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Kinetic Study of Organic Acid Formations and Growth of *Anaerobiospirillum succiniciproducens* During Continuous Cultures

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Succinic acid-producing *Anaerobiospirillum succiniciproducens* was anaerobically grown in glucose-fed continuous cultures using glucose as a carbon source, and the metabolic flexibility of *A. succiniciproducens* in response to varying glucose concentrations and dilution rates was examined. Both succinic acid (SA) and acetic acid (AA) formation was growth-associated, and their growth-rate-related coefficients ($K_{SA/X}$, $K_{AA/X}$) and nongrowth-rate-related coefficients ($K'_{SA/X}$, $K'_{AA/X}$) were slightly influenced by glucose concentrations. A high glucose concentration (38 g/l) and high growth rate (0.63 h^{-1}) did not induce by-product formation.

Keywords: Anaerobic continuous culture, succinic acid, *Anaerobiospirillum succiniciproducens*, acetic acid

Succinic acid is a dicarboxylic acid produced as an intermediate of the tricarboxylic acid cycle and also as one of the fermentation products of anaerobic metabolism [27]. Succinic acid has recently emerged as an important chemical because it can be used for the manufacturing of synthetic resins and biodegradable polymers and as an intermediate for the synthesis of various chemicals [17]. To date, most succinic acid has been produced by chemical processes that use fossil resources. However, owing to the environmental benefits and increasing concerns of petroleum shortages, fermentative production of succinic acid from a renewable biomass by anaerobic bacteria has attracted great interest [6, 17]. Among the succinic acid-producing microorganisms, which include *Anaerobiospirillum succiniciproducens* [1, 23], *Actinobacillus succinogenes* [2, 16], *Mannheimia succiniciproducens* [9, 15], *Corynebacterium glutamicum* [20], and *Escherichia coli* [24], the strict anaerobic bacterium *A. succiniciproducens* has been considered one of the most attractive succinic acid producers because it is able to utilize several renewable resources such as whey (lactose) [11], glycerol [14], wood hydrolysates [10], and galactose [8]. Therefore, batch and fed-batch cultures of *A. succiniciproducens* have been carried out to better understand the environmental and physiological responses of the cell [12, 13, 19, 23].

Although a fundamental knowledge of this bacterium when produced by batch culture is of value, it is not sufficient and provides a rather limited understanding of succinic acid formation and cell growth. Continuous cultivation is a preferred system for biocommodity chemical production and kinetic studies [18, 25]. During continuous fermentation of glucose by *A. succiniciproducens*, succinic and lactic acids formation was found to strongly depend on the level of CO_2 [23]. Recently, internal and external membrane cell recycle systems using *A. succiniciproducens* were reported and physiological and metabolic changes under high-cell density conditions were described [7, 18]. However, information on steady-state parameters is still limited in regard to optimizing continuous succinic acid production using *A. succiniciproducens*. Furthermore, the formation of acetic acid as a by-product needs to be examined as well, because it affects the process of separating succinic acid from culture media [12].

The aim of this study was to establish a better understanding of the cell growth kinetics and succinic and acetic acids formation of *A. succiniciproducens* during continuous culture and to determine the optimal operation conditions for the continuous production of succinic acid with minimal formation of acetic acid as a by-product. *Anaerobiospirillum succiniciproducens* (ATCC 29305) was obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). Cells were grown in sealed anaerobic bottles containing 100 ml of minimal salts medium1 (AnS1), 10 g/l glucose, 2.5 g/l yeast extract, and 2.5 g/l polypeptone with CO_2 as the gas phase. The AnS1 medium contained per liter: 3 g K_2HPO_4, 1 g NaCl, 1 g $(NH_4)_2SO_4$, 0.2 g $CaCl_2 \cdot 2H_2O$, 0.2 g

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$MgCl_2 \cdot 6H_2O$, and 1 g Na_2CO_3. The medium was heat sterilized (15 min at 121°C) in an anaerobic bottle with a nitrogen headspace. The pH of the sterile media was adjusted to pH 6.5 by the addition of concentrated H_2SO_4. The nitrogen headspace was replaced by CO_2, and $Na_2S \cdot 9H_2O$ was added to a final concentration of 1 mg/l to establish strict anaerobic conditions. After 15 min, the reduced medium was inoculated with 2.5 ml of glycerol stock culture and incubated at 39°C for 12–13 h.

Continuous cultures were carried out at 39°C in a jar bioreactor (2.5 l; Korea Fermenter Company, Incheon, Korea) containing 400 ml of minimal salts medium2 (AnS2), 19 or 38 g glucose, 2.5 or 5 g yeast extract, and 5 or 10 g polypeptone. The AnS2 medium contained per liter: 3 g K_2HPO_4, 2 g NaCl, 5 g $(NH_4)_2SO_4$, 0.2 g $CaCl_2 \cdot 2H_2O$, 0.4 g $MgCl_2 \cdot 6H_2O$, 5 mg $FeSO_4 \cdot 7H_2O$, and 3 g Na_2CO_3. The pH was maintained of 6.5 using 1.5 M Na_2CO_3. Feeding solutions were purged with oxygen-free CO_2 gas for 24 h in order to establish anaerobic conditions before use. When the residual glucose concentration dropped to 2 g/l during the batch operations, a reduced feeding solution containing glucose was added at different dilution rates while an equal volume of spent medium was removed from the bioreactor. The new steady states were confirmed by the constant concentrations of cells, glucose, and end-products in the bioreactor for three consecutive samples taken at 6- or 8-h intervals after at least 5 turnovers. Foaming was controlled by the addition of Antifoam 289 (Sigma Chemical Co., St. Louis, MO, U.S.A.). CO_2 gas sparging rate and agitation speed were maintained at 0.25 vvm and 200 rpm, respectively. All chemicals used were of reagent grade and were obtained from either Junsei Chemical Co. (Tokyo, Japan) or Sigma Chemical Co. Gas was scrubbed free of oxygen by passing through a gas purifier (P.J. Cobert Associates, Inc., St. Louis, MO, U.S.A.). The concentrations of glucose, succinic acid, and acetic acid were measured by high-performance liquid chromatography (Hitachi L-3300 RI monitor, L-4200 UV-VIS detector, D2500 chromato-integrator; Tokyo, Japan) equipped with an ion-exchange column (Aminex HPX-87H, 300 mm×7.8 mm; Hercules, CA, U.S.A.) and 0.012 N H_2SO_4 was used as the mobile phase. Cell growth was monitored by measuring the absorbance at 660 nm (OD_{660}) using a spectrophotometer (Ultrospec3000; Pharmacia Biotech, Sweden). Dry cell weight (DCW) was calculated from a curve relating the OD_{660} to DCW: an OD_{660} of 1.0 represented 0.33 g DCW per liter. The theoretical maximum biomass yield, cell maintenance coefficient, and specific glucose uptake rate were determined according to a previously developed method [22]. Yields of succinic and acetic acids were defined as the amount of succinic and acetic acids produced from one gram of glucose consumed. Similarly, biomass yield was defined as the amount of biomass produced from one gram of glucose consumed. Carbon recovery calculations were carried out to verify data consistency at each steady state obtained during the continuous cultures. In these calculations, cell carbon was calculated with $CH_2O_{0.5}N_{0.21}$ [23] and succinic acid was assumed to have 3 mol carbon/mol product [succinate – (CO_2)] due to the relationship between CO_2 fixation and succinic acid formation during fermentation [26]. The yield and carbon recovery calculations were corrected by base dilution factors (*ca.* 8%–10%) due to the addition of 1.5 M Na_2CO_3 for pH control during cultivation.

Anaerobic continuous cultures were carried out at various dilution rates in complex mediums containing 19 or 38 g/l of glucose. These concentrations were chosen because they showed better performance in terms of cell growth

Table 1. Steady-state parameters and yields during anaerobic single-stage continuous fermentation of 19 g/l glucose by *A. succiniciproducens* at various dilution rates[a].

D (h^{-1})	X (g/l)	S (g/l)	SA (g/l)	AA (g/l)	$Y_{X/S}$ (g/g)	$Y_{SA/S}$ (g/g)	$Y_{AA/S}$ (g/g)	CR
0.056	0.96	0	15.0	3.8	0.068	0.88	0.22	0.95
0.10	1.06	0	14.7	3.8	0.081	0.87	0.22	0.94
0.18	1.30	0	14.7	3.8	0.082	0.87	0.22	0.95
0.22	1.35	0	14.1	3.9	0.088	0.84	0.23	0.94
0.27	1.45	2.1	13.7	3.9	0.089	0.86	0.25	0.98
0.29	1.42	2.6	13.1	3.9	0.091	0.85	0.25	1.01
0.31	1.49	3.1	12.2	3.8	0.11	0.84	0.25	0.97
0.36	1.47	3.4	11.7	3.6	0.11	0.83	0.25	0.95
0.43	1.47	3.8	11.3	3.3	0.11	0.86	0.25	0.94
0.52	1.49	6.4	10.2	3.0	0.068	0.85	0.25	1.02
0.58	1.45	6.7	9.7	2.8	0.081	0.87	0.25	1.00
0.63	1.27	8.9	8.3	2.3	0.082	0.86	0.24	1.05

[a] Initial glucose concentration in the feed, 19 g/l; pH 6.5.

D, dilution rate; X, cell concentration; S, glucose; SA, succinic acid; AA, acetic acid; $Y_{X/S}$, biomass yield; $Y_{SA/S}$, succinic acid yield; $Y_{AA/S}$, acetic acid yield; CR, carbon recovery.

Table 2. Steady-state parameters and yields during anaerobic single-stage continuous fermentation of 38 g/l glucose by *A. succiniciproducens* at various dilution rates[a].

D (h^{-1})	X (g/l)	S (g/l)	SA (g/l)	AA (g/l)	$Y_{X/S}$ (g/g)	$Y_{SA/S}$ (g/g)	$Y_{AA/S}$ (g/g)	CR
0.032	1.38	0.2	29.6	7.5	0.042	0.87	0.22	0.93
0.064	1.60	4.7	26.5	6.8	0.054	0.90	0.23	0.99
0.11	1.32	12	19.5	5.1	0.059	0.88	0.23	0.95
0.15	1.29	13.5	18.5	4.8	0.062	0.89	0.23	0.93
0.22	1.16	14	18.1	4.8	0.057	0.90	0.24	0.95
0.41	1.1	16.3	15.9	3.9	0.061	0.89	0.22	0.98
0.54	0.79	25.8	9.2	2.3	0.065	0.86	0.22	1.0

[a]Initial glucose concentration in the feed, 38 g/l glucose; pH 6.5.
D, dilution rate; X, cell concentration; S, glucose; SA, succinic acid; AA, acetic acid; $Y_{X/S}$, biomass yield; $Y_{SA/S}$, succinic acid yield; $Y_{AA/S}$, acetic acid yield; CR, carbon recovery.

and succinic acid formation of *A. succiniciproducens* during batch cultures [12]. When 19 g/l of glucose was in the feed media (Table 1), the steady-state cell concentration was nearly constant (1.46±0.02 g-DCW/l) at dilution rates (D) between 0.27 and 0.58 h^{-1}. However, the cell concentrations were lower at low (<0.22 h^{-1}) and high (>0.58 h^{-1}) D. At higher D=0.22 h^{-1}, glucose started to accumulate and reached a concentration of 8.9 g/l at D=0.64 h^{-1}. Accordingly, the steady state-concentration of succinic acid and acetic acid decreased from 15 to 8.3 g/l and from 3.8 to 2.3 g/l, respectively, with increasing D. Cell yield ($Y_{X/S}$) increased from 0.068 to 0.11 g-DCW/g-glucose when D increased from 0.056 to 0.43 h^{-1}, and thereafter, it decreased to 0.082 g-DCW/g-glucose at D=0.64 h^{-1}. Succinic acid yields ($Y_{SA/S}$) were maintained at 0.86±0.02 g-succinic acid/g-glucose, and acetic acid yields ($Y_{AA/S}$) remained at 0.24±0.02 g-acetic acid/g-glucose. The gram ratio of $Y_{SA/S}/Y_{AA/S}$ was in good agreement with the values previously obtained in batch cultures [12].

When 38 g/l of glucose was used in the feed (Table 2), the cell concentration decreased with increasing D. Consequently, glucose was incompletely consumed at even low D, and it continued to accumulate with increasing D. The steady-state concentration of succinic acid and acetic acid decreased from 27.6 to 9.2 g/l and from 7.5 to 2.3 g/l, respectively, with increasing D. $Y_{X/S}$ was much lower than the value obtained when 19 g/l of glucose was used in the feed: 0.042–0.065 g-DCW/g-glucose vs. 0.068–0.11 g-DCW/g-glucose. Higher $Y_{SA/S}$ (0.88–0.90 g-succinic acid/g-glucose) and lower $Y_{AA/S}$ (0.23–0.24 g-acetic acid/g-glucose) were observed.

The correlation between the specific rate of succinic acid production (q_{SA}) and specific growth rate (μ or D) was examined. As shown in Fig. 1A, succinic acid formation was clearly associated with D and significantly affected by glucose concentration in the feed medium. The growth-rate-related coefficient ($K_{SA/X}$) for succinic acid formation at 19 g/l and 38 g/l of glucose was 5.1 g-succinic acid/g-

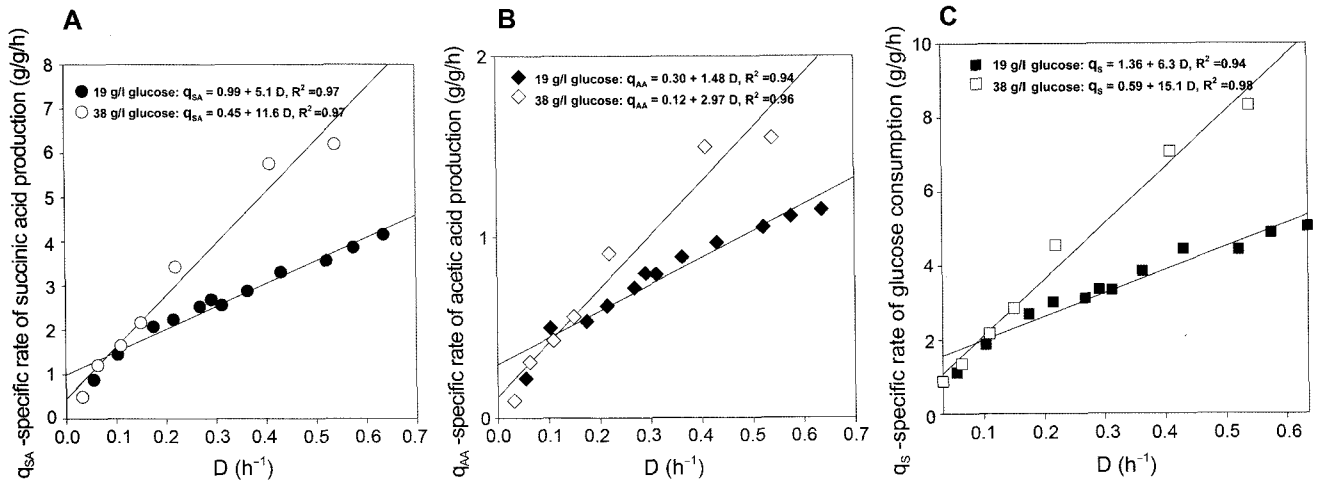

Fig. 1. Representation of the specific rate of succinic acid production (**A**), acetic acid production (**B**), and glucose consumption (**C**) versus the dilution rate used during an anaerobic continuous fermentation of glucose by *A. succiniciproducens*.
Open symbols are 19 g/l glucose and close symbols are 38 g/l glucose.

DCW (from the equation q_{SA}=0.99+5.1D) and 11.6 g-succinic acid/g-DCW (from the equation q_{SA}=0.45+11.6D), respectively. The nongrowth-rate-related coefficient ($K'_{SA/X}$) for succinic acid formation at 19 g/l and 38 g/l of glucose was 0.99 g-succinic acid/g-DCW and 0.45 g-succinic acid/g-DCW, respectively. The value of $K_{SA/X}/K'_{SA/X}$ at 38 g/l of glucose was 5 times higher than the value at 19 g/l of glucose, indicating that succinic acid production was favored at higher glucose concentration.

Next, the correlation between the specific rate of acetic acid production as a by-product (q_{AA}), which is also a growth-associated product, and D was examined (Fig. 1B). The growth-rate-related coefficient ($K_{AA/X}$) for acetic acid formation at 19 g/l and 38 g/l of glucose was 1.48 g-acetic acid/g-DCW (from the equation q_{AA}=0.30+1.48D) and 2.97 g-acetic acid/g-DCW (from the equation q_{AA}= 0.12+2.97D), respectively. The nongrowth-rate-related coefficient ($K'_{AA/X}$) at 19 g/l and 38 g/l of glucose was 0.30 g/g-DCW and 0.12 g/g-DCW, respectively. The value of $K_{AA/X}/K'_{AA/X}$ at 38 g/l of glucose was 5 times higher than the value at 19 g/l of glucose, indicating that acetic acid production was also favored at higher glucose concentration. The value of $K_{SA/X}/K_{AA/X}$ was 3.5 at 19 g/l of glucose and 3.9 at 38 g/l of glucose, respectively, whereas the value of $K'_{SA/X}/K'_{AA/X}$ was 1.5 and 8.25 at 19 g/l and 38 g/l of glucose, respectively.

Fig. 1C shows the correlation between the specific rate of glucose consumption (q_S) and D. From this relationship, the maximum theoretical biomass yields can be calculated using the equation q_S=f(D). The maximum theoretical biomass yields were calculated to be 0.159 g-DCW/g-glucose (=1/6.3) and 0.066 g-DCW/g-glucose (=1/15.1) at 19 g/l and 38 g/l of glucose, respectively. Cell maintenance coefficients [intercept of the lines q_S=f(D)] were 1.36 g/g/h at 19 g/l of glucose and 0.59 g/g/h at 38 g l^{-1} of glucose. Because succinic acid was a growth-associated product, as mentioned above, the volumetric productivity of succinic acid increased with increasing D. The maximum productivities of succinic acid obtained were 5.5 g/l/h (0.58 h^{-1}) and 6.1 g/l/h (0.41 h^{-1}) at 19 g/l and 38 g/l of glucose, respectively (Fig. 2).

During anaerobic continuous culture of *A. succiniciproducens* in glucose-fed media, the steady-state kinetic parameters and yields were significantly influenced by glucose levels in the feed medium and specific growth rates or dilution rates. As expected [11], production of both succinic acid and acetic acid was found to be growth-associated and their growth- and nongrowth-rate-related coefficients were also slightly influenced by the glucose concentration in the feed media. The growth-rate-related coefficient for succinic acid ($K_{SA/X}$=11.6) at 38 g/l of glucose was 2.2 times higher than that ($K_{SA/X}$=5.1) at 19 g/l of glucose. Similarly, the growth-rate-related coefficient for acetic acid ($K_{AA/X}$= 2.97) at 38 g/l of glucose was twice higher than the value

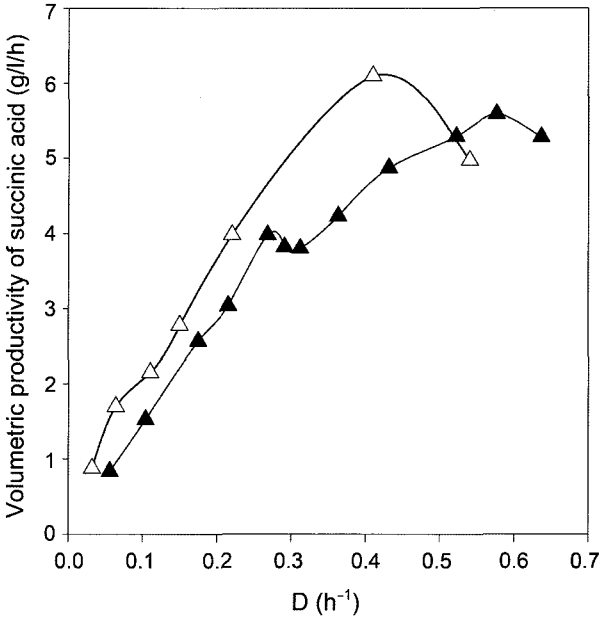

Fig. 2. Succinic acid volumetric productivity versus dilution rate during anaerobic continuous fermentation of glucose by *A. succiniciproducens*.
Symbols are 19 g/l glucose (▲) and 38 g/l glucose (△).

($K_{AA/X}$=1.48) at 19 g/l of glucose. Interestingly, a high glucose concentration and high growth rate (=D) did not induce the formation of by-products, which has been observed for *Saccharomyces* (ethanol) [4] and *Escherichia coli* (formic acid) [5]. This result is in good agreement with previous studies that have used batch cultures [12]. These combined results suggest that *A. succiniciproducens* efficiently controls the overflow of carbon flux towards succinic and acetic acids production without additional by-products formation.

In contrast to the increase in $K_{SA/X}$ and $K_{AA/X}$ with increasing glucose concentration in the feed medium, the biomass yields ($Y_{X/S}$) were shown to decrease with increasing glucose concentration (Tables 1 and 2). This decrease in biomass yield may be attributed to the increasing osmolarity [3] of the media and accumulation of succinic acid and acetic acid [13]. The calculated maximum biomass yields and cell maintenance coefficients of *A. succiniciproducens* were relatively lower than those of other anaerobic bacteria [21].

In many anaerobic fermentation processes for organic acid or alcohol production, the product yield is the key factor affecting the total cost of the process. Although the succinic acid and acetic acid yields were slightly affected by glucose concentration and the specific growth rate (Tables 1 and 2), the succinic acid/acetic acid mass ratio over a range of D's was similar to values obtained using batch fermentation of carbohydrates [12, 13]. The $K_{SA/X}/K_{AA/X}$ (=3.5–3.9) and $K'_{SA/X}/K'_{AA/X}$ (=3.3–3.8) values can

explain the significant changes observed in the succinic acid/acetic acid mass ratios during the batch cultures of *A. succiniciproducens* under different culture conditions.

In conclusion, a comparison of the kinetic parameters and yields at different glucose concentrations shows that the production of succinic and acetic acids is growth associated and is enhanced by increasing the glucose concentration in the feed medium without additional by-product formation. The higher maintenance requirement and lower biomass yield relative to other anaerobic bacteria are unique to *A. succiniciproducens*. Although these results will be important in the design of novel bioprocesses for succinic acid production by *A. succiniciproducens*, further studies will be required to increase the acid-tolerance of this system or to reduce acetic acid formation.

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