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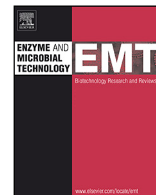

Succinic acid production from hemicellulose hydrolysate by an *Escherichia coli* mutant obtained by atmospheric and room temperature plasma and adaptive evolution

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ABSTRACT

Atmospheric and room temperature plasma and adaptive evolution were combined to generate *Escherichia coli* mutants, which can simultaneously and efficiently utilize glucose and xylose to produce succinic acid in chemically defined medium under exclusively anaerobic condition. Compared to the parent strain BA305, a *pflB*, *ldhA*, *ppc*, and *ptsG* deletion strain overexpressing ATP-forming phosphoenolpyruvate (PEP) carboxykinase (PEPCK), the sugar consumption rate and succinic acid productivity of mutant BA408 were significantly improved with a marked increase in the key enzyme activities. Subsequent anaerobic fermentation of BA408 with corn stalk hydrolysate produced a final succinic acid concentration of 23.1 g L^{-1} with a yield of 0.85 g g^{-1} sugar mixture. The observed synthesis of succinic acid from the corn stalk hydrolysate showed a great potential usage of renewable biomass as a feedstock for an economical succinic acid production using *E. coli*.

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1. Introduction

As a promising platform chemical, succinic acid, a C4-dicarboxylic acid has been extensively used in agricultural, food processing and pharmaceutical industries. Currently, the commercial succinic acid production is mostly chemically synthesized from butane derived from petroleum. However, due to the declining oil reserves, rising prices, and the environmental pollution, the bio-based succinic acid production has attracted more and more attention [1–5].

In order to develop a bio-based industrial production of succinic acid, the medium used must be low-cost and succinic acid could be separated easily. Furthermore, the producing organism must be able to utilize a wide range of low-cost feedstocks to produce succinic acid. China is an agricultural country where the annual biomass wastes, such as corn stalk, wheat straw, and other straw wastes, exceed 0.7 billion tons [6]. The low cost and high carbohydrate content of these biomass wastes make them potential substrates for bioconversion to value-added platform chemicals.

Among succinic acid producers such as *Actinobacillus succinogenes*, *Anaerobiospirullum succiniciproducens* [7], *Corynebacterium glutamicum* [8], and recombinant *Escherichia coli* [9,10], the bacterium *E. coli* has the advantages of fast growth, clear genetic background, and a relatively high succinic acid production rate. Nevertheless, the main problem of hemicellulose hydrolysate fermentation by *E. coli* is the delayed and incomplete utilization of pentose. If the preferred carbon source glucose existed in the fermentation medium, the transport and metabolism of other sugars would be inhibited unless the glucose was exhausted [11]. However, simultaneous consumption of sugars in a sugar mixture is in favor of a fermentative production process because simultaneous consumption of sugars can eliminate diauxic growth, thereby reducing the operating time and increasing the productivity [12].

As a result of a glucose phosphotransferase mutation and the low ATP demand mutant strain *E. coli* AFP184 can simultaneously utilize xylose and glucose for succinic acid production [13]. Meanwhile *E. coli* BA305, a *pflB*, *ppc*, and *ptsG* deletion strain overexpressing the ATP-forming PEPCK from *Bacillus subtilis* 168, can also utilize xylose and glucose simultaneously for succinic acid production due to high ATP supply. However the two strains both need to be incubated in complex medium supplemented with organic nitrogen sources such as tryptone, yeast extract, and corn steep liquor. The addition of these organics increased the cost and the difficulty in separation.

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Table 1
The major composition of corn stalk hydrolysate after being sterilized at 121 °C for 15 min.

Sugars	Concentration[a] ($g\,L^{-1}$)
Total sugar[b]	60.2 ± 1.2
Xylose[c]	49.1 ± 0.4
Glucose[c]	5.7 ± 0.5
Arabinose[c]	2.1 ± 0.7

[a] Each value is the mean of three parallel replicates ± standard deviation.
[b] The total reducing sugar concentration was measured by the 3,5-dinitrosalicyclic acid method.
[c] Glucose, xylose, and arabinose were quantified by high-performance liquid chromatography.

To accomplish the efficient and economical bioconversion of renewable cellulosic materials to succinic acid, many biotechnologies have been applied to generate *E. coli* mutants, such as genetic engineering techniques, metabolic engineering technology, and microbial mutation breeding. On account of the mutative efficiency issues and the safety of the operators, a physical mutation method, atmospheric and room temperature plasma (ARTP) has been developed for microbial mutation breeding [14–17].

In the present work, *E. coli* BA305 was chosen as the parent strain to generate mutants, which can simultaneously and efficiently utilize xylose and glucose for succinic acid production in chemically defined medium by ARTP and adaptive evolution. The succinic acid production, sugar consumption, key enzymes activities, ATP concentration, and corn stalk hydrolysate fermentation were investigated.

2. Materials and methods

2.1. Materials

E. coli BA305 (China General Microbiological Culture Collection Center, CGMCC No. 2012103) was used in this study. The Luria–Bertani (LB) medium contained the following: tryptone ($10\,g\,L^{-1}$), yeast extract ($5\,g\,L^{-1}$), and NaCl ($5\,g\,L^{-1}$). The chemically defined (CD) medium contained the following: citric acid ($3.0\,g\,L^{-1}$), $Na_2HPO_4{\cdot}7H_2O$ ($3.0\,g\,L^{-1}$), KH_2PO_4 ($8.0\,g\,L^{-1}$), $(NH_4)_2HPO_4$ ($8.0\,g\,L^{-1}$), NH_4Cl ($0.2\,g\,L^{-1}$), $(NH_4)_2SO_4$ ($0.75\,g\,L^{-1}$), $MgSO_4{\cdot}7H_2O$ ($1.0\,g\,L^{-1}$), $CaCl_2{\cdot}2H_2O$ ($10.0\,mg\,L^{-1}$), $ZnSO_4{\cdot}7H_2O$ ($0.5\,mg\,L^{-1}$), $CuCl_2{\cdot}2H_2O$ ($0.25\,mg\,L^{-1}$), $MnSO_4{\cdot}H_2O$ ($2.5\,mg\,L^{-1}$), $CoCl_2{\cdot}6H_2O$ ($1.75\,mg\,L^{-1}$), H_3BO_3 ($0.12\,mg\,L^{-1}$), $Al_2(SO_4)_3{\cdot}5H_2O$ ($1.77\,mg\,L^{-1}$), $Na_2MoO_4{\cdot}2H_2O$ ($0.5\,mg\,L^{-1}$), Fe(III) citrate ($16.1\,mg\,L^{-1}$), and thiamine ($20\,mg\,L^{-1}$) [18]. The final concentration of $100\,\mu g\,mL^{-1}$ of ampicillin was added at the beginning of every culture or fermentation.

Tryptone and yeast extract were from Oxoid Ltd. (Cambridge, UK). Other chemicals used were analytical grade and were from either Sinochem (Shanghai, China) or Fluka Chemical (Buchs, Switzerland). CO_2 was purchased from Nanjing Special Gases Factory (Nanjing, China).

Corn stalk with a moisture content of 5.6% (w/v) was obtained from Shandong Zhengde Foods Ltd., China. Before mixing with 2% (v/v) sulfuric acid at a ratio of 1:5 (w/v), the corn stalk was ground with a commercial plant grinder and passed through 20- to 40-mesh screens. The suspension was 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 then filtered through filter paper to remove any solid material. After treatment at 121 °C for 15 min, the corn stalk hydrolysate contained $60.2\,g\,L^{-1}$ reducing sugar as shown in Table 1.

2.2. Helium-based ARTP mutation of E. coli BA305

The ARTP system consists of a co-axial type plasma generator and a slider. The atmospheric-pressure helium plasma jet was issued from the coaxial-type plasma generator, which mainly consists of different chemically active species [19]. The slider was used to adjust the distance between the plasma torch nozzle exit and the treated sample. The detailed descriptions of the experimental setup can be found in the previous paper [20].

For mutation, *E. coli* BA305 cells grown in LB medium were harvested in the exponential phase and diluted to an optical density at 600 nm (OD_{600}) of 1 with sterile physiologic saline solution. $10\,\mu L$ of the suspension was dipped onto a sterilized stainless steel plate (8 mm/diameter). The metal plates containing the bacterial cells were then treated by the helium plasma jet on the vessel. The apparatus was operated at the helium gas flow rate of Q_{He} = 15 slpm (standard liters per minute) and RF power input of 100 W to obtain a uniform and low-temperature glow discharge plasma. The plasma treatment times ranged from 10 s to 30 s. The survival rate could be calculated using the number of colonies scored in CD medium agar plates without plasma treatment as 100%. When the sample was treated for 15 s, the survival rate of *E. coli* BA305 was 9.6 ± 1.2%. According to the previous reports, a survival rate percentage of 10% was set as the criterion for mutant generation [16,21]. Thus 15 s was chosen as the optimal treatment time for mutation.

After treatment of the bacterial cells, the metal plate was put into a test tube containing 2 mL sterile physiologic saline solution and shaken for 2 min to wash off the cells. Then $50\,\mu L$ of the elute was spread on a CD medium agar plate with $8\,g\,L^{-1}$ xylose and $2\,g\,L^{-1}$ glucose. After 48 h anaerobic cultivation, single colonies were selected.

2.3. Adaptive evolution

The adaptive evolution was carried out by sequentially subculturing in a small and pH controlled fermentation vessel. Selection was begun using $15\,g\,L^{-1}$ sugar mixture containing $12\,g\,L^{-1}$ xylose and $3\,g\,L^{-1}$ glucose with serial transfers at 96-h intervals. A bicarbonate solution was used to maintain pH. Anaerobic condition was established by sparging the culture with CO_2 at a flow rate of $0.2\,L\,min^{-1}$. Seed cultures were prepared by inoculating strain from fresh agar plate into 25 mL screw-cap tubes containing 10 mL LB with $10\,g\,L^{-1}$ sugar mixture. After incubating for 12 h (37 °C, 200 rpm), all of the culture was used to inoculate the fermentation vessel containing 100 mL CD medium with $15\,g\,L^{-1}$ sugar mixture. At the end of the seventh transfer (28 days), the culture kept an optical density of 3 at 600 nm (OD_{600}). Then the sugar concentration was increased to $30\,g\,L^{-1}$ and cell solution was transferred with modest improvement in cell growth and a little decline in succinic acid yield. A 1:10 dilution of the samples was plated on agar plates for mutant screening.

2.4. Fermentation

A seed inoculum of 0.5 mL of an overnight 5-mL LB culture was added to a 100-mL flask containing 50 mL of LB medium, and incubated at 37 °C and 200 rpm for 8 h. Subsequently, 3-mL aliquot was added to a 100-mL sealed bottle containing 30 mL CD medium with $20\,g\,L^{-1}$ and $16\,g\,L^{-1}$ magnesium carbonate hydroxide (maintained the pH at 6.8). The culture was incubated at 37 °C and 200 rpm for 48 h. Based on the succinic acid production and sugar consumption rate of these mutants, the best strain was selected for confirmation of the stability of succinic acid production.

Anaerobic fermentations of sugar mixture and corn stalk hydrolysate were carried out in a 3-L bioreactor containing 1.5 L CD medium. A 10% (v/v) inoculum was used to start the anaerobic culture. The anaerobic culture was supplemented with $30\,g\,L^{-1}$ sugar mixture and $24\,g\,L^{-1}$ magnesium carbonate hydroxide. Anaerobic condition was established by sparging the culture with CO_2 at a flow rate of $0.2\,L\,min^{-1}$. The pH, temperature, and agitation were maintained at 6.8, 37 °C, and 200 rpm, respectively.

2.5. Analytical methods

The OD_{600} was measured to monitor cell growth by using an ultraviolet–visible spectrophotometer and correlated to the dry cell weight: DCW ($g\,L^{-1}$) = $0.4 \times OD_{600}$. The total concentration of reducing sugars was measured by 3,5-dinitrosalicylic acid method [22].

Glucose, xylose, and organic acids were quantified by high-performance liquid chromatography (Chromeleon server monitor, P680 pump, Dionex, USA). To determine the amount of glucose, xylose, and arabinose, a refractive index detector, RI101 (Shodex, USA), and an ion exchange chromatographic column (Aminex HPX-87H, 7.8 mm × 300 mm, Bio-Rad, USA) were used at a wavelength of 215 nm, The mobile phase was 5 mM H_2SO_4 with a flow rate of $0.6\,mL\,min^{-1}$ at 55 °C. To determine the organic acids, a UV detector, UVD 170U, and an ion exchange chromatographic column (Prevail Organic Acid 5 µm, 250 mm × 4.6 mm, Grace, USA) were used at a wavelength of 215 nm, and 25 mM KH_2PO_4 (adjusted to a pH of 2.5 by H_3PO_4) was used as the mobile phase with a flow rate of $1\,mL\,min^{-1}$.

The total soluble phenolic compounds were estimated by a modification of Folin–Ciocalteu's method using vanillic aldehyde and tannin as standards [23]. The intracellular concentration of NADH and NAD^+ were assayed using the cycling method [24]. To measure the intracellular ATP concentration, 1 mL of cold 30% (w/v) trichloroacetic acid was added to the samples (4 mL) and mixed thoroughly. The ATP concentrations were then measured using the BacTiter-Glo™ Microbial Cell Viability assay kit on the GloMax®-Multi + Detection System (Promega, Madison WI, USA).

2.6. Enzyme assays

Cells were harvested by centrifugation at 6080 × *g* for 10 min to determine the enzyme activities and washed with 2 mL $100\,mmol\,L^{-1}$ of Tris–HCl (pH 7.5) twice. Subsequently, the cells were resuspended in the same buffer and 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 by using an ultrasonic disruptor (GA92-IID, Shangjia, Wuxi, China). The cell debris was removed by centrifugation (16,060 × *g* for 60 min at 4 °C), and the crude cell extracts were immediately used to determine the enzyme activities [25,26].

The activities of malate dehydrogenase (MDH) and PEPCK were measured by spectrophotometer monitoring the disappearance of NADH, which has a millimolar extinction coefficient of $6.22\,cm^{-1}\,mM^{-1}$ at 340 nm [27]. One unit of specific activity

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