The Journal of Microbiology (2010) Vol. 48, No. 3, pp. 290-296
DOI 10.1007/s12275-010-9262-2

Kinetic Evaluation of Products Inhibition to Succinic Acid Producers *Escherichia coli* NZN111, AFP111, BL21, and *Actinobacillus succinogenes* 130Z^T

Qiang Li[1,2], Dan Wang[1,2], Yong Wu[2,3], Maohua Yang[1,2], Wangliang Li[1], Jianmin Xing[1]*, and Zhiguo Su[1]

[1]*National Key Laboratory of Biochemical Engineering, Institute of Process Engineering, Chinese Academy of Sciences, Beijing 100190, P. R. China*
[2]*Graduate School of the Chinese Academy of Sciences, Beijing 100049, P. R. China*
[3]*Key Laboratory of Green Process and Engineering, Institute of Process Engineering, Chinese Academy of Sciences, Beijing 100190, P. R. China*

(Received August 20, 2009 / Accepted February 1, 2010)

Succinic acid is one of the platform compounds and its production via natural feedstocks has drawn worldwide concerns. To evaluate the inhibitory effects of fermentation products on the growth of *Actinobacillus succinogenes* 130Z^T and *Escherichia coli* NZN111, AFP111, BL21, fermentations with addition of individual products in medium were carried out. The cell growth was inhibited when the concentrations of formate, acetate, lactate, and succinate were at range of 8.8-17.6 g/L, 10-40 g/L, 9-18 g/L, and 10-80 g/L, respectively. For these two species of bacteria, *E. coli* was more resistant to acid products than *A. succinogenes*, while both endured succinate rather than by-products. As a result of end product inhibition, succinate production yield by *A. succinogenes* decreased from 1.11 to 0.49 g/g glucose. Logistic and Monod mathematical models were presented to simulate the inhibition kinetics. The Logistic model was found more suitable for describing the overall synergistic inhibitory effects.

***Keywords*:** *A. succinogenes*, *E. coli*, fermentation, inhibition, kinetics

Succinic acid is valued as one of the famous industrial building block compounds and has drawn worldwide concerns for its applications in biodegradable polymers, foods, fine chemicals, and pharmaceuticals. Traditional petrochemical production of succinic acid requires high pressure, high temperature, and costly catalysts. Therefore, much attention has recently been paid to the microbial conversion (Luque *et al.*, 2009).

In most of succinic acid fermentation processes, the production was not efficient. Efforts have been paid to enhance the conversing yield from platform organisms by several groups (McKinlay *et al.*, 2007). *A. succinogenes* is an acidogenic strain which can produce 74 g/L succinic acid in vials (Guettler *et al*., 1999), whose mutant FZ53 can produce 106 g/L succinic acid (Guettler *et al*., 1996). In a repeat-batch biofilm bioreactor, the titers of succinic acid were about 40-50 g/L with the strain *A. succinogenes* or *E. faecalis* RKY1 (Wee *et al.*, 2002; Urbance *et al*., 2004). Titers of succinic acid produced by *M. succiniciproducens* LPK7 (Lee *et al*., 2006), were 52.4 g/L. 40 g/L succinic acid can be produced by recombinant *E. coli* AFP184 (Andersson *et al*., 2007). Presently, *C. glutamicum* pCRA717 could produce 146 g/L succinic acid under the fed-batch fermentation, which was the highest titer reported presently (Okino *et al*., 2008).

In industrial scale of organic acids fermentation processes such as citric or lactic acids, the final product titers can typically reach nearly 200 g/L (Delhomme *et al.*, 2009). The product inhibition was one of the major reasons resulting the succinic acid production via fermentation economically infeasible compared with a synthetic method based on the petroleum feed stock. Song *et al.* (2008) proposed a Monod model for glucose inhibition and a Luedeking-Piret model for organic acids accumulation by *M. succiniciproducens*. Lin *et al*. (2008) examined the inhibition potentials of substrate and products on *A. succinogenes* in succinic acid fermentation with a modified Monod equation. Corona-González *et al.* (2008) presented a study of glucose conversion to succinic acid using several initial concentrations of glucose in a batch fermentation of *A. succinogenes*, and modeled the growth inhibition by glucose and the organic acids with Jerusalimsky equations.

This paper presented the inhibitory effects by major products in the process of succinic acid fermentation by *E. coli* mutants for the first time, and compared the similarity and discrepancy of the product inhibitory effects together with *A. succinogenes*. Mathematical models were used to simulate the growth behavior of different strains and verified with experimental data. Finally, fermentation strategies were suggested to optimize the fermentation process of succinic acid.

Materials and Methods

Microorganisms

A. succinogenes 130Z (ATCC 55618) (Guettler *et al*., 1999) was obtained from the American Type Culture Collection. *E. coli* NZN111 and AFP111 were kindly obtained from Professor P. Clark (Southern Illinois University, USA). *A. succinogenes*, recombinant *E. coli* NZN111 and AFP111 were facultatively anaerobic strains which were used for the production of succinic acid. *E. coli* BL21, purchased from Invitrogen (USA), was a basic *E. coli* strain for expression of recombinant proteins.

Cultivation and fermentation conditions

All chemicals used in this study were of analytical level and were

* For correspondence. E-mail: jmxing@home.ipe.ac.cn; Tel: +86-010-6255-0913; Fax: +86-010-6255-0913

purchased from either OXOID (England) or Sinopharm Chemical Reagent Beijing Co., Ltd (China) unless otherwise described. *E. coli* strains were cultivated at 37°C, 160 rpm in a 100 ml flask containing 50 ml growth medium (glucose 2 g/L, tryptone 10 g/L, yeast extract 5 g/L, NaCl 5 g/L, KH_2PO_4 2.5 g/L). *A. succinogenes* cultivation was conducted at 37°C, 160 rpm in a 100 ml flask containing 50 ml growth medium (pancreatic digest of casein 17 g/L, soy peptone 3 g/L, glucose 2 g/L, NaCl 5 g/L, KH_2PO_4 2.5 g/L). Inoculation volume was 5% (v/v). Succinic acid, formic acid, acetic acid, and lactic acid at different concentrations were added individually to each medium, respectively. The initial pH of the sterilized medium was adjusted to 7.0 with solid NaOH in order to avoid volume fluctuation.

The fermentation medium contained per L in flask: 30 g glucose, 15 g yeast extract, 2 g urea, 2 g $MgCl_2 \cdot 6H_2O$, 1.5 g $CaCl_2$, 0.07 g $MnCl_2$, 4.4 g Na_2HPO_4, 3.3 g NaH_2PO_4. Succinic acid at different initial concentrations was added to the medium. Glucose was separately sterilized at 115°C for 30 min and added to the medium. The initial pH of the sterilized medium was adjusted to 7.0 with solid NaOH. Batch cultivation for *A. succinogenes* and *E. coli* in flask fermentation was conducted at 37°C, 40 rpm in a rotary shaker filled with CO_2 gas in a 1 L flask containing 0.5 L flask medium. Inoculation volume was 5% (v/v). 50 g $MgCO_3$ was added into the media to control the pH. Each experiment was repeated three times and the average values were calculated.

Analytical methods

Biomass concentrations were estimated from the optical density (OD) of the fermentation broth with a spectrophotometer (723N, Shanghai Precision & Scientific Instrument Co. Ltd, China) at a wavelength of 660 nm for *A. succinogenes* and 600 nm for *E. coli*. Absorbances were related to dry cell concentrations by a calibration curve. For dry-weight determination of organisms, 5 ml samples were removed from the culture, centrifuged, washed twice with distilled water, and dried to a constant weight at 60°C. At an OD_{660} of 1.0, *A. succinogenes* had a concentration of 0.30 g dry cell weight/L; while at an OD_{600} of 1.0, *E. coli* had a concentration of 0.38 g dry cell weight/L. Concentrations of glucose were measured by SBA 40C bio-sense analyzer (Biology Institute, Shandong Academy of Sciences, China). The concentrations of organic acids, were measured by high-performance liquid chromatography Agilent 1200 (Agilent Co. Ltd, USA) equipped with an Agilent model DAD UV-VIS detector and the ZORBAX SB-Aq column (250 mm×4.6 mm, Agilent). The column was operated at 25°C. The mobile phase was pH 2.7, 20 mmol/L KH_2PO_4 at a flow rate of 1 ml/min. The injection volume was 10 μl.

Kinetic modeling

Inhibition experiments were carried out with organic acids at different initial concentrations. μ_{max} (maximum specific growth rate) (h^{-1}) and the yield of succinic acid were calculated. μ_{max} was calculated as the following first order cell growth kinetics equation:

$$C = \frac{1}{\mu}\frac{dC}{dt} \tag{1}$$

where C was the cell mass dry weight (g/L) and t was the time of the process (h). During a batch culture, the specific cell growth rate, μ, changes with time because of diminished nutrient and/or accumulated inhibitory products.

The famous Monod model is already well-described in the literature to represent bacterial growth (Yang and Tsao, 1994). The Logistic mode1 (Kacena *et al*., 1999; Peleg *et al*., 2007), originally proposed by Verhulst, has been a most illustrative model of organisms' growth dynamic. Continuous logistic equation can be expressed mathematically as follow:

$$\frac{dC}{dt} = rC\left[\frac{K-C}{K}\right] \tag{2}$$

Where C was the momentary number of bacterium, dC/dt was the momentary rate, r was a proportionality constant, K was the habitat's capacity quantified as the number of bacteria that it can hold, and initial value $C(t_0)=C_0$. Constants r and K were affected by many environmental factors such as pH, temperature, substrates or inhibitory concentrations. Logistic model can be also expressed by following modified equation of numerical integration:

$$C = \frac{k_1 + k_2\left(\frac{t}{t_0}\right)^a}{1+\left(\frac{t}{t_0}\right)^a} \tag{3}$$

Where C was the momentary growth number of bacterium, t was the time, a was the power of the equation, k_1 and k_2 were the parameters related to the r and K. The kinetic parameters of models were estimated using Matlab.

Results

Product inhibition for *A. succinogenes* and *E. coli*

The growth curves of *A. succinogenes* varied dramatically at different initial inhibitory concentrations of succinic acid, formic acid, lactic acid and acetic acid, respectively. Initial inhibitory concentration was defined as the value that cell growth was inhibited when the products accumulated to a certain titers (Colin *et al*., 2000). According to the time profiles of cell growth in 50 ml/100 ml flask (Fig. 1), the initial inhibitory concentrations were 40 g/L, 8.8 g/L, 10 g/L, and 9 g/L for succinic, formic, acetic, and lactic acids, respectively. Thus, by-products inhibition for cell growth was more significant.

Recombinant *E. coli* can produce mixed-acids such as acetate, formate, lactate, and conventionally a certain titer of succinate. Table 1 listed the initial inhibitory concentrations and tolerated final concentrations for the growth of *A. succinogenes* 130 Z and *E. coli.* Tolerated final concentration was defined as the value that cell growth was totally inhibited when the products accumulated to a certain titers. With similarlity to *A. succinogenes*, *E. coli* can tolerate succinic acid at much higher titers than other by-product acids, while by-product inhibition was obviously more significant as shown in Figs. 2-4. As shown in Fig. 2C, Fig. 3C, and Fig. 4C, similar growth pattern/trend was observed when lactic acid was added into the medium for fermentations using *E. coli* strains. The 9 g/L lactic acid supplementation could promote *E. coli* growth after a lag phase of 20 h cultivation. The lactate promotion was not seen in the growth of *A. succinogenes.* The tolerance level as regards to the titers of formic acid, acetic acid, and lactic acid was much less than that of succinic acid.

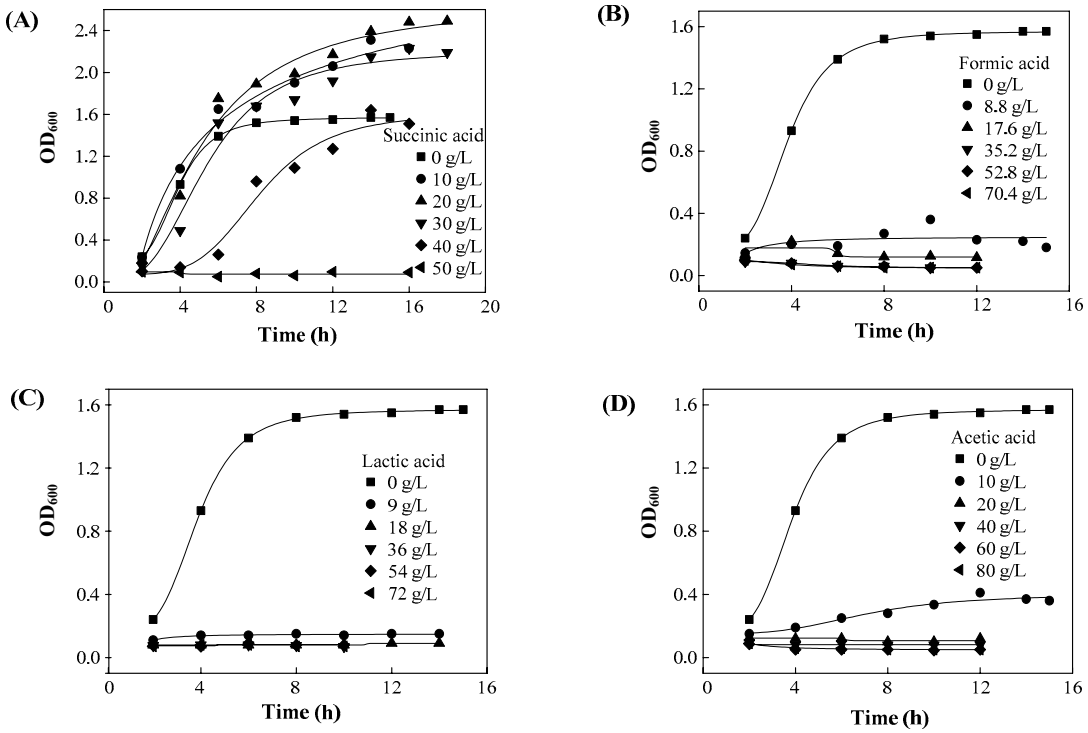

Fig. 1. Growth curves of *A. succinogenes* 130Z with succinic acid (A), fomic acid (B), lactic acid (C), and acetic acid (D) added respectively at variable concentrations. Experimental (symbols); simulated (lines)

Batch cultivation of *A. succinogenes* and *E. coli* for cell growth, substrate consumption, and organic acids production

Batch fermentation *A. succinogenes* and *E. coli* was conducted in 1 L flask with 20 g/L initial glucose. Table 2 compared the final metabolites yields and succinate productivities for *A. succinogenes* 130Z^T, *E. coli* NZN111, AFP111, and BL21. In the batch anaerobic fermentation process, *A. succinogenes* produced the largest succinate yield 1.75±0.05 mol of product per mol of glucose, while BL21 produced the lowest succinate yield 0.18±0.02 mol of product per mol of glucose. The by-products by *E. coli* varied dramatically. Taking BL21 for example, acetic and formic acids were the major organic acid products.

Kinetic expression for specific growth

Kinetic modeling was considered as an indispensable step in developing a fermentative process because the models can be used to determine an optimal operation condition for a target product. Figure 5 showed the kinetic parameter, maximum exponential growth rate, calculated under different initial succinic acid concentration. The maximum specific growth curves of *A. succinogenes* and *E. coli* showed the optimized values at the different initial succinic acid concentrations. For *A. succinogenes*, *E. coli* NZN111, and AFP111, the μ_{max} reached the maximum values at the range of 10-20 g/L titers of succinate. When the succinate was over 60 g/L, the μ_{max} reduced sharply. For *E. coli* BL21, the value of μ_{max} was observed at higher succinate titers. The values of the parameters for the overall kinetic model of cell growth carried out by *A. succinogenes* and *E. coli* had been calculated. The values of correlation coefficients (R^2) were used to compare the validity of modeling. The values of R^2 for Logistic mode1 were higher than those for Monod mode1 (data not shown). Therefore, the Logistic model accurately represented the experimental data, and the obtained parameters can be used for a quantitative comparison of the inhibition response against products concentration varied.

Table 1. Products initial inhibitory concentrations and tolerated final concentrations for the growth of *A. succinogenes* 130 Z and *E. coli*

Organic acid metabolites	*A. succinogenes*		*E. coli* NZN111		*E. coli* AFP111		*E. coli* BL21	
	IIC (g/L)	TFC (g/L)	IIC (g/L)	TFC (g/L)	IIC (g/L)	TFC (g/L)	IIC (g/L)	TFC (g/L)
Succinic acid	<40	50	<20	80	<20	80	<40	>80
Formic acid	<8.8	35.2	<8.8	52.8	<8.8	35.2	<8.8	35.2
Acetic acid	<10	20	<20	60	<20	40	<20	60
Lactic acid	<9	18	<9	18	<9	18	<9	18

IIC, initial inhibitory concentrations; defined as the value that cell growth was inhibited when the products accumulated to a certain titers.
TFC, tolerated final concentrations; defined as the value that cell growth was totally inhibited when the products accumulated to a certain titers.

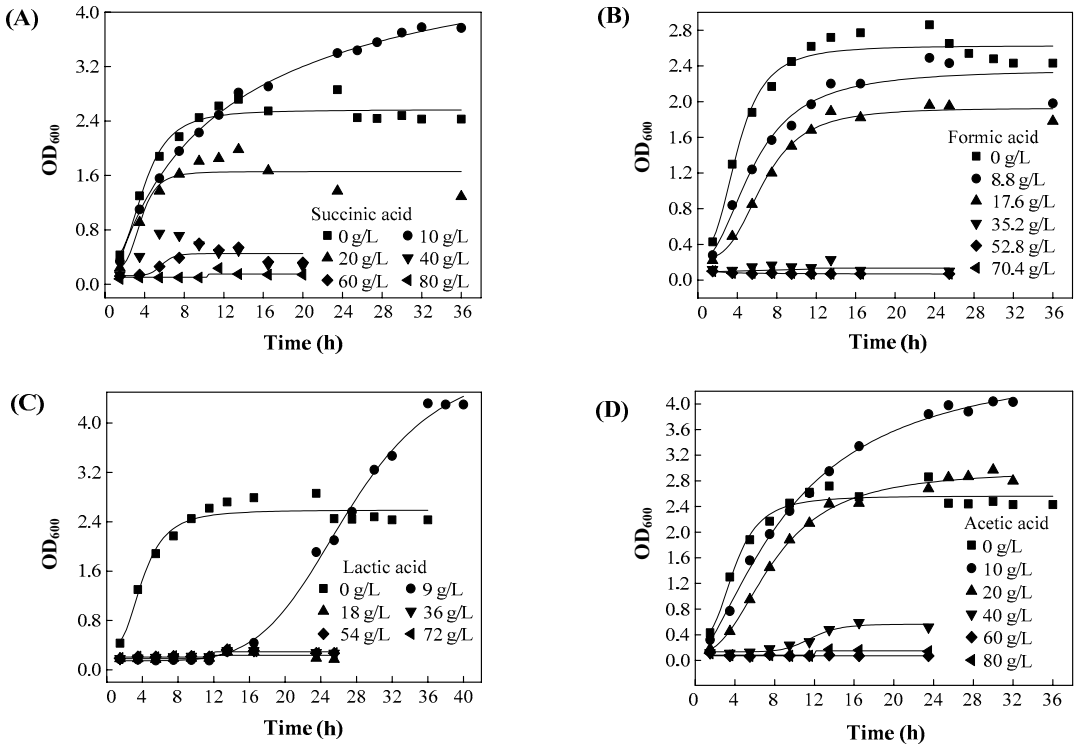

Fig. 2. Growth curves of *E. coli* NZN111 with succinic acid (A), fomic acid (B), lactic acid (C), and acetic acid (D) added respectively at variable concentrations. Experimental (symbols); simulated (lines)

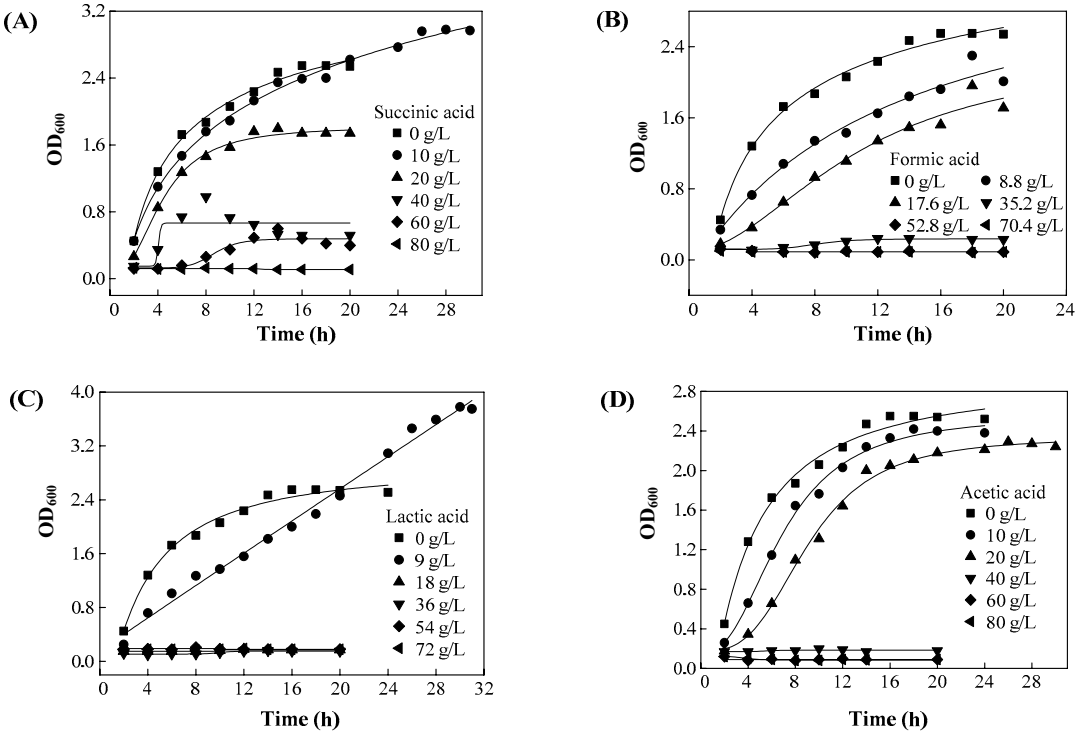

Fig. 3. Growth curves of *E. coli* AFP111 with succinic acid (A), fomic acid (B), lactic acid (C), and acetic acid (D) added respectively at variable concentrations. Experimental (symbols); simulated (lines)

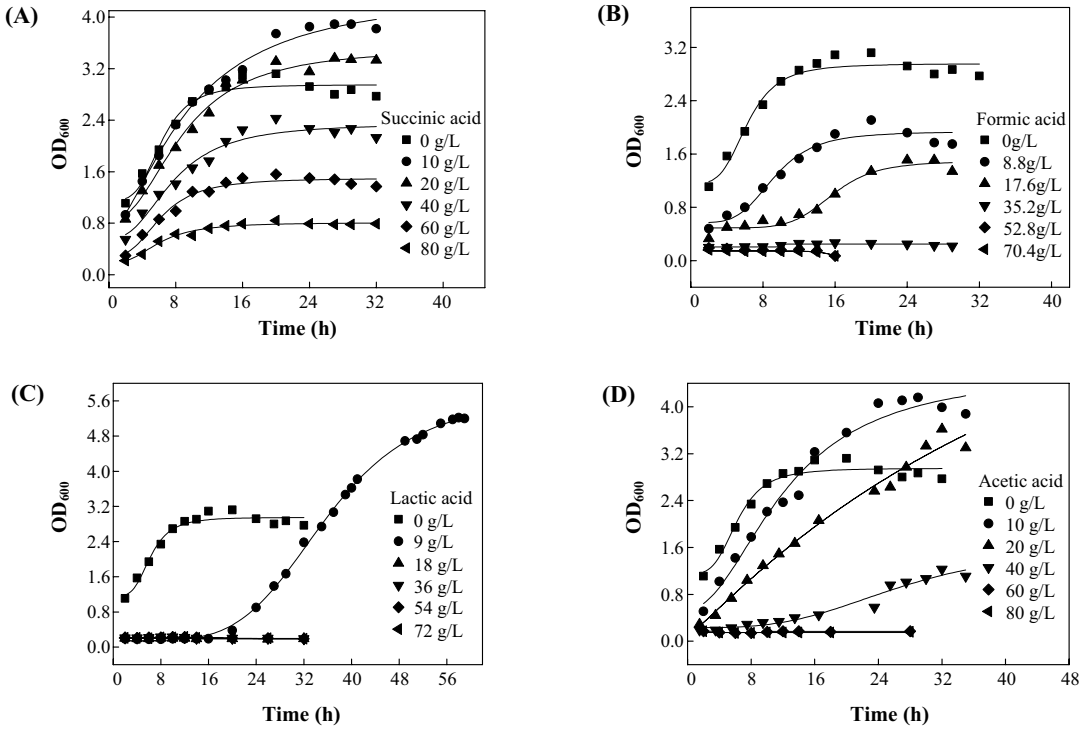

Fig. 4. Growth curves of *E. coli* BL21 with succinic acid (A), fomic acid (B), lactic acid (C), and acetic acid (D) added respectively at variable concentrations. Experimental (symbols); simulated (lines)

Succinic acid production affected by product inhibition

Initial succinic acid concentrations had a great effect on the final succinic acid production by *A. succinogenes*. Time profiles of glucose depletion and succinic acid produced in 1 L flask batch culture of *A. succinogenes* with different initial succinic acid concentration 0 g/L, 20 g/L, 40 g/L, and 50 g/L were shown in Fig. 6. *A. succinogenes* could grow and ferment quickly at 0 g/L initial concentrations of succinic acid. However, succinic acid at high initial titers of 40 g/L can inhibit cell growth and fermentation. For 20 g/L initial titers of succinic acid, the glucose utilization was delayed, and the succinic acid yield on glucose decreased from 1.11 g/g to 0.49 g/g.

Discussion

In this study, the modeling growth curves of *E. coli* and *A. succinogenes* took a slow or fast 'S' shape pattern. The proportionality constant k in the logistic equation was temperature dependent but its magnitude was also affected by any other environmental factors that might influence the specific growth rate, notably the pH, substrates, the concentration of salts and so forth. With the maintenance of appropriate substrate concentrations, proper pH values and temperature, the logistic equation can model the products inhibition effects well in the case, which showing very accurate fittings with the experimental values.

In order to simulate the fermentation process, pH values were usually adjusted to neutral. The succinic acid fermentation processes usually yielded succinate rather than free acid in the fermentation broth as a result of pH neutralization. Acids had different proportions of dissociated and undissociated forms at different pH values, and the effects of these two forms of acids were different as well. At the low pH values, the acids could be in the forms of free acid. However, the essence of free acid inhibition was notably low pH inhibition. The inhibitory effects could be adequately quantified by the dissociated acid forms only at neutral pH value.

As discussed in the previous paragraph, models expressing the cell growth inhibition kinetics varied in the literature reported. In many modeling cases, the acid products were

Table 2. Comparison of batch fermentations for *A. succinogenes* 130Z^T and *E. coli* NZN111, AFP111, and BL21

Strain	Yield (mol of product/mol of glucose)				Succinate productivity (g/L·h)	Cell concn (g DCW[a]/L)
	Succinate	Lacitate	Acetate	Formate		
130Z^T	1.75±0.05	0.94±0.06	1.16±0.04	1.29±0.08	0.31±0.02	5.35±0.04
NZN111	0.32±0.04	0.02±0.01	0.01±0.00	0.01±0.00	0.11±0.01	2.93±0.04
AFP111	0.94±0.06	0.04±0.01	0.52±0.05	0.01±0.00	0.23±0.01	3.74±0.03
BL21	0.18±0.02	0.44±0.04	0.72±0.06	0.77±0.03	0.06±0.00	2.1±0.02

[a] DCW, dry cell weight

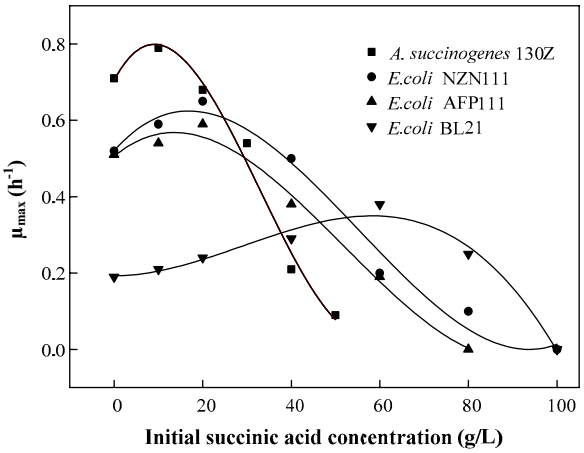

Fig. 5. Effect of succinic acid on μ_{max}.

assumed to be as the sum of the amounts of individual acids to simplify the modeling process, because the dissociation constants of these weak organic acids was considered not different (Lin *et al*., 2008; Song *et al*., 2008), for example, acetic acid (pK_a of 4.8), formic acid (pK_a of 3.8), lactic acid (pK_a of 3.9), and succinic acid (pK_{a1} of 5.6, pK_{a2} of 4.2). However, the resistance of both *E. coli* and *A. succinogenes* to these acids inhibition was different considering the inhibition effects according to the experimental results and the modeling data. This discrepancy could be due to inhibitory effects caused by the types of products. For example, *E. coli* endured acid products at higher titers than *A. succinogenes*, while both of them endured succinic acid at higher titers than by-product acids.

The accumulation of acid products over inhibitory concentrations caused the deterioration of cell growth and production. Each acid had individual inhibition features because acid inhibition was a complex phenomenon affecting many cellular structure and processes, particularly membrane composition, solute transportation, or the other membrane-associated processes. Acids had different antimicrobial activity or toxicity to the levels of degree (Goncalves *et al*., 1997; Park and Zeiku, 1999). According to chemiosmotic models or energetic coupling models, acids accumulated may disturb the pH gradient across the cell membrane for ATP metabolism, or block the functions of key enzymes (Goncalves *et al*., 1997; Park and Zeiku, 1999; McKinlay and Vieille, 2008).

Acetate had substantially negative effect on the cell growth such as some yeast (Oliva *et al*., 2006) and fungi (Kang *et al*., 2003). *E. coli* showed much more resistant against acetate than *A. succinogenes* (Table 1). For *E. coli* BL21, it can tolerate nearly 40 g/L of acetate. BL21 was known to have a higher activity of the glyoxylate shunt and more importantly acetyl CoA synthase, which would likely be related to its elevated acetate resistance. Moreover, when *E. coli* BL21 was cultivated aerobically, acetate tolerance was found to be inversely correlated to the dissolved oxygen (DO) levels (Phue and Shiloach, 2005). Lactic acid supplementation was conducive to improved growth in the case *E. coli* strains, but not for *A. succinogenes*, which might due to the individual metabolic flux. Lactate metabolism in *E. coli* was very well defined, including its role in redox balancing and energetics. Thus, *E. coli* can be extensively metabolically engineered, with target gene deletions and/or insertions to yield a specific carbon flux distribution conducive to succinic acid production.

However, the physiological states of cells with externally products supplementation differ from those in real fermentation processes. The experimental set-up exposed the cells to the inhibitor initially at a target concentration. So the addition of the inhibitory products took place at the beginning of the fermentation. However, these inhibitory products were formed and released into the medium gradually. The cells were exposed to an increasing amount of acids in their salt forms and become acclimatized to the inhibitory titers of products. The impact of the acid products on succinic acid fermentation implied that *A. succinogenes* can not effectively ferment the glucose under the high initial succinic acid titers. Succinic acid yield on glucose would decrease by 56% when adding 20 g/L succinic acid at the beginning of cultivation. Fermentation at high titers can be achieved by the application of suitable and optimized genetic or fermentation technologies (Lee *et al*., 2008). Anaerobic-aerobic combined (dual-phase) fed-batch fermentation by *E. coli* AFP111/pTrc99A-pyc can obtain 99.2 g/L succinate titers (Vemuri *et al*., 2002), which is the highest production reported by *E. coli*. However, from the study, lactic acid at very low level could be one of factors to promote cell growth of *E. coli*. The development of novel fermentation processes was another engineering method to increase the yields and titers of succinic acid dramatically. For example, *in situ* product removal (ISPR) has been developed to maintain

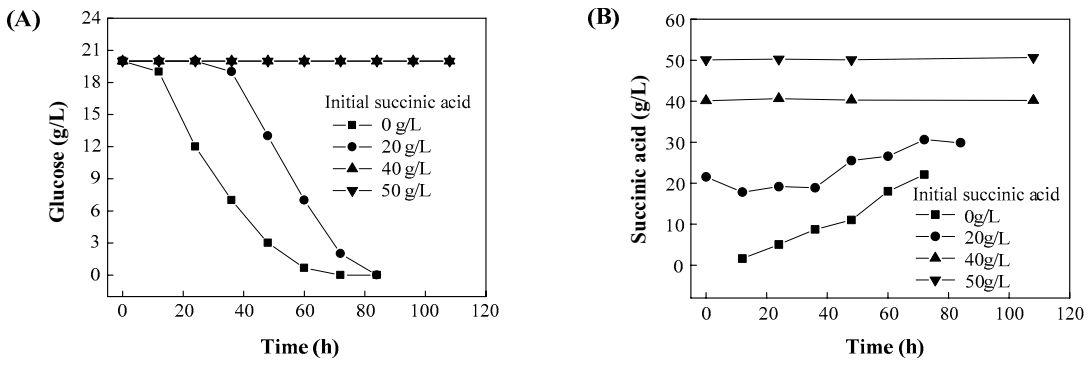

Fig. 6. Glucose and succinic acid curves of *A. succinogenes* 130Z with different initial succinic acid concentrations.

the acid products below the levels of inhibitory titers (Chen and Ju, 2002). Unlike *E. coli*, *A. succinogenes* was not extensively metabolically engineered. Metabolic and genetic engineering tools can also be used to manipulate the metabolic pathways so that target product pathway can be strengthened or by-product pathways can be controlled or even eliminated (Lee *et al*., 2005; Sánchez *et al*., 2005).

Acknowledgements

This work was supported by the Knowledge Innovation Program of the Chinese Academy of Sciences, Grant NO.KSCX2-YW-G-021. We thank Professor David P. Clark (Southern Illinois University, USA) for providing us *E. coli* NZN111 and AFP111.

References

Andersson, C., D. Hodge, K.A. Berglund, and U. Rova. 2007. Effect of different carbon sources on the production of succinic acid using metabolically engineered *Escherichia coli*. *Biotechnol. Progr*. 23, 381-388.

Chen, C.C. and L.K. Ju. 2002. Coupled lactic acid fermentation and adsorption. *Appl. Microbiol. Biotechnol.* 59, 170-174.

Colin, T., A. Bories, and G. Moulin. 2000. Inhibition of *Clostridium butyricum* by 1, 3-propanediol and diols during glycerol fermentation. *Appl. Microbiol. Biotechnol.* 54, 201-205.

Corona-González, R.I., A. Bories, V. González-Álvarez, and C. Pelayo-Ortiz. 2008. Kinetic study of succinic acid production by *Actinobacillus succinogenes* ZT-130. *Process Biochem*. 43, 1047-1053.

Delhomme, C., D. Weuster-Botz, and F.E. Kühn. 2009. Succinic acid from renewable resources as a C4 building-block chemical-a review of the catalytic possibilities in aqueous media. *Green Chem*. 11, 13-26.

Goncalves, L.M.D., A. Ramos, A.S. Almedia, A.M.R.B. Xavier, and M.J.T. Carrondo. 1997. Elucidation of the mechanism of lactic acid growth inhibition and production in batch cultures of *Lactobacillus rhamnosus*. *Appl. Microbiol. Biotechnol*. 48, 346-350.

Guettler, M.V., M.K. Jain, and D. Rumler. 1996. Method for making succinic acid, bacterial variants for use in the process, and methods for obtaining variants. *US Patent* 5,573,931.

Guettler, M.V., D. RumLer, and M.K. Jain. 1999. *Actinobacillus succinogenes* sp. nov., a novel succinic-acid-producing strain from the bovine rumen. *Int. J. Syst. Bacteriol*. 49, 207-216.

Kacena, M.A., G.A. Merrell, B. Manfredi, E.E. Smith, D.M. Klaus, and P. Todd. 1999. Bacterial growth in space flight: logistic growth curve parameters for *Escherichia coli* and *Bacillus subtilis*. *Appl. Microbiol. Biotechnol*. 51, 229-234.

Kang, H.C., Y.H. Park, and S.J. Go. 2003. Growth inhibition of a phytopathogenic fungus, *Colletotrichum* species by acetic acid. *Microbiol. Res.* 158, 321-326.

Lee, S.Y., J.M. Kim, H. Song, J.W. Lee, T.Y. Kim, and Y.S. Jang. 2008. From genome sequence to integrated bioprocess for succinic acid production by *Mannheimia succiniciproducens*. *Appl. Microbiol. Biotechnol*. 79, 11-22.

Lee, S.L., D.Y. Lee, T.Y. Kim, B.H. Kim, J.W. Lee, and S.Y. Lee. 2005. Metabolic engineering of *Escherichia coli* for enhanced production of succinic acid, based on genome comparison and in silico gene knockout simulation. *Appl. Environ. Microbiol.* 71, 7880-7887.

Lee, S.J., H. Song, and S.Y. Lee. 2006. Genome-based metabolic engineering of *Mannheimia succiniciproducens* for succinic acid production. *Appl. Environ. Microbiol*. 72, 1939-1948.

Lin, C.S.K., C.Y. Du, A. Koutinas, R. Wang, and C. Webb. 2008. Substrate and product inhibition kinetics in succinic acid production by *Actinobacillus succinogenes*. *Biochem. Eng. J.* 41, 128-135.

Luque, R., C.S.K. Lin, C.Y. Du, D.J. Macquarrie, A. Koutinas, R.H. Wang, C. Webb, and J.H. Clark. 2009. Chemical transformations of succinic acid recovered from fermentation broths by a novel direct vacuum distillation-crystallisation method. *Green Chem*. 11, 193-200.

McKinlay, J.B. and C. Vieille. 2008. ^{13}C-metabolic flux analysis of *Actinobacillus succinogenes* fermentative metabolism at different $NaHCO_3$ and H_2 concentrations. *Metab. Eng*. 10, 55-68.

McKinlay, J.B., C. Vieille, and J.G. Zeikus. 2007. Prospects for a bio-based succinate industry. *Appl. Microbiol. Biotechnol*. 76, 727-740.

Okino, S., R. Noburyu, M. Suda, T. Jojima, M. Inui, and H. Yukawa. 2008. An efficient succinic acid production process in a metabolically engineered *Corynebacterium glutamicum* strain. *Appl. Microbiol. Biotechnol*. 81, 459-464.

Oliva, J., M. Negro, F. Sáez, I. Ballesteros, P. Manzanares, A. González, and M. Ballesteros. 2006. Effects of acetic acid, furfural and catechol combinations on ethanol fermentation of *Kluyveromyces marxianus*. *Process Biochem*. 41, 1223-1228.

Park, D.H. and J.G. Zeikus. 1999. Utilization of electrically reduced neutral red by *Actinobacillus succinogenes*: physiological function of neutral red in membrane-driven fumarate reduction and energy conservation. *J. Bacteriol*. 181, 2403-2410.

Peleg, M., M.G. Corradini, and M.D. Normand. 2007. The logistic (Verhulst) model for sigmoid microbial growth curves revisited. *Food Res. Int*. 40, 808-818.

Phue, J.N. and J. Shiloach. 2005. Impact of dissolved oxygen concentration on acetate accumulation and physiology of *E. coli* BL21 evaluating transcription levels of key genes at different dissolved oxygen conditions. *Metab. Eng*. 7, 353-363.

Sánchez, A.M., G.N. Bennett, and K.Y. San. 2005. Novel pathway engineering design of the anaerobic central metabolic pathway in *Escherichia coli* to increase succinate yield and productivity. *Metab. Eng*. 7, 229-239.

Song, H., S.H. Jang, J.M. Park, and S.Y. Lee. 2008. Modeling of batch fermentation kinetics for succinic acid production by *Mannheimia succiniciproducens*. *Biochem. Eng. J.* 40, 107-115.

Urbance, S.E., A.L. Pometto, A.A. DiSpirito, and Y. Denli. 2004. Evaluation of succinic acid continuous and repeat-batch biofilm fermentation by *Actinobacillus succinogenes* using plastic composite support bioreactors. *Appl. Microbiol. Biotechnol*. 65, 664-670.

Vemuri, G.N., M.A. Eiteman, and E. Altman. 2002. Succinate production in dual-phase *Escherichia coli* fermentations depends on the time of transition from aerobic to anaerobic conditions. *J. Ind. Microbiol. Biotechnol*. 28, 325-332.

Wee, Y.J., J.S. Yun, K.H. Kang, and H.W. Ryu. 2002. Continuous production of succinic acid by a fumarate-reducing bacterium immobilized in a hollow-fiber bioreactor. *Appl. Biochem. Biotechnol.* 98-100, 1093-1104.

Yang, X.P. and G.T. Tsao. 1994. Mathematical modeling of inhibition kinetics in acetone-butanol fermentation by *Clostridium acetobutylicum*. *Biotechnol. Progr.* 10, 532-538.