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Kinetic study on succinic acid and acetic acid formation during continuous cultures of *Anaerobiospirillum succiniciproducens* grown on glycerol

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Abstract Succinic acid-producing *Anaerobiospirillum succiniciproducens* was anaerobically grown in a glycerol-fed continuous bioreactor in order to investigate the physiological responses of the cell to different pH values (5.9, 6.2, or 6.5) and various dilution rates, *D*. In these experiments, *A. succiniciproducens* showed a pH-dependent glycerol consumption behavior. When pH was maintained at 5.9 or 6.5, glycerol started to accumulate even at a very low *D* of 0.027 h^{-1}. Succinic acid yield was not significantly affected by the pH of the culture or the *Ds*. However, more acetic acid formation was observed when the growth rate of *A. succiniciproducens* was fast on glycerol at pH 6.2 (at $D \geq 0.15$ h^{-1}). The highest obtainable succinic acid/acetic acid ratio was 40:1, which was 10 times higher than that obtained by batch cultures grown on glucose. The maximum obtainable productivity of succinic acid was 2.1 g L^{-1} h^{-1}), which was 14 times higher than that obtained by batch culture.

Keywords Anaerobic continuous culture · *Anaerobiospirillum succiniciproducens* · Glycerol · Succinic acid

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Introduction

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 chemical synthesis [1]. 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. To date, most commercial succinic acid has been produced by chemical processes that use fossil resources. However, due 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 [1, 2]. Among the succinic acid-producing microorganisms, which include *Anaerobiospirillum succiniciproducens* [3, 4], *Actinobacillus succinogenes* [5, 6], *Mannheimia succiniciproducens* [7, 8], *Corynebacterium glutamicum* [9] and *Escherichia coli* [10], 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 [12], wood hydrolysates [13] and galactose [14].

Glycerol is an attractive carbon substrate because it is available from renewable resources in large quantities and can be utilized by a number of microorganisms for the production of biofuels and biochemicals [15, 16]. Currently, glycerol is produced as a surplus by-product in the growing oleochemical industries for the production of soaps, fatty acids, waxes and surfactants [17]. Therefore, in an attempt to utilize glycerol as a starting material for microbial fermentations, several environmentally friendly bioprocesses have been proposed for the production of β-hydroxypropionaldehyde (reuterin) [18], 3-hydroxypropionic acid [19],2,3-butanediol [20] and 1,3-propandiol [21, 22], and PHB [23].

In a previous study, we showed that glycerol can be used as a starting material for the fermentative production of succinic acid by *A. succiniciproducens* [12]. The use of glycerol as a carbon source for succinic acid production has some advantages over the use of glucose. When glucose was used as a carbon source in the culture of *A. succiniciproducens*, acetic acid was produced as by-product at a succinic acid to acetic acid mass ratio (*S/A*) of 4:1 [24]. The formation of acetic acid as a by-product tends to reduce the succinic acid yield and cell growth. However, when glycerol was used as the sole carbon source in the culture of *A. succiniciproducens*, unwanted acetic acid formation was significantly suppressed and thus more succinic acid was produced. Consequently, the *S/A* ratio increased up to 37:1, which is 9 times higher than that obtained using glucose [12]. In addition to a higher succinic acid yield, the benefits of suppressing acetic acid formation include an easy and cost-saving recovery of succinic acid from culture media [8].

Although utilizing glycerol for the production of succinic acid by *A. succiniciproducens* has the advantages described above, the detailed physiological and metabolic responses of *A. succiniciproducens* grown on glycerol has not been studied in a continuous culture. Therefore, in this study, we quantify, for the first time, the kinetics of cell growth and succinic acid/acetic acid formations of *A. succiniciproducens* grown on glycerol during continuous cultures at different pH values and various dilution rates or specific growth rates.

Materials and methods

Organism and growth conditions

Anaerobiospirillum succiniciproducens (ATCC 29305) was obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in sealed anaerobic bottles containing 100 mL minimal salts medium 1 (AnS1), 5 g L^{-1} glycerol, 10 g L^{-1} yeast extract and 5 g L^{-1} polypeptone with CO_2 as a gas phase. The AnS1 medium contains 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 $MgCl_2{\cdot}6H_2O$, and 3 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 medium was adjusted to 6.5 by the addition of concentrated H_2SO_4. The nitrogen headspace was replaced by CO_2, and $Na_2S{\cdot}9H_2O$ (final concentration: 1 mg L^{-1}) was added to ensure strict anaerobic conditions. After 15 min, the reduced medium was inoculated with 2.5 mL glycerol stock culture and incubated at 39 °C for 20–24 h.

Continuous cultures were carried out at 39 °C with a working volume of 500 mL in a jar bioreactor (2.5 L, Korea Fermentor Company, Incheon, Korea), and the AnS2 medium was used as a feeding solution. The AnS2 medium contains per liter: 11 ± 0.3 g glycerol, 25 g yeast extract (Difco, Detroit), 3 g K_2HPO_4, 1 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 5 g 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 glycerol concentration dropped to 1 g L^{-1} during the batch operations, a reduced feeding solution was added at various 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, glycerol, and end-products in the bioreactor for three consecutive samples taken at 6 or 8-h intervals after at least 5 turnovers. The pH was maintained at 5.9, 6.2 or 6.5 using 2 M Na_2CO_3, as described in the "Results and discussion" section. Foaming was controlled by adding Antifoam 289 (Sigma Chemical Co., St. Louis, MO). 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).

Analytical methods

The concentrations of glycerol, 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 × 7.8 mm, Hercules, CA) 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 L^{-1}.

Estimation of kinetic parameters and yields

The theoretical maximum biomass yield, maintenance energy coefficient and specific glycerol uptake rate were determined according to the method of Pirt [25]. Yields of succinic and acetic acid were defined as the amount of succinic and acetic acid produced from one gram of glycerol consumed. Similarly, biomass yield was defined as the amount of biomass produced from one gram of glycerol 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 $CH_2O_{0.5}N_{0.21}$ [4] 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. 5–8%) due to the addition of 2 M Na_2CO_3 for pH control during cultivation.

Results and discussion

Steady-state parameters and yields

In a continuous culture of obligate anaerobes, the production of end-products or their profile can be significantly affected by the operating conditions such as the dilution rate, D, and culture pH [27, 28]. Thus, to investigate the metabolic/physiological responses of *A. succiniciproducens* to culture pH and D, continuous cultures of *A. succiniciproducens* were anaerobically carried out at different controlled pHs (5.9, 6.2, and 6.5) using media containing 11 ± 0.3 g L^{-1} glycerol as the carbon source. When the pH of continuous culture was maintained at 5.9 (Table 1), the steady-state cell concentration decreased from 0.48 to 0.42 g DCW L^{-1} when D was increased from 0.022 to 0.042 h^{-1}. Unexpectedly, glycerol started to accumulate at a D of 0.027 h^{-1}, indicating a pH of 5.9 is not an optimal condition for growth of *A. succiniciproducens* on glycerol. Accordingly, the steady-state concentration of succinic acid and acetic acid decreased from 16.1 to 14.0 g L^{-1} and 0.5 to 0.48 g L^{-1}, respectively, with increasing D. HPLC analysis revealed no traces of other end-products such as propanediol or butanediol, which is a common end-product during the fermentation of glycerol by anaerobes [29–31]. Steady-state cell yields ($Y_{X/S}$) of 0.044 ± 0.002 g DCW g^{-1} glycerol were obtained at pH 5.9. In addition, a succinic acid yield ($Y_{SA/S}$) and acetic acid yield ($Y_{AA/S}$) of 1.41–1.45 g g^{-1} and 0.045–0.050 g g^{-1}, respectively, were obtained at this pH, resulting in a gram S/A ratio of 31:1–29:1. The high S/A ratio and accumulation of glycerol indicate that even though pH 5.9 is not optimal for *A. succiniciproducens* growth, there were no significant metabolic changes with regard to succinic acid and acetic acid formations. Because *A. succiniciproducens* grown at pH 5.9 did not consume glycerol completely at even very low D (0.027 h^{-1}), we investigated the metabolic response of the cell at a pH of 6.2.

When the culture pH was maintained at 6.2 (Table 2), the steady-state cell concentration increased from 0.49 to 0.62 g DCW L^{-1} and glycerol was completely consumed at a $D \leq 0.14$ h^{-1}. At $Ds \geq 0.19$ h^{-1}, glycerol started to accumulate and accordingly the cell concentration started to decrease and reached 0.35 g DCW L^{-1} at a D of 0.25 h^{-1}. The steady-state concentration of succinic acid decreased from 16.0 g L^{-1} at a D of 0.022 h^{-1} to 8.0 g L^{-1} at a D of 0. 25 h^{-1}. However, the steady-state concentration of acetic acid unexpectedly increased from 0.41 to 0.51 g L^{-1}. Consequently, the S/A ratio decreased from 40:1 at D of 0.032 h^{-1} to 15.7:1. These results indicate that a pH of 6.2 is favorable for *A. succiniciproducens* growth and rapidly growing *A. succiniciproducens* tends to produce more acetic acid to properly balance the physiological/metabolic changes or requirements. Based on the proposed catabolic pathway of *A. succiniciproducens* [12], rapidly growing cells need to produce more acetic acid to generate ATP. Even though a metabolic change in *A. succiniciproducens* was observed at pH 6.2, no traces of other end-products were detected. $Y_{X/S}$ increased from 0.043 to 0.055 g DCW g^{-1} with increasing Ds, irrespective of incomplete glycerol consumption at a D of 0.19 and 0. 25 h^{-1}. $Y_{SA/S}$ decreased from 1.42 to 1.23 g g^{-1}. In contrast, $Y_{AA/S}$ increased from 0.035 g g^{-1} at $Ds \leq 0.14$ h^{-1} to 0.77 g g^{-1} at a D of 0.25 h^{-1}.

When the culture pH was increased to 6.5 (Table 3), *A. succiniciproducens* did not consume glycerol completely at even a low D of 0.027 h^{-1}, which was similar to that observed at pH 5.9. Therefore, the cell concentration continually decreased from 0.44 to 0.27 g DCW L^{-1} with increasing Ds. This pH-dependent consumption of glycerol, as shown in Tables 1, 2 and 3, suggests that glycerol fermentation by *A. succiniciproducens* may be sensitive to culture pH values as reported for other glycerol-assimilating anaerobes [32]. For example, glycerol consumption by *Klebsiella pneumoniae* was reported to be very sensitive to culture pH during an anaerobic continuous 1,3-propandiol fermentation [21]. Furthermore, glycerol consumption of *A. succiniciproducens* is more sensitive to culture pH than glucose consumption [8, 12, 24]. Steady-state concentrations of succinic acid and acetic acid decreased from 16 to 9.4 g L^{-1} and 0.5 to 0.43 g L^{-1}, respectively, with increasing Ds. There were no traces of other end-products detected at pH 5.9 and 6.2. $Y_{X/S}$ remained between 0.041 and 0.048 g DCW g^{-1} over all Ds examined. Interestingly, while $Y_{SA/S}$ remained between 1.46 and 1.50 g g^{-1}, $Y_{AA/S}$ continually increased from 0.047 to 0.062 g g^{-1} with increasing Ds, indicating that the cell underwent physiological changes at this pH and D.

Specific rates of succinic acid formation vs. dilutions or specific glycerol consumption rates

The correlation between the specific rate of succinic acid production (q_{SA}) and specific growth rate (μ or D) at different pH values was first examined. As shown in Fig. 1, succinic acid production was linearly associated with D at all pH values even though the number of data point sets obtained pH 5.9 and 6.5 were smaller than those obtained at pH 6.2. The growth rate-related coefficient ($K_{SA/X}$) for succinic acid was

Table 1 Steady-state parameters and yields of growth and end-products during an anaerobic single-stage continuous fermentation of glycerol by *A. succiniciproducens* at different dilution rates (initial glycerol concentration in the feed, 11 g L^{-1}; pH 5.9)

D (h^{-1})	X (g L^{-1})	S (g L^{-1})	SA (g L^{-1})	AA (g L^{-1})	$Y_{X/S}$ (g g^{-1})	$Y_{SA/S}$ (g g^{-1})	$Y_{AA/S}$ (g g^{-1})	CR
0.022	0.48	0.0	16.1	0.50	0.044	1.45	0.045	1.22
0.027	0.46	0.96	14.5	0.50	0.046	1.44	0.050	1.20
0.042	0.42	1.10	14.0	0.48	0.042	1.41	0.048	1.19

D dilution rate, *X* cell concentration, *S* glycerol, *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 of growth and end-products during an anaerobic single-stage continuous fermentation of glycerol by *A. succiniciproducens* at different dilution rates (initial glycerol concentration in the feed, 11.3 g L^{-1}; pH 6.2)

D (h^{-1})	X (g L^{-1})	S (g L^{-1})	SA (g L^{-1})	AA (g L^{-1})	$Y_{X/S}$ (g g^{-1})	$Y_{SA/S}$ (g g^{-1})	$Y_{AA/S}$ (g g^{-1})	CR
0.022	0.49	0.0	16.0	0.41	0.043	1.42	0.035	1.18
0.032	0.50	0.0	16.1	0.40	0.044	1.42	0.035	1.18
0.042	0.52	0.0	16.0	0.40	0.046	1.42	0.035	1.19
0.053	0.51	0.0	15.2	0.42	0.045	1.35	0.035	1.14
0.064	0.55	0.0	15.7	0.41	0.049	1.39	0.035	1.17
0.10	0.61	0.0	15.3	0.40	0.054	1.35	0.035	1.17
0.14	0.62	0.0	15.5	0.43	0.055	1.37	0.035	1.17
0.19	0.46	3.0	11.1	0.50	0.055	1.34	0.068	1.16
0.25	0.35	4.8	8.0	0.51	0.054	1.23	0.077	1.09

D dilution rate, *X* cell concentration, *S* glycerol, *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 3 Steady-state parameters and yields of growth and end-products during an anaerobic single-stage continuous fermentation of glycerol by *A. succiniciproducens* at different dilution rates (initial glycerol concentration in the feed, 10.7 g L^{-1}; pH 6.5)

D (h^{-1})	X (g L^{-1})	S (g L^{-1})	SA (g L^{-1})	AA (g L^{-1})	$Y_{X/S}$ (g g^{-1})	$Y_{SA/S}$ (g g^{-1})	$Y_{AA/S}$ (g g^{-1})	CR
0.022	0.44	0.0	16.0	0.50	0.041	1.50	0.047	1.25
0.027	0.42	1.4	14.0	0.42	0.041	1.50	0.043	1.24
0.032	0.39	2.7	12.7	0.51	0.048	1.48	0.062	1.31
0.042	0.27	4.3	9.4	0.43	0.042	1.46	0.062	1.25

D dilution rate, *X* cell concentration, *S* glycerol, *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

34.9, 21.3 and 34.3 g (g DCW)$^{-1}$ at pH 5.9, pH 6.2 and pH 6.5, respectively. These values were 2–3 times higher than those obtained when glucose was used as the carbon source [33]. The non-growth rate-related coefficient ($K'_{SA/X}$) for succinic acid at pH 6.2 was 0.4 g (g DCW)$^{-1}$ h^{-1}, but the $K'_{SA/X}$ values at pH 5.9 and 6.5 were zero. Based on the finding that succinic acid was a growth-associated product, a theoretical succinic acid yield was calculated from the slopes of the curves (that is, the representation of the specific rates of succinic acid production, q_{SA}, as a function of the specific rate of glycerol consumption, q_S), as shown in Fig. 2. The calculated theoretical succinic acid yields were 1.54 g g^{-1} at pH 5.9, 1.55 g g^{-1} at pH 6.2, and 1.48 g g^{-1} at pH 6.5, respectively.

Specific rates of acetic acid formation versus dilutions or specific glycerol consumption rates

As previous studies indicated, acetic acid was produced as a by-product at a *S/A* mass ratio of 3.7:1–4.0:1 when glucose was used the carbon source [24, 34]. However, when glycerol was used as a carbon source the highest obtainable *S/A* mass ratio was reported to be 32:1 [12]. Acetic acid formation was also found to be growth-associated during continuous culture at pH values of 5.9, 6.2, and 6.5 (Fig. 3). The growth rate-related coefficients ($K_{AA/X}$) for acetic acid at pH 5.9 and pH 6.5 were 1.85 and 1.27 g (g DCW)$^{-1}$, respectively. Interestingly at pH 6.2, the $K_{AA/X}$ depended on *D*: at *Ds* of $\leq$0.14 h^{-1} $K_{AA/X}$ was

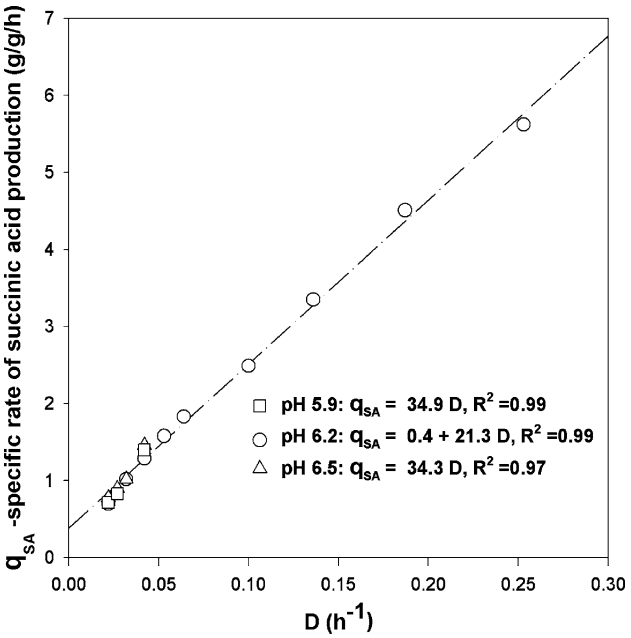

Fig. 1 Representation of the specific rate of succinic acid production versus dilution rate at different pH values (*square* pH 5.9, *circle* pH 6.2, *triangle* pH 6.5) during anaerobic continuous culture of *A. succiniciproducens*

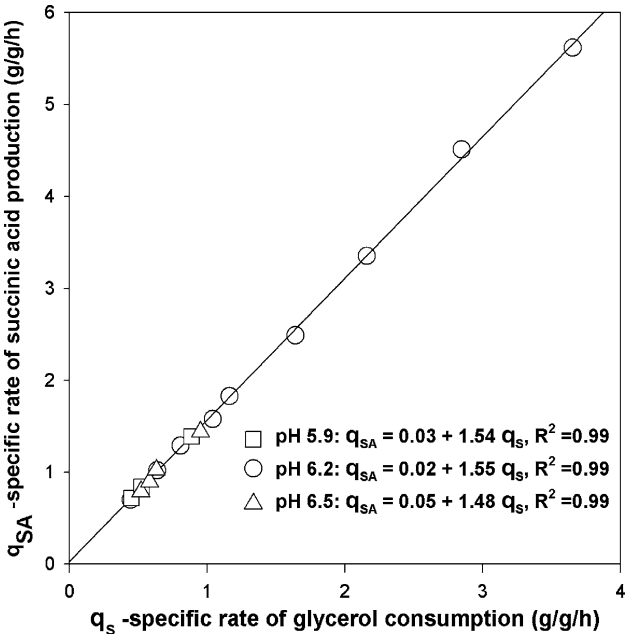

Fig. 2 Representation of the specific rate of succinic acid production versus the specific rate of glycerol consumption at different pH values (*square* pH 5.9, *circle* pH 6.2, *triangle* pH 6.5) during anaerobic continuous culture of *A. succiniciproducens*

0.66 g (g DCW)$^{-1}$ and at $Ds \geq 0.15$ h^{-1} $K_{AA/X}$ was 2.30 g (g DCW)$^{-1}$. This kinetic change may be mainly due to the increasing requirement of ATP to grow faster [4]. The non-growth rate-related coefficients ($K'_{AA/X}$) for acetic acid at pH 5.9 and 6.5 were calculated to be zero. The theoretical acetic acid yields were calculated to be 0.057, 0.08 and 0.045 g (g DCW)$^{-1}$ at pH 5.9, pH 6.5 and pH 6.2 and lower D, and 0.18 g (g DCW)$^{-1}$ at pH 6.2 and higher D (Fig. 4).

Growth parameters and succinic acid productivity

The theoretical maximum biomass yields ($Y_{X/S}$), which were calculated from the inverse of the slope ($1/Y_{S/X}$) of the $q_S = f(D)$ curves, were 0.045 g (g DCW)$^{-1}$ (=1/22.2), 0.048 g (g DCW)$^{-1}$ (=1/20.9) and 0.046 g (g DCW)$^{-1}$ (=1/21.7) at pH 5.9, pH 6.2 and pH 6.5, respectively, even though the number of data point sets obtained pH 5.9 and 6.5 were smaller than those obtained at pH 6.2. Cell maintenance energy coefficients, which were determined from the intercept of the $q_S = f(D)$ curves, approached zero at pH 5.9 and 6.5, and 0.1 g (g DCW)$^{-1}$ h^{-1} at pH 6.2 (Fig. 5), which are much lower than those obtained using glucose [33]. The calculated zero values of cell maintenance energy coefficients might be attributed to very slow growth of *A. succiniciproducens* on glycerol. Cell maintenance coefficients of rapidly growing *A. succiniciproducens* on glucose were calculated to be 1.36 g (g DCW)$^{-1}$ h^{-1} at 19 g L^{-1} of glucose and 0.59 g (g DCW)$^{-1}$ h^{-1} at 38 g L^{-1} of glucose [33]. The maximum obtainable productivity of succinic acid was 2.1 g L^{-1} h^{-1} at a D of

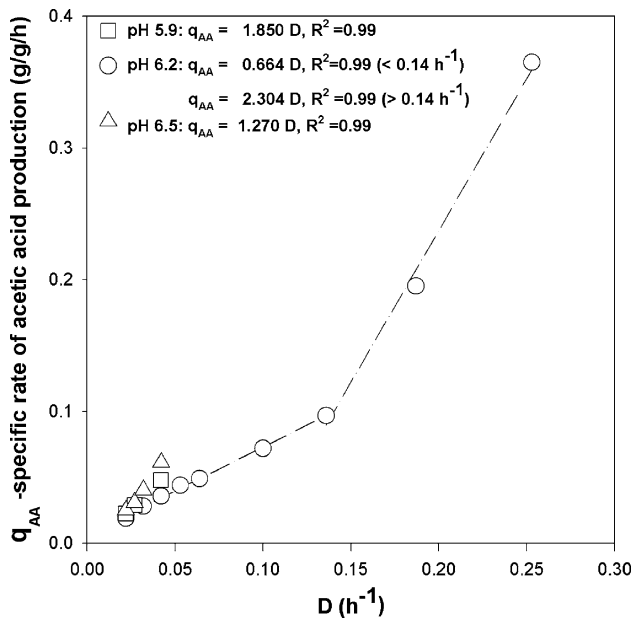

Fig. 3 Representation of the specific rate of acetic acid production versus the dilution rate at different pH values (*square* pH 5.9, *circle* pH 6.2, *triangle* pH 6.5) during anaerobic continuous culture of *A. succiniciproducens*

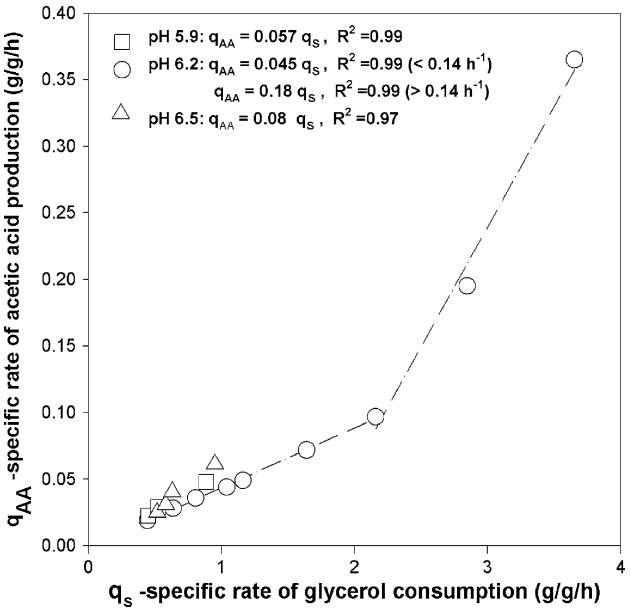

Fig. 4 Representation of the specific rate of acetic acid production versus the specific rate of glycerol consumption at different pH values (*square* pH 5.9, *circle* pH 6.2, *triangle* pH 6.5) during anaerobic continuous culture of *A. succiniciproducens*

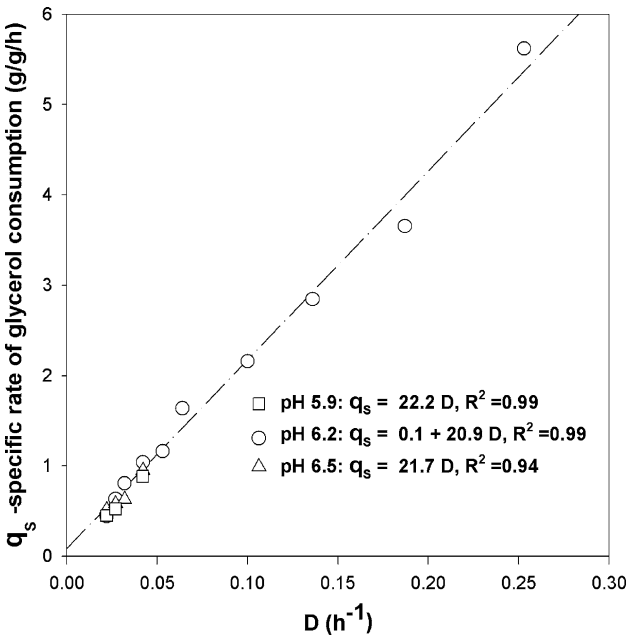

Fig. 5 Representation of the specific rate of glycerol consumption versus the dilution rate at different pH values (*square* pH 5.9, *circle* pH 6.2, *triangle* pH 6.5) during anaerobic continuous culture of *A. succiniciproducens*

0.14 h^{-1} and pH of 6.2, which was 14 times higher than that obtained by batch culture with glycerol as the carbon source [12].

In conclusion, the results obtained from the continuous fermentation of glycerol by *A. succiniciproducens* proved that glycerol is a promising substrate for succinic acid production. When glycerol was used as a carbon source during fermentation a high yield and productivity of succinic acid was obtained with less production of acetic acid as a by-product. Other end-products such as butanediol or propanediol were not detected during continuous cultures at pH 5.9, 6.2, or 6.5. However, more acetic acid formation was observed when *A. succiniciproducens* grew rapidly (at $D \geq 0.15$ h^{-1}). These kinetic data will prove important in the design of novel bioprocesses for succinic acid production from waste glycerol by *A. succiniciproducens*.

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