dioxide gas to remove dissolved oxygen. This culture (weight of cells was 1.8 g l^{-1}) was used for continuous culture selection and transferred to CD medium supplemented with 0.2 % xylose, 30 µg ml^{-1} kanamycin, and 25 µg ml^{-1} chloramphenicol (10 % (v v^{-1}) inoculation).

The metabolic evolution strategies were carried out in a continuous culture system, comprising a 500-ml glass test tube having a variety of tube fittings with an operating volume of 200 ml [44]. Agitation resulted from sterile CO_2 being sparged into the bottom of the vessel. The fermentation conditions were 120 rpm, no oxygenation 37 °C, and pH 7.0 (controlled by Na_2CO_3 additions). The continuous culture condition was initiated by feeding fresh CD medium supplemented with 15 g l^{-1} xylose at a dilution rate of 0.015 h^{-1}. After the culture reached an optical density of 2 at 600 nm (OD_{600}) and at least four residence times, the feed flow was increased a dilution rate of 0.03 h^{-1}. This procedure was repeated until the dilution rate reached 0.06 h^{-1}. When the cell density increased, then the samples were removed from the continuous culture system. A 1:10 dilution of the samples was plated on agar plates for mutant screening.

Mutant screening

The mutants were cultivated at 37 °C under anaerobic conditions (N_2:H_2:CO_2 = 8:1:1) in an anaerobic workstation (Bug box; RUSKINN, UK). The colonies showing pump and genetic stability on the agar medium were selected after eight generations.

Fermentation

A seed inoculum of 200 µl from an overnight 5-ml LB culture was added to a 500-ml flask containing 50 ml of LB medium for aerobic growth at 37 °C and 200 rpm. After incubating for 8 h, a 10 % inoculum (approximately 0.16 g l^{-1} DCW) was used to start the anaerobic culture in screw-cap bottles containing 30 ml of CD medium supplemented with 30 g l^{-1} of xylose and 24 g l^{-1} magnesium carbonate hydroxide at 37 °C and 200 rpm.

Anaerobic fermentation was performed in a 3-l bioreactor (Bioflo 110, USA) with 1.5 l of CD medium. The culture conditions were the same as those in the sealed bottles

described above, supplemented with 35 g l^{-1} total reductive sugar and 30 g l^{-1} magnesium carbonate hydroxide.

Corn stalk hydrolysate fermentation culture conditions were the same as those for the mixed sugar fermentations described above.

Analytical methods

The OD_{600} was measured and correlated to the dry cell weight (DCW): DCW (g l^{-1}) = 0.4 × OD_{600}. Sugars and organic acids were measured by high-performance liquid chromatography (Chromeleon server monitor, P680 pump, Dionex, Sunnyvale, CA, USA). To determine glucose, xylose, and arabinose, a refractive 14 index detector, RI101 (Shodex, Showa Denko America, Inc., New York, NY, USA), and an ion-exchange chromatographic column (Aminex 15 HPX-87H, 7.8 mm × 300 mm, Bio-Rad, Hercules, CA, USA) and a refractive index detector, RI101 (Shodex, Showa Denko America, Inc., New York, NY, USA) using the following parameters: a wavelength of 215 nm, and the mobile phase of 5 mM H_2SO_4 with a flow rate of 0.6 ml min^{-1} at 55 °C. To quantify organic acids, a UV detector, UVD170U, and an ion-exchange chromatographic column (Prevail organic acid 5 μm, 250 × 4.6 mm Grace, Deerfield, IL, USA) were used with a mobile phase which is 25 mM KH_2PO_4 (adjusted to pH of 2.5 by H_3PO_4) at a flow rate of 1 ml $min^{-1.}$

Enzyme assays

ATP is an indicator of metabolically active cells. For ATP assays, 1 ml of cold 30 % (w v^{-1}) trichloroacetic acid was added to the samples (4 ml) and mixed thoroughly. The BacTiter-Glo Microbial Cell Viability Assay kit on the GloMax-Multi + Detection System (Promega, Madison, WI, USA) was used to measure the intracellular ATP concentrations.

The intracellular concentrations of NADH and NAD^+ were assayed using a cycling method [24].

Specifically, cells were harvested by centrifugation at 8,000 × *g* for 10 min at 4 °C, and washed with 100 mM Tris–HCl (pH 7.5) and then resuspended in the same buffer containing 1 mM TPP. Cells were then sonicated on ice for 10 min (a working period of 3 s in a 3-s interval for each cycle) at a power output of 300 W using an ultrasonic disruptor (GA92-IID, Shangjia, Wuxi, China). Cell debris was removed by centrifugation at 20,000 × *g* for 15 min at 4 °C, and the crude cell extracts immediately used to assay enzyme activities. One unit (U) of specific activity was defined as the amount of enzyme needed to oxidize 1 μmol of NADH per minute per milligram of protein. The total protein concentration was measured using the Bradford method [5]. The activities of PEPCK and PPC were measured by monitoring spectrophotometrically the disappearance of NADH [39, 43]. MDH activity was measured spectrophotometrically at the end of anaerobic fermentations [4].

Results and discussion

Screening of xylose-utilizing mutants by ARTP

The ARTP mutation system, which is based on low-pressure plasmas generated under vacuum, has been widely used to achieve mutation on a molecular level [31]. This system can generate diverse breakage in plasmid DNA and oligonucleotides by varying in the plasma dosage [40]. This system has been successfully applied to achieve mutations in *Streptomyces avermitilis*, the enabling generation of a mutant with higher avermectin productivity [41].

In the present study, the ARTP jet, driven by a radio frequency (RF) power supply with water-cooled and bare-metallic electrodes, was used to mutate *E. coli* strain AFP111. For effective mutation and selection of the mutant, a high rate of cell lethality is required. Figure 1 shows the effects of different plasma treatment time points on the lethality rate of AFP111 under aerobic conditions. When the sample was treated for 30 s under these conditions, no colonies could survive. Based on earlier reports, a lethality rate of 90 % was considered optimal [14, 22]. Thus, 15 s was chosen as optimal for mutation (Fig. 1). Measurements were performed after 36 h of cultivation when cells entered stationary phase. After 15 generations of culture, only three mutants, DW111, DC111, and DX111, survived, and were used for subsequent fermentation in sealed bottles. As presented in Table 3, the mutants showed an obvious production of succinic acid and a slight increase

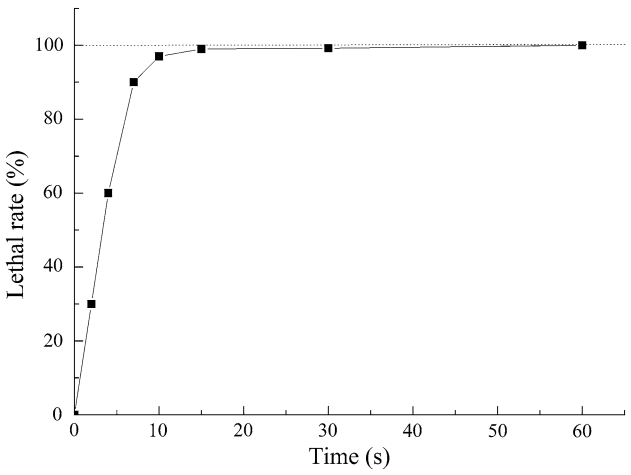

Fig. 1 Effects of plasma treatment times on the lethality rate of irradiated AFP111 under aerobic condition on LB medium. The results were performed in duplicate

Table 3 Exclusively anaerobic fermentations of the ARTP mutants in CD medium after 72-h cultivation

Strains	OD_{600}	Xylose consumed (g l^{-1})	Succinic acid (g l^{-1})	Acetic acid (g l^{-1})	Succinic acid yield (mol mol^{-1})
AFP111	0.36 ± 0.02	ND	ND	ND	ND
DW111	1.21 ± 0.03	5.1 ± 0.04	1.7 ± 0.03	0.8 ± 0.01	0.43 ± 0.03
DX111	2.01 ± 0.01	6.5 ± 0.05	3.5 ± 0.01	1.2 ± 0.06	0.68 ± 0.02
DC111	2.26 ± 0.08	10.5 ± 0.06	6.6 ± 0.04	1.5 ± 0.03	0.78 ± 0.03

Each value is the mean of three parallel replicates ± standard deviation

ND not detected

in cell concentration. Under anaerobic conditions, the concentration of succinic acid in DC111 is 2.88-fold and 0.89-fold higher than that in DW111 and DX111, respectively (Table 3).

Metabolic evolution of DC111

As microorganisms have the ability to rapidly adapt to different environmental conditions, ME has been used with great success to improve yields and reduce costs in industrial settings. ME can lead to the activation of latent metabolic pathways, increase substrate/product tolerance, and improve fitness [11]. Therefore, DC111 was subjected to continuous culture under an anaerobic environment to generate strains better adapted to grow and produce more succinic acid. As shown in Fig. 2, the fermentation media were inoculated with 10 % (v v^{-1}) seeds, and a dilution rate of 0.015 h^{-1} was established. With dilution, rates increased as cell density reached a plateau after three retention times. At 402 h, the OD_{600} increased to 2.42 and the dilution rate increased to 0.06 h^{-1}, suggesting that the bacteria had adapted to the environment. The mutants were obtained under sterile conditions and spread on CD agar plates supplemented with 0.2 % xylose. After eight generations of culture, four mutants, DC112–DC115, were isolated. The original strain W1485 and five mutants were characterized under anaerobic conditions using the CD medium with 30 g l^{-1} of xylose. Table 4 shows a comparison between the products of the generated mutants and the original strain under anaerobic conditions. Amongst the mutants, strain DC115 was found to perform the best, exhibiting an ability to consume xylose and produce elevated titers of succinic acid. Furthermore, the succinic acid yield rate of DC115 was 3.47 times higher than that of W1485.

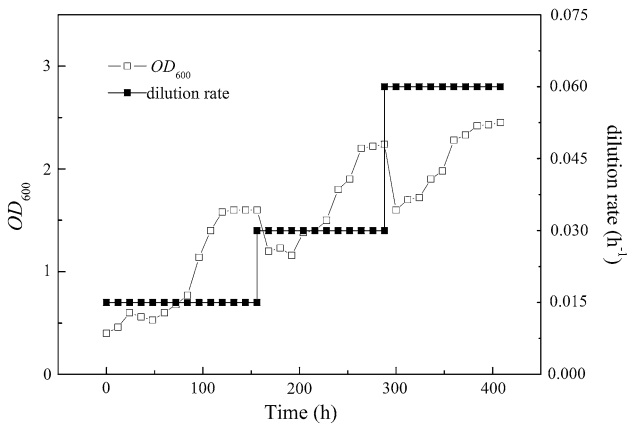

Fig. 2 Process of continuous culture. Cultivation conditions were as described in the "Methods" section. Dilution rate (*filled squares*), OD 600 (*empty square*)

As shown in Fig. 3, the ability to consume xylose was restored in DC115 when compared with that in the wild-type ancestor W1485. Furthermore, AFP111 failed to grow with xylose as the carbon source, as reported earlier. Although DC115 grew much more slowly than W1485, it eventually attained approximately the same final cell density. Thus, DC115 was selected for further studies.

Key enzyme analysis of the mutant DC115

PEP is converted to oxaloacetate (OAA) as the first step in succinic acid production. In an *E. coli ppc* deletion mutant, this reaction is catalyzed by the ATP-generating PCK, which restores cell growth and enhances succinic acid production [18, 35]. Previous studies have suggested that the enzyme activity of PCK and PPC can be investigated. In the present study, the specific enzyme activity of PPC in DC115 and AFP111 were 0.35 and 0.05 U mg^{-1}, respectively, whereas the specific enzyme activity of PCK in DC115 cells (1.03 U mg^{-1}) was higher than that in AFP111 (0.22 U mg^{-1}). This finding suggested that conversion of PEP to OAA is main catalyzed by the ATP-generating direction, meeting the ATP requirement for xylose metabolism under anaerobic conditions. As expected, the ATP measurements have proved that the high ATP supply in DC115 restored the xylose utilization. In AFP111 and AFP184, one molecule xylose needs two and 1.33 ATP respectively for its transport and phosphorylation [12, 30]. As a result, AFP184 can ferment xylose and has strong growth characteristics. Conversely, AFP111 cannot utilize xylose for biomass generation and succinic acid production. Therefore, one of the most important reason about the

Table 4 Exclusively anaerobic fermentations of the ME mutants in CD medium after 72-h cultivation

Strains	OD_{600}	Xylose consumed ($g\ l^{-1}$)	Succinic acid ($g\ l^{-1}$)	Acetic acid ($g\ l^{-1}$)	Formic acid ($g\ l^{-1)}$	Lactic acid ($g\ l^{-1}$)	Ethanol ($g\ l^{-1}$)	Succinic acid yield ($mol\ mol^{-1}$)
W1485	3.54 ± 0.02	28.5 ± 0.4	4.2 ± 0.02	5.9 ± 0.01	2.8 ± 0.03	1.9 ± 0.02	4.2 ± 0.03	0.19 ± 0.05
DC111	2.26 ± 0.08	10.5 ± 0.06	6.5 ± 0.04	1.5 ± 0.06	ND	ND	ND	0.78 ± 0.05
DC112	2.18 ± 0.05	16.1 ± 0.4	10.2 ± 0.02	2.6 ± 0.03	ND	ND	ND	0.81 ± 0.04
DC113	2.20 ± 0.08	15.3 ± 0.1	9.6 ± 0.05	2.6 ± 0.01	ND	ND	ND	0.80 ± 0.08
DC114	2.36 ± 0.02	14.5 ± 0.2	9.0 ± 0.03	2.3 ± 0.04	ND	ND	ND	0.79 ± 0.02
DC115	2.80 ± 0.06	18.0 ± 0.3	12.1 ± 0.02	2.9 ± 0.03	ND	ND	ND	0.85 ± 0.08

Each value is the mean of three parallel replicates ± standard deviation

ND not determined

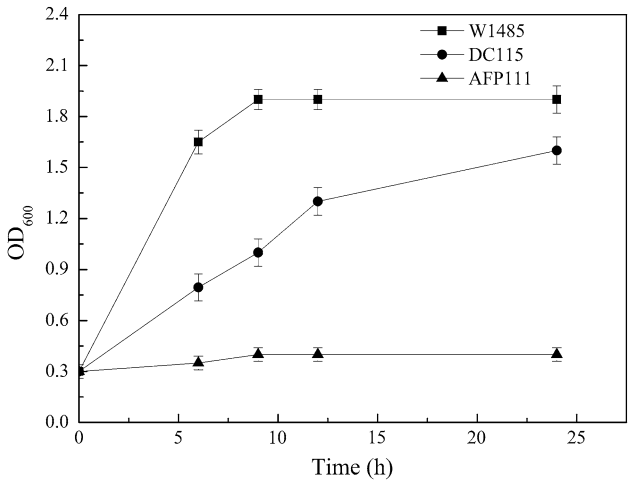

Fig. 3 Anaerobic growth of strains in CD medium. Strains DC115 (*filled circles*), its immediate parent AFP111 (*filled triangles*), and the wild-type ancestor W1485 (*filled squares*) were grown anaerobically at 37 °C, in 100 ml of CD medium containing 15 g l^{-1} xylose in a sealed bottle under an atmosphere of CO_2

xylose metabolism is the ATP supply. The ATP concentration of DC115 was 1.33-fold higher than AFP111 during xylose fermentation (Table 5). Thus, the enhancement of ATP was one reason why the DC115 can utilize xylose to generate succinic acid.

Malate dehydrogenase (MDH) (EC1.1.1.37), an NADH-dependent enzyme, catalyzes the conversion of OAA to malate, which is the second step in succinic acid production, simultaneously regenerating NAD^+ [25]. The results of the present study indicate that the specific enzyme activities of MDH in DC115 and AFP111 were 0.65 and 0.04 U mg^{-1}, respectively (Table 5). The higher enzyme activity of MDH in DC115 suggested that it enhanced the metabolic flux from OAA towards succinic acid and was favorable for NAD^+ regeneration to restore redox balance.

The NADH/NAD^+ cofactor pair plays a major role in microbial catabolism, in which a carbon source such as xylose is oxidized using NAD^+, producing reducing equivalents in the form of NADH. Under anaerobic conditions and in the absence of an alternate oxidizing agents, regeneration of NAD^+ is achieved through fermentation using NADH to reduce metabolic intermediates such as PEP, pyruvate, or their derivatives, which serve as electron acceptors to maintain the overall redox balance [3]. In the present study, the levels of NADH and NAD^+ were increased in DC115 when compared with those in AFP111, and the total amount of NAD(H) was 1.3-fold higher in DC115 than that in AFP111 (Table 5). As NADH replenishment enhanced succinic acid production in the fermentation process, alterations in the availability of NADH could have had a profound effect on the whole metabolic network of DC115.

Sugar mixture utilization by the mutant DC115 during anaerobic fermentation

Xylose is the major component of corn stalk hydrolysate, and fermentation of this sugar by microorganisms in a

Table 5 Determination of the amount of ATP, NADH, NAD^+, NAD(H) and specific activities in crude extracts of the strains

Strain	ATP ($nmol\ g^{-1}$ DCW)	PCK activity ($U\ mg^{-1}$)	PPC activity ($U\ mg^{-1}$)	MDH activity ($U\ mg^{-1}$)	NADH ($\mu mol\ g^{-1}$)	NAD^+ ($\mu mol\ g^{-1}$)	NAD (H) ($\mu mol\ g^{-1}$)
AFP111	426 ± 36	0.22 ± 0.02	0.35 ± 0.02	0.04 ± 0.01	1.41 ± 0.04	2.80 ± 0.03	4.21 ± 0.06
DC115	889 ± 28	1.03 ± 0.03	0.05 ± 0.04	0.65 ± 0.02	4.52 ± 0.03	5.31 ± 0.05	9.83 ± 0.08

Each value is the mean of three parallel replicates ± standard deviation

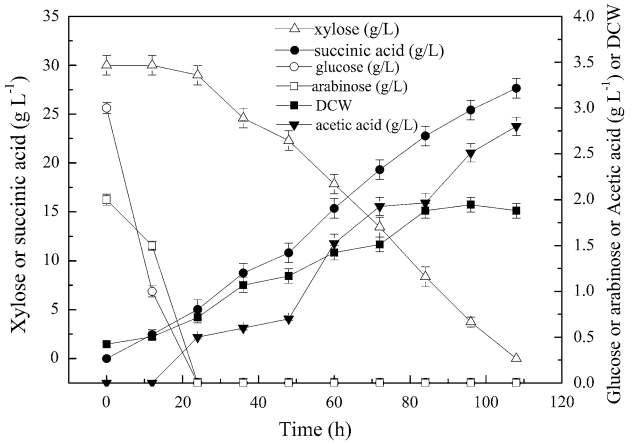

Fig. 4 Anaerobic fermentation of mixed sugars by DC115 in CD medium. Symbols used in the figure: xylose (*empty triangles*), glucose (*empty circles*), arabinose (*empty squares*), DCW (*filled squares*), succinic acid (*filled circles*), and acetic acid (*filled triangles*)

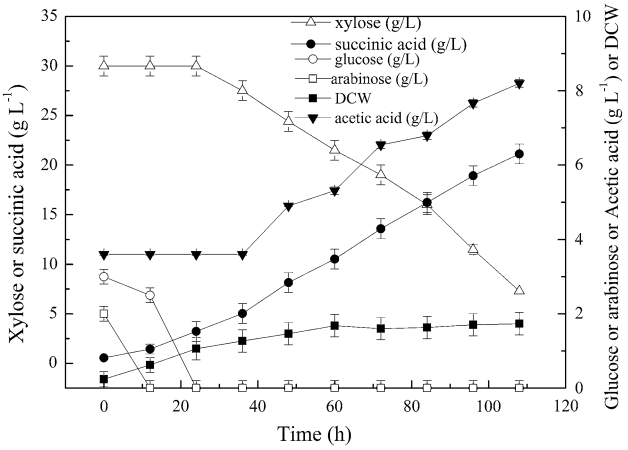

Fig. 5 Anaerobic fermentation of corn stalk hydrolysate by DC115 in CD medium. Symbols used in the xylose (*empty triangles*), glucose (*empty circles*), arabinose (*empty squares*), DCW (*filled squares*), succinic acid (*filled circles*) and acetic acid (*filled triangles*)

rapid and efficient manner to produce succinic acid is desirable. Under anaerobic conditions in CD medium, AFP111 failed to metabolism xylose. To overcome this limitation, the mutant DC115 was generated through ARTP and ME, and was grown in CD medium containing sugar mixture. The sugar mixture included 3 g l^{-1} of glucose, 2 g l^{-1} of arabinose, and 30 g l^{-1} of xylose, consistent with the ratios of the quantities of sugars in the corn stalk hydrolysate. DC115 was found to co-metabolize glucose and arabinose, and utilize xylose only after glucose–arabinose depletion. In addition, glucose–xylose co-metabolism was completely inhibited by the presence of arabinose. A previous study has shown that this effect is attributed to the preferential pattern of pentose consumption exhibited by *E. coli* [13, 17]. As shown in Fig. 4, in 108 h, the concentration and yield of succinic acid reached 27.7 g l^{-1} and 0.79 g g^{-1} sugar mixture, respectively, and at the end of fermentation, 2.5 g l^{-1} of acetic acid was produced. Acetic acid, the oxidized byproduct, is formed because fermentations require a balanced production and consumption of reducing equivalents [34].

Corn stalk utilization by the mutant DC115 during anaerobic fermentation

Corn stalk represents a renewable resource for the production of biofuels and valuable chemicals. As corn stalk has a relatively high amount of hemicellulose, we attempted to use it to prepare xylose and glucose. Previous studies have shown that corn stalk hemicellulose could be used as a carbon source to produce succinic acid by using engineered *E. coli* [30]. The corn stalk hemicellulose hydrolysate utilized in the present study contained approximately 88 % xylose, 8 % glucose, and 4 % arabinose. Anaerobic fermentation of corn stalk hydrolysate was carried out to produce succinic acid by using DC115 in a 3-l stirred bioreactor with an initial sugar concentration of 35 g l^{-1}, containing 30 g l^{-1} of xylose, 3 g l^{-1} of glucose, and 2 g l^{-1} of arabinose. As shown in Fig. 5, at the beginning of the fermentation process, 3.6 g l^{-1} of acetic acid was detected in the medium, along with 1.8 g l^{-1} of total soluble phenolic compounds (TPC). In 108 h, 3 g l^{-1} of glucose, 2 g l^{-1} of arabinose, and 22.7 g l^{-1} of xylose were consumed, and a succinic acid concentration of 21.1 g l^{-1} was achieved with a succinic acid yield of 0.76 g g^{-1} sugar. There was no significant difference in the sugar utilization patterns between mixed sugar fermentation and hydrolysate fermentation. The only byproduct formed during the fermentation process was 8.2 g l^{-1} of acetic acid. However, when compared with the fermentation of mixed sugars, the sugar consumption rates decreased and the concentration of the byproduct (acetic acid) increased during the hydrolysate fermentation. Corn stalk hydrolysate contains many byproducts formed during the process of hydrolysis, which might be inhibitory for the microorganisms [23]. Hence, acetic acid and TPC may act as inhibitors of the fermentation process.

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