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ORIGINAL PAPER

Optimization of culture conditions in CO_2 fixation for succinic acid production using *Actinobacillus succinogenes*

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Abstract The culture conditions in CO_2 fixation by *Actinobacillus succinogenes* for succinic acid production were investigated by a model of available CO_2 in a 3-l fermentor. The results from the model analysis showed that the available CO_2 for succinic acid production in the fermentation broth is the sum of HCO_3^-, CO_3^{2-}, and CO_2 influenced by external culture conditions such as medium components, CO_2 partial pressures, and temperature. The optimized conditions for CO_2 supply in a 3-l fermentor were determined as follows: CO_2 partial pressure and stirring speed were maintained at 0.1 MPa and 200 r min^{-1}, respectively, with a pH of 6.8 and a temperature of 37°C; 0.15 mol l^{-1} $NaHCO_3$ was added. Under the optimized conditions, a CO_2 fixation rate of 0.57 g l^{-1} h^{-1} was obtained, and a succinic acid concentration of 51.6 g l^{-1} with a yield of 75.8% was reached. These results suggest that optimized conditions of CO_2 supply are effective in high succinic acid production and thus have potential applications in succinic acid production and CO_2 fixation.

Keywords Optimized · CO_2 · Succinic acid · Fermentation · Fixation

Yong-lan Xi and Ke-quan Chen contributed equally to this study.

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Introduction

Succinic acid is a common natural organic acid often found in humans, animals, plants, and microorganisms that plays an important role in biological metabolism [28]. Recent studies show that succinic acid can also be used as a C4 platform chemical for the synthesis of 1,4-butanediol, tetrahydrofuran, γ-butyrolacetone and other bulk chemicals, and polybutylene succinate (PBS), which aids in the degradation of polyester [3, 22]. Because of the depletion of fossil fuel resources and the strong demand for environmentally friendly energy, the biological production of succinic acid has attracted great interest. A wide variety of strains have been used for succinic acid production; the most studied strains are *Mannheimia succiniciproducens* [10, 19], *Actinobacillus succinogenes* [7], *Anaerobiospirillum succiniciproducen* [9, 18], and *Escherichia coli* [11, 27].

As 1 mol CO_2 is theoretically required for the synthesis of 1 mol succinic acid, CO_2 should play an important role in succinic acid production [14, 16, 25]. The CO_2 levels could regulate the PEP carboxykinase pathway used for succinic acid production by *A. succiniciproducens* [21], *A. succinogenes* [25]and *M. succiniciproducens* [23]. Higher

succinic acid production is obtained at a higher CO_2 level. Wang et al. [26] showed that in *E. coli*, the use of HCO_3^-, such as $MgCO_3$ and $NaHCO_3$, could increase the activity of phosphoenolpyruvate carboxylase from 0.2 to 1.13 Umg^{-1} protein to increase succinic acid production further.

The extracellular CO_2 environment is important in maintaining suitable CO_2 levels for succinic acid production. A four-process explicit model describing the CO_2 transfer and utilization was proposed for recombinant *E. coli* AFP111. The model predicted that at CO_2 concentrations below 30–40%, the system becomes limited by gas phase CO_2, whereas at higher CO_2 concentrations, the system is limited by PEP carboxylase enzyme kinetics [14]. A modeling of dissolved CO_2 concentration in a medium for *M. succiniciproducens* was also proposed to investigate the extracellular environment of CO_2 fixation for succinic acid production [23].

Actinobacillus succinogenes has been considered one of the most promising strains for industrial succinic acid production because of its ability to produce a comparatively large amount of succinic acid, use a wide range of carbon sources, and tolerate high concentrations of organic acids [5, 12, 30]. However, the extracellular culture conditions for succinic acid production by *A. succinogenes* have not been investigated in detail. In this paper, we investigated the effects of the extracelluar environmental conditions in CO_2 fixation for succinic acid production by *A. succinogenes* based on the model of available CO_2.

Materials and methods

Chemicals and materials

All chemicals were of reagent grade received from either Sinochem (Shanghai, P.R. China) or Fluka Chemical (Buchs, Switzerland). N_2 and CO_2 were obtained from Nanjing Special Gases Factory (Nanjing, P.R. China).

Microorganism and growth conditions

A. succinogenes NJ113 (China General Microbiological Culture Collection Center, CGMCC no. 1716) was used in all experiments. Cells were grown in 100-ml sealed anaerobic bottles containing 50 ml medium. The medium for inoculum cultures was composed of (per liter): 10.0 g glucose, 5.0 g yeast extract, 10.0 g $NaHCO_3$, 8.5 g $NaH_2PO_4{\cdot}H_2O$, and 15.5 g K_2HPO_4. Medium was heat sterilized at 121°C for 15 min. Anaerobic bottles were inoculated with 1 ml of a −70°C glycerol stock culture and incubated at 37°C.

For anaerobic bottle cultivation, exponentially growing cells were inoculated into 100-ml sealed anaerobic bottles filled with 30 ml of BM medium containing the following (per liter): 3.0 g KH_2PO_4, 0.2 g $MgCl_2{\cdot}6H_2O$, 0.2 g $CaCl_2$, 1.0 g NaCl, 50 g glucose, and 10 g yeast extract. The pH of the medium was maintained by the addition of 50 g/l $MgCO_3$. The anaerobic bottle cultivation was carried out in a rotary shaker at 37°C and 180 rpm. Batch fermentation was conducted in a 3-l fermentor (Bioflo 110, USA) with an initial broth volume of 1.2 l fermentation medium containing the following (per liter): 3.0 g KH_2PO_4, 0.2 g $MgCl_2{\cdot}6H_2O$, 0.2 g $CaCl_2$, 1.0 g NaCl, 5.0 g corn steep liquor, and 10.0 g yeast extract. The glucose was separately sterilized and added to the medium at a final concentration of 50 g l^{-1}. While investigating the effect of the CO_2 fixation process on one culture condition, the other conditions remained unchanged during the fermentation process. Batch fermentation were firstly carried out in a 3-l fermentor at 0.1 MPa, 37°C, pH of 6.8 and 400 r min^{-1} stirring speed without supplementation of carbonate. The CO_2 partial pressures, culture pH, supplementation of carbonate, culture temperature, and agitation rate of the CO_2 fixation for succinic acid production were investigated by batch fermentation. The pH was controlled by automatically adding 10 mol l^{-1} NaOH. The results of all batch experiments reported are the averages from duplicate experiments and were calibrated for alkali neutralization.

Model of available CO_2

The dissolved CO_2 in the broth was made according to the previous study [23], which was mainly based on chemical reaction equilibrium and phase equilibrium when the CO_2 dissolves in the broth. The model was as follows:

Chemical reaction equilibrium

CO_2 and carbonate have the following chemical reaction equilibrium in the steady-state condition:

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \quad K_1 = \frac{[H_2CO_3]}{[CO_2]} \tag{1}$$

$$H_2CO_3 \leftrightarrow HCO_3^- + H^+ \quad K_2 = \frac{[HCO_3^-][H^+]}{H_2CO_3} \tag{2}$$

$$HCO_3^- \leftrightarrow H^+ + CO_3^{2-} \quad K_3 = \frac{[CO_3^{2-}][H^+]}{[HCO_3^-]} \tag{3}$$

As the carbonic acid is very unstable in solution and easily broken down into HCO_3 and H^+, Eqs. 1 and 2 can be combined into Eq.4.

$$CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$$
$$K_4 = K_1 \times K_2 = \frac{[HCO_3^-][H^+]}{[CO_2]} \tag{4}$$

α_0 is expressed as the proportion of CO_2 in the total carbonate and has the following expression:

$$\alpha_0 = \frac{[CO_2]}{[CO_2] + [HCO_3^-] + [CO_3^{2-}]} \quad (5)$$

The K_3 and K_4 were substituted into the above expression, may:

$$\alpha_0 = \frac{1}{1 + K_4/[H^+] + K_4K_3/[H^+]^2} \quad (6)$$

Supposed :$C_T = [CO_2] + [HCO_3^-] + [CO_3^{2-}]$,there

$$[CO_2] = \frac{C_T}{1 + K_4/[H^+] + K_4K_3/[H^+]^2} \quad (7)$$

where K_3 and K_4 are dissociation constants with $K_4 = 5.35 \times 10^{-7}$,$K_3 = 6.12 \times 10^{-11}$(39°C)$[H^+]$ is the concentration of H^+ in solution, which is be derived from the pH value of the medium.

Phase equilibrium

In the steady-state condition, CO_2 stays balanced between the gas phase and the liquid phase. According to Henry's law:

$$[CO_2] = P_{CO_2}/H_0 \quad (8)$$

where $[CO_2]$ is the CO_2 concentration dissolved in a liquid (mol l^{-1}), P_{CO2} is the CO_2 partial pressure in a gas mixture (KPa), and H_0 is the Henry's constant for CO_2 in a pure solvent (kPa m^3 $kmol^{-1}$).

A culture medium contains various kinds of salts (i) and organic substances (j), which is the result of CO_2 gas dissolved in the liquid phase. According to Eberhard Rischbieter and Adrian Schumpe and others, to amend the Eberhard Rischbieter and Adrian Schumpe formula, the equation in the existence of organic amendments to the media and ion system was proposed as:

$$\begin{aligned} \log(H/H_0) &= \log\left(C_{G,0}/C_G\right) = \sum_i h_G C_i + \sum_j K_{n,j} C_{n,j} \\ &= \sum_i \left(hi + h_{G,0} + h_{G,T}(T/K - 298.15)\right) C_i \\ &\quad + \sum_j \left(b_n + b_{G,0} + b_{G,T}(T/K - 298.15)\right) C_{n,j} \end{aligned} \quad (9)$$

where h is the ion coefficient, and h_i is the inorganic ion coefficient in medium (298.15 K)(m^3 $kmol^{-1}$): Na^+, 0.1143; K^+, 0.0922; Mg^{2+}, 0.1694; H^+, 0; Cl^-, 0.0318; HPO_4^{2-}, 0.1499; OH^-, 0.0839; HCO_3^-, 0.0967; CO_3^{2-}, 0.1423; CH_3COO^-, 0.9000; $H_2PO_4^-$, 0.0906. The $h_{G,0}$ of CO_2 is -0.0172 m^3 $kmol^{-1}$; $h_{G,T}$ is -0.338×10^{-3} m^3 $kmol^{-1}$ K^{-1}. C_i is the ion concentration (g l^{-1}). b is the organic constant; the b_n values of yeast extract, glucose, and corn steep liquor in medium were (288–323 K)(m^3 kg^{-1}): 7.9×10^{-4}, 6.68×10^{-4}, 2.11×10^{-4}. The values of $b_{G,0}$ and $b_{G,T}$ concerning CO_2 were respectively -1.86×10^{-4} m^3 kg^{-1} and 0.01×10^{-4} m^3 kg^{-1} K^{-1}. C_n is the concentration of organic matter(mol l^{-1}).

Using Henry's constant H and P_{CO2} expressed C_T was as follows:

$$C_T = [CO_2]/\alpha_0 = P_{CO_2}/(\alpha_0 \times H) \quad (10)$$

Analytical methods

The dry cell weight (DCW) was computed from a curve relating optical density at 660 nm (OD_{660}) to dry weight. An OD_{660} of 1.0 represented 520 mg of dry weight per liter. Glucose was analyzed by an SBA-40C biosensor analyzer (Institute of Biology, Shandong Province Academy of Sciences, P.R. China). Fermentation products, including succinic acid, acetic acid, and formic acid, were analyzed by high-performance liquid chromatography (Chromeleon server monitor, UVD 170U detector, P680 pump, Dionex, USA). To determine the fermentation products, an ion exchange chromatographic column (Prevail organic acid column, Grace, USA) was used, and 25 mM KH_2PO_4 (adjusted to pH 2.5 by H_3PO_4) was used as the mobile phase with a flow rate of 1 ml/min.

Enzyme assays

For preparation of cell extracts, 30 ml of cells was taken from the mid-exponential phase in a 50-ml centrifuge tube and harvested by centrifugation for 10 min at 10,000 *g* and 4°C. After centrifugation, the supernatant was decanted, and the cell pellet was washed twice with 100 mM Tris-HCl (pH 7.0) containing 20 mM KCl, 5 mM $MnSO_4$, 2 mM DTT, and 0.1 mM EDTA. The washed cell pellet was resuspended in the same buffer, and the supernatant was placed in an ice bath for 20 min ultrasonication. Afterwards, the supernatant was centrifuged for 20 min at 10,000 *g* and 4°C. The supernatant was then transferred into another centrifuge tube and stored at −80°C until required.

Protein concentration in the cell-free extracts was determined by Bradford assay using bovine serum albumin as standard [4]. The average protein concentration in the cell extract prepared was 17.10 mg ml^{-1}.

PEP carboxy kinase (PCK) activity assay

300 mM Tris-HCl (pH 6.6), 300 mM $MgCl_2$, 150 mM $MnCl_2$, 1.125 M $NaHCO_3$, 12 U ml^{-1} malate dehydrogenase, 100 mM ADP·Na_2, 3 mM NADH, 150 mM PEP, and cell extracts. All the substrates were placed in a water bath for 20 min at 37°C. The initial reaction rate was measured by an online UV-Vis spectrophotometer at 340 nm.

Pyruvic acid kinase (PYK) activity assay

For the assay, 300 mM Tris-HCl (pH 7.5), 300 mM $MgCl_2$, 750 mM KCl, 12 U ml^{-1} lactic acid dehydrogenase, 100 mM ADP·Na_2, 3 mM NADH,150 mM PEP, and cell extracts were used. All the substrates were placed in a water bath for 20 min at 37 °C. The initial reaction rate was measured by online UV-Vis spectrophotometer at 340 nm.

Definition of activity units

A unit of enzyme activity, defined as 1 nmol substrates, was catalyzed for 1 min and then converted to the product volume. The number of units of the enzyme activity was contained in per mg of protein.

$$\text{Enzyme activity unit } (\text{Uml}^{-1}) = \frac{K_S \times K' \times 1000 \times V_T}{V_{C.E.}}$$

where K_s is the standard curve slope of NADH expressed as dc/dA_{340} (dc is the molarity of NADH; dA340 is the initial reaction rate, which was measured by online UV-Vis spectrophotometer at 340 nm); K′ is the absorbance of the measured sample that changes with the slope of the curve over time; V_T is the total volume of the reaction solution, and $V_{C.E.}$ the sample volume of the cell extracts.

Specific activity (U mg^{-1}) = Enzyme activity unit/Protein concentration.

CO_2 fixation rate (g l^{-1} h^{-1}), SA (succinic acid) productivity (g l^{-1} h^{-1}), and SA yield (%)

The CO_2 fixation rate (g l^{-1} h^{-1}) was calculated as SA mass concentration (g l^{-1})/Fermentation time (h) × 0.373 (0.373 for the synthesis of succinic acid reactive CO_2 conversion factor). SA productivity (g l^{-1} h^{-1}) was calculated as SA mass concentration (g l^{-1})/Fermentation time(h). SA yield (%) was calculated as SA mass concentration (g l^{-1})/Consumption of glucose (g l^{-1}).

Results and discussion

Available CO_2 for *A. succinogenes*

CO_2 can directly permeate cell membranes and be used as a substrate, whereas HCO_3^- and CO_3^- need ATP to bind to protein for transport [29]. Bacteria utilize CO_2 in the presence of HCO_3^-, CO_3^{2-}, and CO_2 [14]. Therefore, the dissolved CO_2 (dCO_2) in the broth is directly related to the supply of intracellular CO_2 [2] and consistently complied with Henry's law, which maintained a proportion with the CO_2 partial pressure. Furthermore, HCO_3^- and CO_3^{2-} can be dissociated from CO_2, which can be converted to dCO_2 when the CO_2 utilization rate is greater than the dissolution rate of CO_2 in the process of biomass fermentation. The available CO_2 in the broth, C_T (the total carbon content), should be the sum of dCO_2, HCO_3^-, and CO_3^{2-}. In conclusion, as shown in the model, the extracellular availability of CO_2 could be influenced by the medium composition, CO_2 partial pressure, carbonate content, temperature, and other factors when an external CO_2 exchange exists.

Effect of culture medium composition on dissolved CO_2

The inorganic salts in the medium and the presence of the organic components had a negative effect on Henry's constant, consequently influencing the dCO_2 in the broth. Henry's constant at 37°C is $H_0 = 3.989 \times 10^3$ kPa m^3 $kmol^{-1}$[6]. Therefore, the following equation is obtained:

$$\log(H^*/H_0) = \log\left(C_{G,0}/C_G\right) = 0.0438$$

$$H^* = 1.105 \times H_0 = 4.412 \times 10^3 \text{ kPa m}^3 \text{ kmol}^{-1}.$$

The dCO_2 in the medium is calculated to be 0.0259 mol l^{-1} at 37°C and 0.1 MPa, 10.5% lower than that found in pure water. A previous study shows dCO_2 in the fermentation medium is 6.1% lower than that found in pure water [23]. This might be the difference from the medium components and temperature.

Effect of CO_2 partial pressures on CO_2 fixation and succinic acid production

In the model, Henry's law shows that different gas partial pressures cause different dissolved gasses. Therefore, the effects of different CO_2 partial pressures ranging from 0 to 0.10 MPa (CO_2 and N_2 are mixed in proportion) on dCO_2, C_T, and succinic acid fermentation were investigated (Table 1). As shown in Table 1, when the CO_2 gas was not supplied to the broth, the succinic acid fermentation was severely inhibited. By increasing the partial pressure of CO_2, dCO_2 and C_T increased. The ratio of succinic acid to acetic acid (SA/Ac) also increased, indicating an increase in the succinic acid pathway flux and a decrease in the by-product acetic acid flux. When the CO_2 gas partial pressure reached 0.1 MPa, the CO_2 fixation rate and succinic acid production rate reached 1 time and the SA/Ac increased to 1.3 times compared to the gas partial pressure reaching 0.025 MPa. Therefore, CO_2 is not only a substrate for succinic acid production, but is also necessary for cell growth. Unfortunately, because the maximum gas partial pressure of the fermentor in this study was 0.10 MPa, the higher CO_2 partial pressures were not considered.

Table 1 Effect of different CO_2 partial pressures on the CO_2 fixation by NJ113

CO_2: partial pressures (MPa)	0.00	0.025	0.05	0.075	0.10
dCO_2 (mM)	0.00 ± 0	5.67 ± 0.12	11.3 ± 0.20	17.0 ± 0.19	22.7 ± 0.16
C_T(mM)	0.00 ± 0	24.8 ± 0.23	49.5 ± 0.39	74.5 ± 0.64	99.9 ± 0.87
DCW_{max} (g l^{-1})	0.72 ± 0.03	2.28 ± 0.11	2.45 ± 0.09	2.87 ± 0.10	3.05 ± 0.13
CO: fixation rate (g l^{-1} h^{-1}	–	0.20 ± 0.01	0.27 ± 0.03	0.36 ± 0.01	0.42 ± 0.03
SA productivitv (g l^{-1} h^{-1})	–	0.55 ± 0.03	0.73 ± 0.03	0.97 ± 0.02	1.14 ± 0.02
SA/Ac	–	2.1 ± 0.11	3.2 ± 0.13	4.2 ± 0.20	4.7 ± 0.16
SA yield (%)	–	55.4 ± 0.05	64.1 ± 0.03	69.6 ± 0.05	71.4 ± 0.04

DCW dry cell weight, – not detected, *SA* succinic acid, *Ac* acetic acid, *SA yield* g of SA formed/g of glucose utilized

Effect of carbonate supplementation on CO_2 fixation and succinic acid production

According to the model, when CO_2 is consumed by the bacteria, HCO_3^- and CO_3^{2-} can be converted to dCO_2. To investigate the effect of the supplementation of carbonate on the availability of CO_2 and succinic acid production, fermentations were carried out with supplementation of $CaCO_3$, $NaHCO_3$, and $MgCO_3$ in the medium. As the solubility of sodium bicarbonate in pure water reaches to 1.5 mol l^{-1} (39°C) [24], the added $NaHCO_3$ can be completely dissociated from HCO_3^-. Although calcium carbonate is a slightly soluble substance, its solubility is only 0.0053 mol l^{-1} (39°C) [17]; the maximum solubility of $MgCO_3$ in water at 40°C is reported to be 139 mM [15]. As shown in Table 2, when the supplement of $CaCO_3$ increased from 0.1 to 0.2 mol l^{-1}, no significant effects on C_T and succinic acid fermentation were observed. In contrast, when the $NaHCO_3$ was supplemented, the C_T and succinic acid fermentation improved. When 0.15 mol l^{-1} $NaHCO_3$ was supplemented, C_T, cell growth, and succinic acid productivity were enhanced. The supplementation of 0.1^{-1} ~ 0.2 mol l^{-1} of $MgCO_3$ had little influence on the CO_2 fixation rate, SA productivity, or SA/Ac. Previous studies showed the effects of sodium ions on succinic acid production [20], and the presence of a large number of sodium ions may have an impact on cell osmolarity [1, 13]. Thus, the indistinct influence of $NaHCO_3$ supplementation on succinic acid production, as compared to that of dCO_2, might be due to the high content of sodium ions present in the medium.

Effect of pH on CO_2 fixation and succinic acid production

Although pH does not directly affect dCO_2 in the fermentation broth, it can affect the C_T and the PEP carboxylase kinase activity, which is the key enzyme for CO_2 fixation in *A. succinogenes*. Fermentations were carried out at pH levels of 6.2, 6.8, and 7.4 to investigate their effects on cell growth and succinic acid production. Assays of PCK and PYK at different pH levels were also performed.

Table 3 shows that the lowest C_T content and the worst cell growth were obtained at a pH of 6.2. In contrast, the C_T content at pH 7.4 was 7.3 times higher than that at pH 6.2. However, the optimum pH for succinic acid production was 6.8. The C_T at pH 7.4 was the highest, but it was not the optimum pH. The key enzyme of the fixed CO_2 synthesized at pH 7.4 was not suitable. Samuelov reported that the activity of PEP carboxylase kinase at pH 6.2 was 35 times more than at pH 7.4 in *A. succiniciproducens* [21]. As shown in Fig. 1, PEP is an important node in the *A. succinogenes* metabolic pathway. By catalyzing with the PCK activity, PEP transfers to the oxaloacetate and then generates the succinic acid. However, upon catalization by the PYK activity, the PEP will convert to pyruvic acid, and further transform into formic acid, acetic acid, and other by-products [8, 20]. The analysis of key enzyme activities in the C4 and C3 pathways showed that the PYK activity increased by increasing the pH value while PCK did not. Previous studies showed that PCK plays the most important role in the carboxylation reaction, generating ATP in *M. succiniciproducens* [23]. This high PCK activity suggested that the availability of enough CO_2 was obligatory not only for the production of succinic acid, but also for the growth of the bacteria by generating ATP, while the enzyme PYK did not. This may be true in *Actinobacillus succinogenes*. In addition, the external medium pH affects the cytoplasmic pH, which may determined the PCK activity [25]. At pH 6.8, the PCK activity was the highest, and the cell dry weight and the CO_2 fixation rate reached their maximum. The maximum PYK activity was obtained at pH 7.4, which was 1.65 times higher than at pH 6.8. However, the PCK at pH 7.4 was 0.66 times higher than that at pH 6.8 (Fig. 2). The SA/Ac at pH 6.8 was 2.19 times higher than at pH 7.4, indicating that more PEP was used in the succinic acid production at pH 6.8.

Table 2 Effect of carbonate supplementation on CO2 fixation by NJ113

Carbonate	$CaCO_3$(mol l^{-1})			$NaHCO_3$ (mol l^{-1})			$MgCO_3$ (mol l^{-1})		
	0.1	0.15	0.2	0.1	0.15	0.2	0.1	0.15	0.2
C_T(mM)	105.2 ± 0.10	105.2 ± 0.10	105.2 ± 0.10	199.9 ± 0.16	249.9 ± 0.20	299.9 ± 0.14	199.9 ± 0.23	238.9 ± 0.31	238.9 ± 0.33
DCW_{max} (g l^{-1})	3.05 ± 0.13	3.10 ± 0.11	3.02 ± 0.16	3.16 ± 0.08	3.25 ± 0.12	3.24 ± 0.16	3.11 ± 0.22	3.18 ± 0.27	3.16 ± 0.19
CO: fixation rate (g l^{-1} h^{-1}	0.42 ± 0.02	0.43 ± 0.03	0.41 ± 0.02	0.48 ± 0.01	0.52 ± 0.01	0.51 ± 0.02	0.46 ± 0.02	0.47 ± 0.01	0.44 ± 0.01
SA productivitv (g l^{-1} h^{-1})	1.13 ± 0.03	1.16 ± 0.03	1.10 ± 0.01	1.29 ± 0.02	1.40 ± 0.01	137 ± 0.01	1.22 ± 0.03	126 ± 0.01	1.19 ± 0.03
SA/Ac	4.7 ± 0.21	4.8 ± 0.19	4.6 ± 0.26	5.0 ± 0.17	5.2 ± 0.11	5.2 ± 0.13	4.9 ± 0.21	4.9 ± 0.13	5.1 ± 0.20
SA yield (%)	71.0 ± 0.06	71.5 ± 0.02	71.6 ± 0.02	74.6 ± 0.01	75.1 ± 0.03	75.7 ± 0.02	72.9 ± 0.03	74.4 ± 0.04	75.2 ± 0.01

The C_T is the sum of the C_T at different pHs plus dissolved carbonate

Table 3 Effect of pH on CO_2 fixation by NJ113

pH	6.2	6.8	7.4
C_T(mM)	42.5 ± 0.76	99.9 ± 1.32	310.3 ± 3.42
DCW_{max} (g l^{-1})	2.96 ± 0.15	3.12 ± 0.16	3.26 ± 0.16
CO: fixation rate (g l^{-1} h^{-1}	0.40 ± 0.01	0.44 ± 0.01	0.36 ± 0.01
SA productivitv (g l^{-1} h^{-1})	0.83 ± 0.02	1.19 ± 0.03	0.86 ± 0.03
SA yield (%)	67.0 ± 0.01	71.8 ± 0.02	51.2 ± 0.01
SA/Ac	3.8 ± 0.24	4.6 ± 0.17	2.1 ± 0.22

The C_T are based on the Herry constent and pH [23]

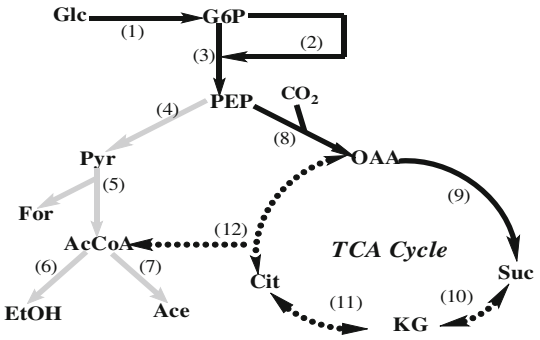

Fig. 1 Simplified metabolic map of the central map of the central metabolism of *A. succinogenes* [16]. *Thin black arrows* represent glucose uptake, pentose phosephate pathway, and Embden-Meyerhoff-Parnas pathway reactions. *Gray arrows* represent C3 pathway reactions. *Thick black arrows* represent C4 pathway reactions. *Dashed arrows* represent TCA-associatated reactions that have not been tested. *1* hexokinase or PEP: glucose phosphotransferase, *2* pentose phosphate pathway, *3* Embden-Meyerhoff-Parnaa pathway, *4* pyruvate kinase and PEP: glucose phosphotransferase, *5* pyruvate-formate lyase, *6* acetaldehyde dehydrogenase and alcohol dehydrogenase, *7* phosphotransacetylase and acetate kinase, *8* PEP carboxykinase, *9* malate dehydrogenase, fumarase, and fumarate reductase, *10* succinyl-CoA synthetase, αKG dehydrogenase, and αKG synthase, *11* isocitrate dehydrogenase and aconitasem, *12* citrate lyase and citrate synthase. Metabolites: *Glc* glucose, *G6P* glucose-6-phosphate, *Pyr* pyruvate, *For* formate, *AcCoA* acetyl-CoA, *EtOH* ethanol, *Ace* acetate, *OAA* oxaloacctate, *Suc* succinate, *Cit* citrate

Effect of temperature on CO_2 fixation and succinic acid production

The model suggests that temperature can affect Henry's coefficient and further affect dCO_2. Moreover, low temperature is conducive to CO_2 dissolution. The temperature also affects cell growth and product synthesis. The culture temperature of the cell growth and succinic acid production is shown in Fig. 3. Poor cell growth and CO_2 fixation rate were observed at 41°C, which could be caused by the CO_2 solubility being the lowest and the temperature being too high for the growth of bacteria. The succinic acid reached the maximum with an incubation temperature of 37°C, whereas the cell dry weight was at its highest with a CO_2

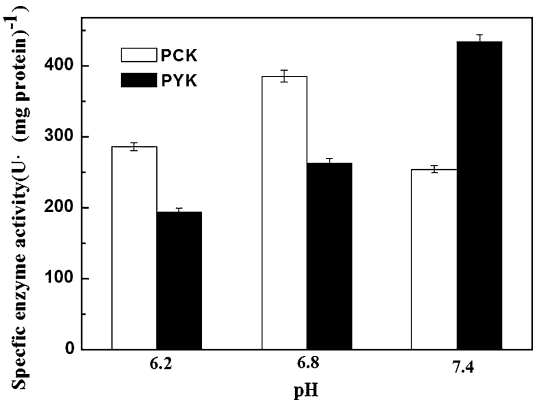

Fig. 2 Effect of pH on the specific enzyme activity of PCK and PYK. Cells were grown in anaerobic conditions with an initial total sugar concentration of 50 g/l for 28 h

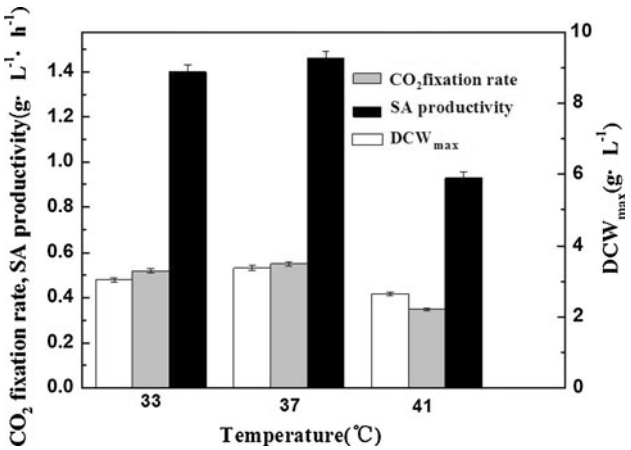

Fig. 3 Effect of temperature on CO_2 fixation by NJ113. *SA* Succinic acid, *DCW* dry cell weight

fixation rate. Thus, the temperature of 37°C is the optimum temperature for bacterial growth and succinic acid production.

Effect of the agitation rate on CO_2 fixation and succinic acid production

Unlike other anaerobic fermentations, the succinic acid anaerobic fermentation process needs fixed CO_2. Therefore, changing the stirring speed can affect the gas-liquid contact area of CO_2 gas and the fermentation broth, affecting the rate of CO_2 fixation. When the stirring speed is within the range of 0–400 r min^{-1}, inspecting the CO_2 fixation rate and succinic acid production rate is necessary.

When the stirring speed was 0, the CO_2 fixation rate minimum was only at 0.30 g l^{-1} h^{-1}. This indicates that the mass transfer between the CO_2 gas and the fermentation liquid was less effective and resulted in the fermentation system's lack of available CO_2 supply. If the CO_2 fixation rate was low, the metabolic flux flowed to the branch of the

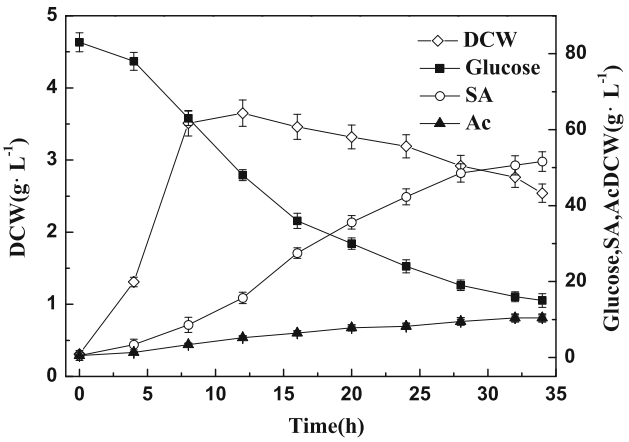

Fig. 4 Succinic acid production by NJ113 with CO_2 fixation in a 3-l fermentor. Cells were grown in anaerobic conditions with an initial total sugar concentration of 80 g/l for 35 h

pathway. The minimum CO_2 fixation rate of 0.48 g l^{-1} h^{-1} was obtained at 100 r min^{-1}. However, after the stirring speed was increased to 200 r min^{-1}, the CO_2 the fixation rate and the succinic acid production rate became stable at 0.53 g l^{-1} h^{-1} and 1.41 g l^{-1} h^{-1}, respectively. Further increasing the stirring speed from 200 to 400 r min^{-1} caused little change in the CO_2 the fixation rate and the succinic acid production rate. Therefore, 200 r min^{-1} was the optimal stirring speed for succinic acid production.

Succinic acid production by NJ113 with CO_2 fixation

Batch fermentation was carried out in a 3-l fermentor at 37°C and a pH of 6.8 with CO_2 partial pressure of 0.1 MPa; 0.15 mol l^{-1} $NaHCO_3$ was added at 200 r min^{-1} stirring speed. The process of succinic acid production by NJ113 with CO_2 fixation is shown in Fig. 4.

The strains in the logarithmic phase fixed the fastest rate of CO_2 production of succinic acid. Cell growth was slow and reached its maximum in the first 12 h. After 12 h, cell growth gradually declined. The glucose consumption rate was also reduced when the fermentation lasted for 34 h, and the residual glucose of 15.0 g l^{-1} was no longer consumed. As a result, the final SA concentration reached 51.6 g l^{-1}, SA/Ac was attained up to 5.0, the CO_2 fixation rate reached 0.57 g l^{-1} h^{-1}, and the SA productivity was 1.52 g l^{-1} h^{-1} with a yield of 75.8%. Compared with the data before the optimization, the yield of succinic acid was enhanced by 35.2%.

Conclusions

CO_2 levels play an important role in the biosynthesis of succinic acid, which not only promotes cell growth, but

also uses glucose as a substrate for succinic acid production. In this paper, the culture conditions related to CO_2 supply were optimized for succinic acid production using *A. succinogenes* NJ113. During batch fermentation, culture medium composition, CO_2 partial pressures, carbonate supplementation, pH, temperature, and agitation had a significant effect on the CO_2 supply. Under optimized culture conditions, the CO_2 fixation rate and SA yield greatly improved.

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References

1. Andersson C, Helmerius J, Hodge D, Berglund KA, Rova U (2009) Inhibition of succinic acid production in metabolically engineered *Escherichia Coli* by neutralizing agent, organic acids, and osmolarity. Biotechnol Prog 25:116–123
2. Baez A, Flores N, Bolivar F, Ramirez OT (2009) Metabolic and transcriptional response of recombinant *Escherichia coli* to elevated dissolved carbon dioxide concentrations. Biotechnol Bioeng 104:102–110
3. Bechthold I, Bretz K, Kabasci S, Kopitzky R, Springer A (2008) Succinic acid: a new platform chemical for biobased polymers from renewable resources. Chem Eng Technol 31:647–654
4. Bradford MM (1976) Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. Anal Biochem 72:248–254
5. Chen KQ, Jiang M, Wei P, Yao JM, Wu H (2010) Succinic acid production from acid hydrolysate of corn fiber by *Actinobacillus succinogenes*. Appl Biochem Biotech 160:477–485
6. Gros JB, Dussap CG, Catte M (1999) Estimation of O_2 and CO_2 solubility in microbial culture media. Biotechnol Progr 15:923–927
7. Guettler MV, Rumler D, Jain MK (1999) *Actinobacillus succinogenes* sp. nov., a novel succinic-acid-producing strain from the bovine rumen. Int J Syst Bacteriol 49:207–216
8. Laivenieks M, Vieille C, Zeikus JG (1997) Cloning, sequencing, and overexpression of the *Anaerobiospirillum succiniciproducens* phosphoenolpyruvate carboxykinase (pckA) gene. Appl Environ Microb 63:2273–2280
9. Lee PC, Lee WG, Lee SY, Chang HN (2001) Succinic acid production with reduced by-product formation in the fermentation of *Anaerobiospirillum succiniciproducens* using glycerol as a carbon source. Biotechnol Bioeng 72:41–48
10. Lee PC, Lee SY, Hong SH, Chang HN (2002) Isolation and characterization of a new succinic acid-producing bacterium, *Mannheimia succiniciproducens* MBEL55E, from bovine rumen. Appl Microbiol Biot 58:663–668
11. Lin H, Bennett GN, San KY (2005) Genetic reconstruction of the aerobic central metabolism in *Escherichia coli* for the absolute aerobic production of succinate. Biotechnol Bioeng 89:148–156
12. Liu YP, Zheng P, Sun ZH, Ni Y, Dong JJ, Zhu LL (2008) Economical succinic acid production from cane molasses by *Actinobacillus succinogenes*. Bioresource Technol 99:1736–1742
13. Liu YP, Zheng P, Sun ZH, Ni Y, Dong JJ, Wei P (2008) Strategies of pH control and glucose-fed batch fermentation for production of succinic acid by *Actinobacillus succinogenes* CGMCC1593. J Chem Technol Biot 83:722–729
14. Lu SY, Eiteman MA, Altman E (2009) Effect of CO_2 on succinate production in dual-phase *Escherichia coli* fermentations. J Biotechnol 143:213–223
15. Mark HF, Other DF, Overberger CG, Seaborg GT (eds) (1981) Kirk-Othmer encyclopedia of chemical technology. Wiley, New York
16. McKinlay JB, Zeikus JG, Vieille C (2005) Insights into *Actinobacillus succinogenes* fermentative metabolism in a chemically defined growth medium. Appl Environ Microb 71:6651–6656
17. Mucci A (1983) The solubility of calcite and aragonite in seawater at various salinities, temperatures, and one atmosphere total pressure. Am J Sci 283:780–799
18. Nghiem NP, Davison BH, Suttle BE, Richardson GR (1997) Production of succinic acid by *Anaerobiospirillum succiniciproducens*. Appl Biochem Biotechnol 63–5:565–576
19. Oh IJ, Lee HW, Park CH, Lee SY, Lee J (2008) Succinic acid production by continuous fermentation process using *Mannheimia succiniciproducens* LPK7. J Microbiol Biotechn 18:908–912
20. Podkovyrov SM, Zeikus JG (1993) Purification and characterization of phosphoenolpyruvate carboxykinase, a catabolic CO_2-fixing enzyme, from *Anaerobiospirillum-Succiniciproducens*. J Gen Microbiol 139:223–228
21. Samuelov NS, Lamed R, Lowe S, Zeikus JG (1991) Influence of CO_2-HCO_3- Levels and pH on growth, succinate production, and enzyme-activities of *Anaerobiospirillum-Succiniciproducens*. Appl Environ Microb 57:3013–3019
22. Song H, Lee SY (2006) Production of succinic acid by bacterial fermentation. Enzyme Microb Tech 39:352–361
23. Song HH, Lee JW, Choi S, You JK, Hong WH, Lee SY (2007) Effects of dissol ved CO_2 levels on the growth of *Mannheimia succiniciproducens* and succinic acid production. Biotechnol Bioeng 98:1296–1304
24. Speight JG (2005) Lange's handbook of chemistry (16th edn). McGraw-Hill, NewYork
25. VanderWerf MJ, Guettler MV, Jain MK, Zeikus JG (1997) Environmental and physiological factors affecting the succinate product ratio during carbohydrate fermentation by *Actinobacillus* sp. 130Z. Arch Microbiol 167:332–342
26. Wang D, Li Q, Li WL, Xing JM, Su ZG (2009) Improvement of succinate production by overexpression of a cyanobacterial carbonic anhydrase in *Escherichia coli*. Enzyme Microb Tech 45:491–497
27. Wu H, Li ZM, Zhou L, Ye Q (2007) Improved succinic acid production in the anaerobic culture of an *Escherichia coli PflB ldhA* double mutant as a result of enhanced anaplerotic activities in the preceding aerobic culture. Appl Environ Microb 73: 7837–7843
28. Zeikus JG, Jain MK, Elankovan P (1999) Biotechnology of succinic acid production and markets for derived industrial products. Appl Microbiol Biot 51:545–552
29. Zhang K, Kurano N, Miyachi S (2002) Optimized aeration by carbon dioxide gas for microalgal production and mass transfer characterization in a vertical flat-plate photobioreactor. Bioproc Biosyst Eng 25:97–101
30. Zheng P, Fang L, Xu Y, Dong JJ, Ni Y, Sun ZH (2010) Succinic acid production from corn stover by simultaneous saccharification and fermentation using *Actinobacillus succinogenes*. Bioresource Technol 101:7889–7894