Thermodynamic Analysis of Product Formation in Mesophilic Acidogenesis of Lactose

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Received 1 June 2003; accepted 8 April 2004

Published online 18 August 2004 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/bit.20190

Abstract: Thermodynamic analysis on the acidogenesis of lactose was performed to evaluate the different acidogenic patterns and mechanisms by using Gibbs free energy calculation. Batch acidogenesis of lactose was investigated by using an enriched culture at 37°C, pH 5.5 and varied substrate levels. In addition to usual acidogenic products, *i*-butyrate, valerate, *i*-valerate, caproate, and propanol were also produced at a significant level. Thermodynamic analysis shows that valerate might be formed through the reaction requiring hydrogen as electron donor and consuming of propionate and carbon dioxide. Caproate was most likely produced directly from butyrate, hydrogen, and carbon dioxide. The minimum amount of Gibbs free energies needed to sustain isomerization of butyrate and valerate were approximately 5.7–5.8 and 4.5–4.6 kJ/mol, respectively. Propanol was produced from acetate, hydrogen, and carbon dioxide with a minimum amount of Gibbs free energy of 41.8–42.0 kJ/mol. Formation of butanol was controlled more by substrate level or population dynamics than by thermodynamics.

Keywords: acidogenesis; alcohols; lactose; thermodynamic; volatile fatty acids (VFA)

INTRODUCTION

The application of thermodynamic laws to biochemical processes provide a theoretical basis for analysis of experimental results and an important tool in understanding bacterial growth and energy metabolism (Heijnen and van Dijken, 1992; Sandler and Orbey, 1991; Welch, 1993). The second law of thermodynamics regulates that microbial conversions can only sustain microbial growth if the reaction is exergonic, and that the amount of energy generated during a biochemical conversion equals the Gibbs free energy change of the chemical reaction. A well-known application of the thermodynamic principles for biotechnological processes is the observed correlation between the microbial yield and Gibbs free energy changes of microbial conversions (Heijnen and van Dijken, 1992). Furthermore, the mechanisms of formation of intermediate compounds can be analyzed using thermodynamic considerations (Smith and McCarty, 1989a).

Microbial conversions in an anaerobic reactor for wastewater treatment proceed close to thermodynamic equilibrium. Several anaerobic fermentation reactions are energonic under standard conditions (Thauer et al., 1977). Combined with the fact that methanogens only utilize a limited range of simple substrates, the degradation of substrates in an anaerobic reactor becomes dependent on a mixture of fermenting and methanogenic bacteria (Stams, 1994). Only if the methanogenic bacteria keep the concentration of fermentation products low, then the fermentation is exergonic and the reaction can be pulled to the product side. Because of their mutual dependence these mixed cultures are referred to as syntrophic cultures (Schink, 1992). The discovery of interspecies hydrogen transfer between an ethanol-oxidizing organism and a hydrogen-consuming methanogen demonstrated the importance of thermodynamics in understanding how anaerobic ecosystems function (Iannotti et al., 1973). Since then, a number of interspecies-hydrogen-based syntrophic associations for oxidation of propionate, butyrate, *i*-valerate, and benzoate have been isolated (Schink, 1992; Stams, 1994).

Perturbation of ethanol- and propionate-fed continuous stirred tank reactor (CSTR) for methanogenesis was energetically investigated by Smith and McCarty (1989a, 1989b). They found that reduced products, such as propanol and C_4-C_7 volatile fatty acids (VFA), were formed in the transients following perturbation with ethanol and propionate. The formation resulted from a coupling of the reduction of propionate and oxidation of ethanol to acetate.

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Contract grant sponsors: Natural Science Foundation of China; The Ministry of Science & Technology, China
Contract grant numbers: No. 20122203; No. 2001AA515050

The C_4-C_7 VFA were produced through back-reaction of beta-oxidation in the presence of hydrogen. It was observed that during an organic and hydraulic loading increase to a batch-fed methanogenic CSTR, the concentrations of propionate and valerate varied over the course in response to the concentrations of their oxidation products, such as acetate and hydrogen (Hickey and Switzenbaun, 1991). They suggested that the accumulation of higher-molecular-weight VFA (HMW-VFA) was thermodynamically and not kinetically controlled. In a perturbation study using a glucose-fed methanogenic CSTR, build-up of hydrogen dramatically changed the distribution of glucose metabolic intermediates, and the fermentation of glucose quickly shifted from a butyrate-type fermentation to the propionate-type one (Xing et al., 1997). Fang and Jia (1999) studied the formation of interim by-products during the methanogenic degradation of butyrate. Their chemical energy analysis indicated that the formation of caproate, one of the HMW-VFA, was independent of the increase of hydrogen partial pressure.

However, all of the above thermodynamic analyses on anaerobic reactors are focused on the methanogenesis, while little information is available on bioenergetic aspects of acidogenesis. In this study, thermodynamic analysis on the acidogensis of lactose was performed to evaluate the different acidogenic patterns and mechanisms by using Gibbs free energy (δG) which was based on the in situ concentrations of the reactants and products.

MATERIALS AND METHODS

Reactor, Wastewater, and Seed Sludge

A 2.8-L upflow acidogenic lactose-fed reactor was used to supply sludge for the batch experiment. The reactor was operated at hydraulic retention time of 12 h, substrate concentration of 12 g chemical oxygen demand (COD)/L, pH 5.50 and 37°C.

Synthetic wastewater was prepared by using lactose as the sole carbon source. The wastewater also contained balanced nutrient, trace metals, and buffering chemicals following the formulation used in a previous study (Fang and Yu, 2001). For influent of a different concentration, the amounts of all organic and inorganic constituents were adjusted pro rata. Batch tests were conducted to investigate the degradation patterns at various substrate levels. Six sets of tests were conducted in duplicate in 157-mL glass serum vials at 37°C using feed solutions containing, respectively, 2, 4, 8, 12, 20, and 30 g COD/L. The feed solutions were first purged with nitrogen to remove any dissolved oxygen. Each vial was seeded with the sludge from the continuous reactor. About 200 mL of sludge sampled from the continuous reactor was washed with stock solution, and followed by centrifugation. After decanting the supernatant, the sludge was resuspended in 1200-mL stock solution. About 100 mL of the mixed solution containing 44 mg of VSS was transferred to each vial using a syringe, and the pH was adjusted to 5.50. All vials were submerged in a 37°C shaking water bath. The vigorous shaking motion ensured complete mixing. At given time intervals, the volume of biogas produced was measured using a syringe, and the pH values, contents of the biogas, and mixed liquor were analyzed.

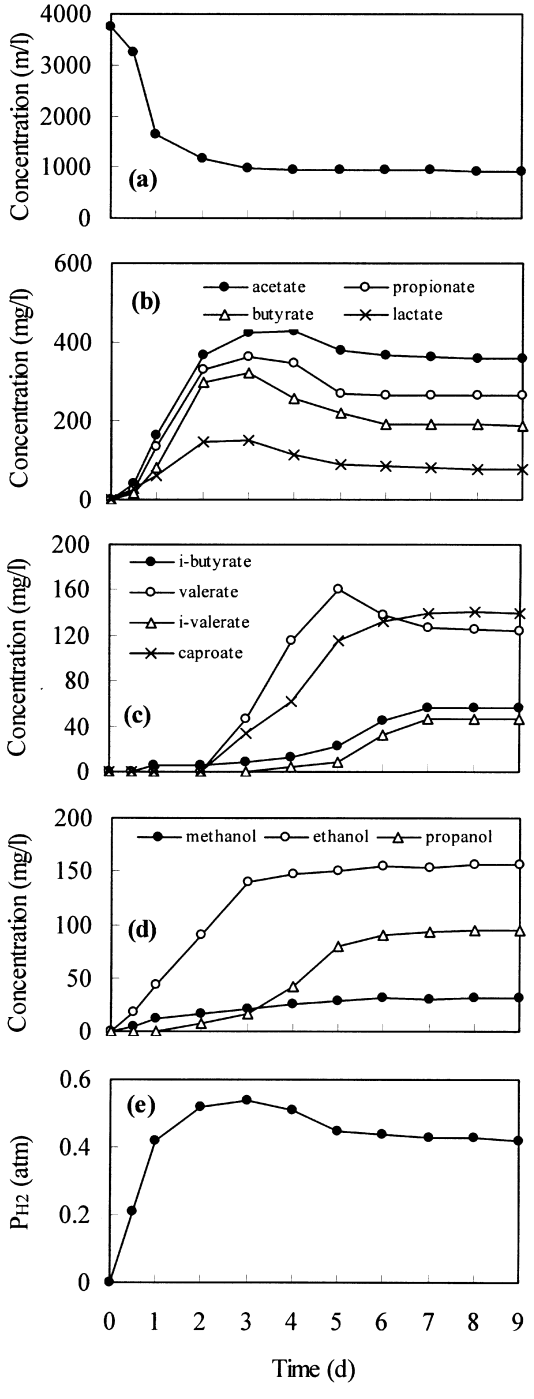

Figure 1. Concentration changes of (a) lactose; (b) acetate, propionate, butyrate, and lactate; (c) *i*-butyrate, valerate, *i*-valerate, and caproate; (d) methanol, ethanol, propanol, and butanol; and (e) P_{H2} in the batch receiving 4 g COD/L of substrate.

Analyses

The contents in the biogas were analyzed by a gas chromatograph (Hewlett Packard, Model 5890 Series II) equipped with a thermal conductivity detector and a 2 m $\times$ 2 mm (i.d.) stainless-steel column packed with Porapak N (80–100 mesh). Injector and detector temperatures were respectively kept at 130°C and 200°C, while column temperature was increased from 90°C to 110°C.

The concentrations of VFA and alcohols in the effluent, including acetate, propionate, butyrate, *i*-butyrate, valerate, *i*-valerate, caproate, lactate, methanol, ethanol, propanol and butanol, were determined by a second gas chromatograph of same model equipped with a flame ionization detector and a 10 m $\times$ 0.53 mm HP-FFAP fused-silica capillary column. Effluent samples were filtered through a 0.2 μm filter, acidified by formic acid, and measured for free acids and alcohols. The initial temperature of the column was 70°C for 4 min and then 140°C for 3 min, and finally 170°C for 4 min. The temperatures of injector and detector were both 200°C. Helium was used as the carrier gas at a flow rate of 25 mL/min. Formate, which could not be detected by gas chromatograph, was measured by the colorimetric method (Lang and Lang, 1972). Lactose was measured using the colorimetric ferric-cyanide method (Dubois et al., 1956). Measurements of COD, pH, and VSS were performed according to the Standard Methods (APHA, 1992).

RESULTS

The acidogenesis of lactose produced four classes of products: (1) organic acids including C_2-C_7 VFA; (2) C_1-C_4 alcohols; (3) biogas; and (4) biomass. In the other studies on acidogenesis of lactose (Chartrain and Zeikus, 1986; Kissalita et al., 1989; Yu and Pinder, 1993), the distributions of acidogenic products were less complex, and very little or no *i*-butyrate, *i*-valerate, caproate, and alcohols were detected from effluent. By contrast, in the present study, a significant amount of alcohols was present in the effluent; *i*-butyrate, *i*-valerate, caproate were also produced in all the runs.

Concentration of acidogenesis products of lactose was monitored at various time intervals. In all batches, the biogas was mainly composed of hydrogen and carbon dioxide, and the mixed liquor was composed of VFA and alcohols. The VFA were mostly acetate, propionate, and butyrate, plus smaller quantities of lactate, *i*-butyrate, valerate, *i*-valerate and caproate; whereas the alcohols were mostly ethanol, propanol and butanol, plus a trace amount of methanol.

To avoid overcrowding, only the results of two batch tests are presented here. Figure 1 illustrates the concentration changes of (a) lactose; (b) acetate, propionate, butyrate, and lactate; (c) *i*-butyrate, valerate, *i*-valerate, and caproate; (d) methanol, ethanol, propanol, and butanol; and (e) P_{H2} in batches treating midstrength wastewaters, using the one of 4 g COD/L as an example. Figure 3 illustrates the corresponding results treating high-strength wastewaters, using the one of 20 g COD/L as an example, for comparison.

Treating Wastewater at 4 g COD/L

Figure 1a illustrates that lactose was degraded rapidly and over 70% of lactose was reduced within 2 days. Similar observation was reported for the methanogenesis (Yu and Pinder, 1993) and acidogenesis (Kissalita et al., 1989) of lactose. By day 3, lactose concentration was decreased to 980 mg/L; afterwards it remained almost unchanged.

Figure 1b illustrates that formation of acetate, propionate, butyrate and lactate was in accord with the degradation of lactose. The concentrations of acetate, propionate, butyrate, and lactate increased rapidly, reaching 423, 365, 322, and 151 mg/L, respectively, by day 3. Thereafter, the concentrations of propionate, butyrate and lactate began to decrease, while the concentration of acetate slightly increased to 427 mg/L on day 4 before declining later. By day 6, the concentration of acetate reduced to 363 mg/L, propionate to 263 mg/L, butyrate to 192 mg/L, lactate to 84 mg/L. After day 6, they leveled off.

On the other hand, Figure 1c illustrates that *i*-butyrate, valerate, *i*-valerate, and caproate were produced later than the above four carboxylic acids. The concentrations of *i*-butyrate, valerate, *i*-valerate, and caproate were insignif-

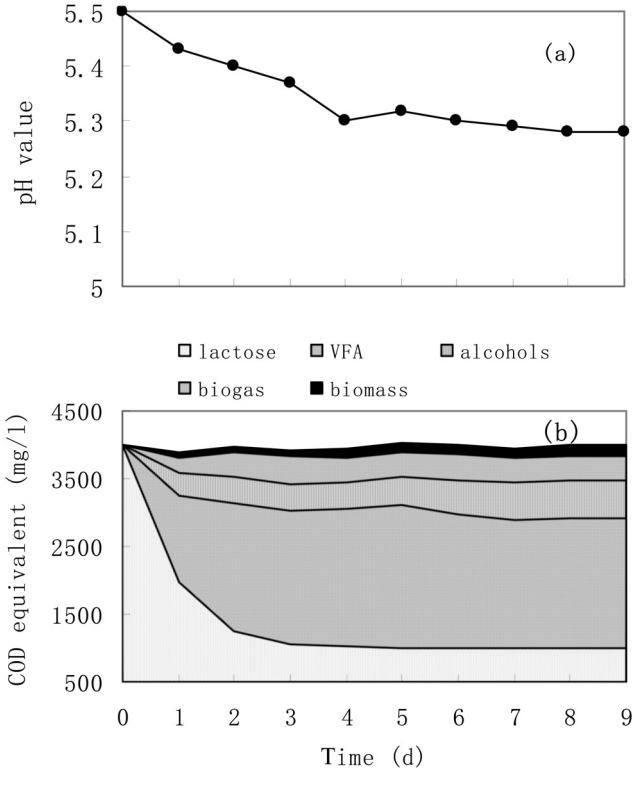

Figure 2. Changes of (a) pH, and (b) COD equivalents of the four groups of compounds present/formed in the batch receiving 4 g COD/L of substrate.

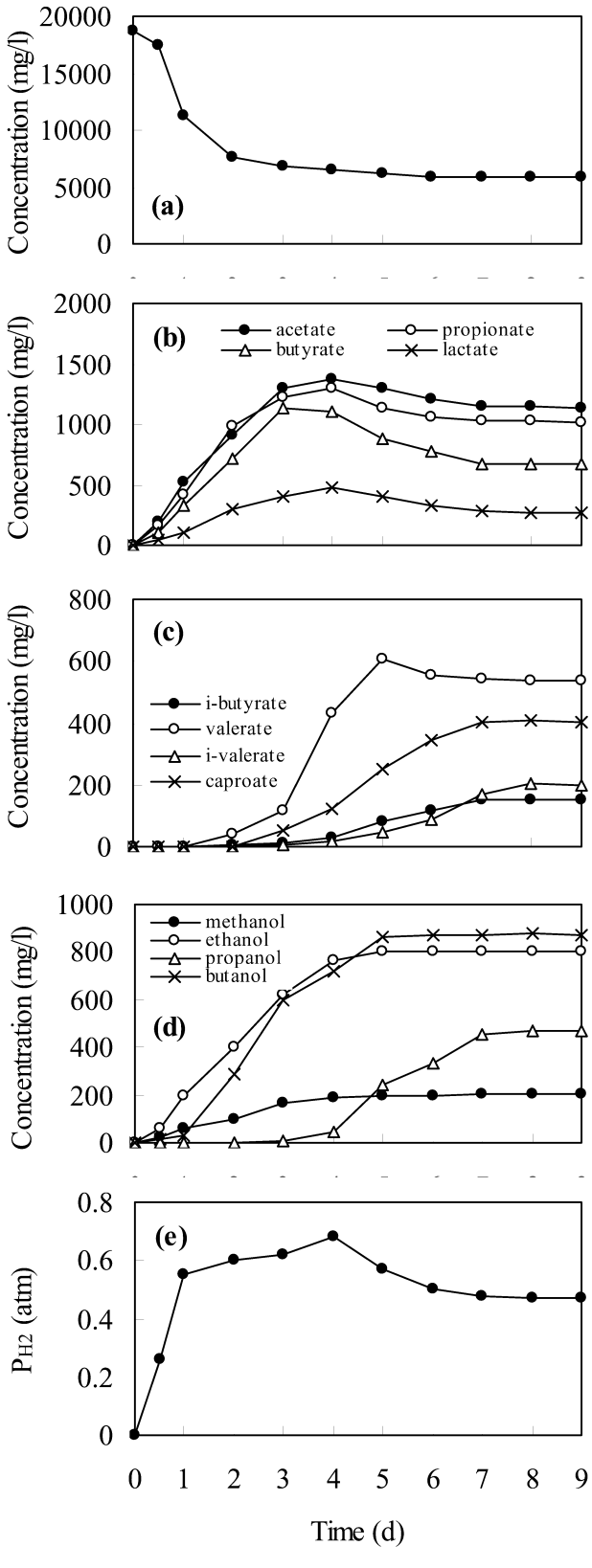

Figure 3. Concentration changes of (a) lactose; (b) acetate, propionate, butyrate, and lactate; (c) *i*-butyrate, valerate, *i*-valerate, and caproate; (d) methanol, ethanol, propanol, and butanol; and (e) P_{H2} in the batch receiving 20 g COD/L of substrate.

icant in the first 3 days. They began to increase thereafter with the decrease of acetate, propionate, butyrate, and lactate concentrations, reaching 22, 160, 10, and 116 mg/L, respectively, by day 5. After that, valerate concentration declined to 127 mg/L, while *i*-butyrate, *i*-valerate, and caproate kept increasing, and reached 56, 46, and 139 mg/L, respectively, by day 7. Nevertheless, the production of *i*-valerate was coincident with the reduction of valerate.

Figure 1d illustrates that concentrations of alcohols were lower than those of VFA. Ethanol was the main alcohol produced, reaching 155 mg/L by day 6. Methanol and propanol were produced at lower concentrations, each reaching about 31 and 94 mg/L by day 9. Butanol was not detected throughout the 9-day experiment.

Figure 1e illustrates that hydrogen was produced in a direct response to the degradation of lactose. It is well known that acidification of carbohydrate, including lactose (Kissalita et al., 1989), produces hydrogen as a by-product. P_{H2} reached 0.21 atm in 12 h, and 0.55 atm when lactose was not degraded anymore by day 3. It then slightly declined afterwards and leveled off by day 7, corresponding to the increase of valerate, caproate, and propanol.

This batch was started at pH of 5.50, but the solution pH decreased by 0.14–0.22 during the experiment (Fig. 2a). This insignificant reduction in pH was mainly attributed to the buffer supplied and the partial conversion of lactose to alcohols rather than to VFA.

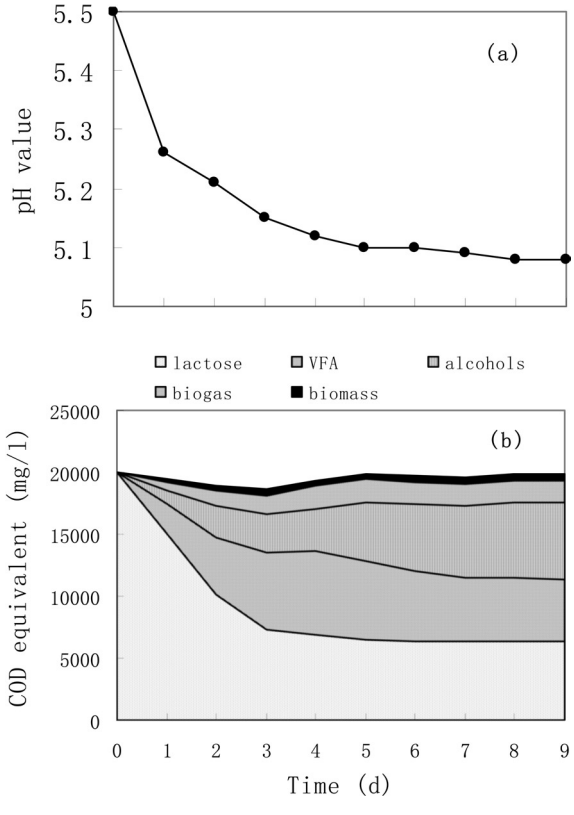

Figure 4. Changes of (a) pH, and (b) COD equivalents of the four groups of compounds present/formed in the batch receiving 20 g COD/L of substrate.

Table I. Valerate-forming reactions and the changes of standard Gibbs free energy.

Reactions	ΔG° (kJ)
A. $CH_3CH_2COO^- + CH_3(CH_2)_2COO^- \rightarrow CH_3(CH_2)_3COO^- + CH_3COO^-$	−0.04
B. $CH_3CH_2COO^- + CH_3COO^- + 2H_2 + H^+ \rightarrow CH_3(CH_2)_3COO^- + 2H_2O$	−49.64
C. $CH_3CH_2COO^- + 2CO_2 + 6H_2 \rightarrow CH_3(CH_2)_3COO^- + 4H_2O$	−143.28

COD is a wastewater parameter indirectly measuring the amount of electrons in substrates available for oxidation. In a strict anaerobic process, no electron acceptor is added to the system. In such a case, although the COD in the substrate can be transformed into VFA, alcohols, hydrogen and biomass, the overall COD should remain unchanged. Assuming that each gram of biomass is equivalent to 1.42 g of COD based on the chemical formula of $C_5H_7NO_2$, the total-COD of the four groups of compounds present/formed during the course of the process are calculated and illustrated in Figure 2b. As illustrated in Figure 2b, about 14% of the substrate COD ended up in the alcohols; VFA accounted for 48% of the COD; biogas and biomass accounted for 9% and 4%, respectively, of the total COD. It should be noted that around 25% of lactose remained unconverted. This poor conversion efficiency of lactose might be associated with the low pH in the solution. Previous studies demonstrate the appropriate pH for the acidogenesis of lactose was beyond 6.5 (Kissalita et al., 1989; Fang and Yu, 2001).

Treating Wastewater at 20 g COD/L

Results of treating high-strength wastewater, using the one of 20 g COD/L as an example, are illustrated in Figure 3. Most of the results in treating lactose-rich wastewater were independent of the wastewater strength, except production of butanol and peaking time of all VFA and alcohols. Figure 3a illustrates that lactose was rapidly degraded within the initial 3 days and leveled off after day 4. Figure 3b illustrates that the formation of acetate, propionate, butyrate, and lactate was directly associated with the degradation of lactose. On the other hand, Figure 3c illustrates that, like in treating midstrength wastewaters, formation of *i*-butyrate, valerate, *i*-valerate, and capraote was coincident with the decrease of acetate, propionate, butyrate and lactate, and that their total amount was not significant as compared to the four lower acids. Figure 3d illustrates that a significant amount of butanol was also produced, in addition to the formation of methanol, ethanol, and propanol.

Plots for the other experiments using different substrate concentrations gave similar results. In all batches, productions of alcohols increased with the substrate concentration. Figure 3d illustrates that, propanol and butanol were produced at a much higher level, as compared to batches receivinging mid-strength wastewaters. Initially the P_{H2} in all batches increased rapidly, due to the acidification of lactose. However, the P_{H2} decreased after reaching a peak pressure (0.68 atm in Fig. 3e). This coincided with the sharp increases in the concentrations of *i*-butyrate, valerate, *i*-valerate, caproate, and propanol and the sharp decreases in the concentrations of acetate, propionate, butyrate, lactate and hydrogen, suggesting that the later group of products was likely to partially converted to the former group in the later stage of the 9-day acidogenic experiment.

As shown in Figure 4a, the solution pH slightly decreased from initial 5.50 to 5.08 at the end of experiment. The total-COD of the four groups of compounds present/formed during the course of the process are calculated and

Table II. Gibbs free energy profiles of the valerate-forming reactions.

	ΔG (kJ) for reaction					
	4 g COD L			20 g COD L		
Time (d)	A	B	C	A	B	C
0.5	NA	NA	NA	NA	NA	NA
1	NA	NA	NA	NA	NA	NA
2	NA	NA	NA	−7.53	−8.64	−139.33
3	−5.57	−7.22	−135.46	−5.57	−5.53	−137.50
4	−2.16	−4.42	−131.91	−2.16	−2.97	−134.79
5	−0.47	−1.94	−128.88	−0.47	−2.07	−132.30
6	−0.41	−1.76	−129.51	−0.41	−1.97	−131.07
7	−0.14	−1.50	−129.58	−0.14	−1.78	−130.84
8	−0.16	−1.39	−129.92	−0.16	−1.79	−130.52
9	−0.14	−1.35	−129.48	−0.14	−1.75	−130.48

Note: NA, not applicable.

Table III. Caproate-forming reactions and the changes of standard Gibbs free energy.

Reactions	ΔG° (kJ)
D. $2CH_3(CH_2)_2COO^- \rightarrow CH_3(CH_2)_4COO^- + CH_3COO^-$	−0.11
E. $CH_3(CH_2)_2COO^- + CH_3COO^- + 2H_2 + H^+ \rightarrow CH_3(CH_2)_4COO^- + 2H_2O$	−48.01
F. $CH_3(CH_2)_2COO^- + 2CO_2 + 6H_2 \rightarrow CH_3(CH_2)_4COO^- + 4H_2O$	−143.34
G. $3CH_3COO^- + 3H_2 + 2H^+ \rightarrow CH_3(CH_2)_4COO^- + 4H_2O$	−86.20

illustrated in Figure 4b. About 31% and 26% of the substrate COD ended up in the alcohols and VFA, respectively, while biogas and biomass accounted for 9% and 3%, respectively, of the total COD. Approximately 31% of lactose remained unconverted.

Production of Valerate

Table I lists three possible valerate-forming reactions suggested by Smith and McCarty (1989a), where ΔG° is the change of Gibbs free energy (Thauer et al., 1977) at pH 7 under standard conditions (i.e., all solutes are at the concentration of 1*M*, and gases have partial Δpressure of 1 atm). The actual ΔG during the reaction, however, is dependent on the concentrations of reactants and products. Taking reaction (A) for example,

$$\Delta G = G^o + 2.303RT\log$$

$$[\{\text{valerate}\}\ \{\text{acetate}\}/\{\text{propionate}\}\ \{\text{butyrate}\}]$$

where *R* is the universal gas constant, 8.314 J/K mol, *T* is the absolute temperature in *K*, { } represents the chemical activity, which approximates molarity at low concentrations, and P_{H2} and P_{CO2} are partial pressures in atm. At 37°C, the term 2.303RT equals 5.934 kJ.

According to the second thermodynamic law, a reaction can take place only if ΔG is negative. Since all terms needed for the calculation have been measured, the actual ΔG values for the five reactions during the experiment can be calculated. Table II lists the ΔG profiles of the four valerate-forming reactions for the batches receiving 4 and 20 g COD/L of lactose, respectively.

As shown in Table II, the ΔG values were consistently negative for reactions (A), (B) and (C), indicating that these three reactions were thermodynamically possible. However, reactions (A) and (B) were unlikely responsible for the formation of valerate, because after day 4 the ΔG values of both reactions were less than 2 kJ/mol, the minimum amount of Gibbs free energy needed to sustain growth and/or conversion of a substrate (Dwyer et al., 1988; Smith and McCarty, 1989b; Hickey and Switzenbaum, 1991). This leaves reaction (C) as the only reaction potentially responsible for the formation of valerate. This reaction requires hydrogen as electron donor and consuming of propionate and carbon dioxide.

Production of Caproate

Table IV lists the ΔG profiles of the four possible caproate-forming reactions calculated from the measured data. The ΔG values of reaction (G) were consistently positive. Thus, it did not occur in this experiment. Between day 3 and day 5, the ΔG values of reactions (D) and (E) were negative, but they became positive after day 5 when the concentration of caproate was still increasing. This indicates the reactions (D) and (E) were unlikely responsible for the formation of caproate. Throughout the experiment the ΔG values of reaction (F) were lower than −128 kJ/mol, suggesting that caproate was most likely produced directly from butyrate,

Table IV. Gibbs free energy profiles of the caproate-forming reactions.

	ΔG (kJ) for reaction							
	4 g COD L				20 g COD L			
Time (d)	D	E	F	G	D	E	F	G
0.5	NA	NA	NA	NA	NA	NA	NA	NA
1	NA	NA	NA	NA	NA	NA	NA	NA
2	NA	NA	NA	NA	NA	NA	NA	NA
3	−4.93	−5.92	−135.85	2.77	−7.55	−11.75	−139.50	−6.31
4	−2.21	−3.53	−132.77	4.83	−1.97	−6.54	−137.59	−2.47
5	−0.07	−0.57	−129.13	8.64	−1.05	−1.49	−133.88	1.44
6	0.87	0.33	−128.75	9.47	0.74	−1.22	−131.39	2.61
7	1.05	0.65	−128.42	9.95	0.23	−0.48	−131.45	2.99
8	1.04	0.69	−128.69	10.02	0.26	−0.46	−130.08	2.92
9	1.05	0.81	−128.24	10.25	0.22	−0.45	−130.12	2.90

Note: NA, not applicable.

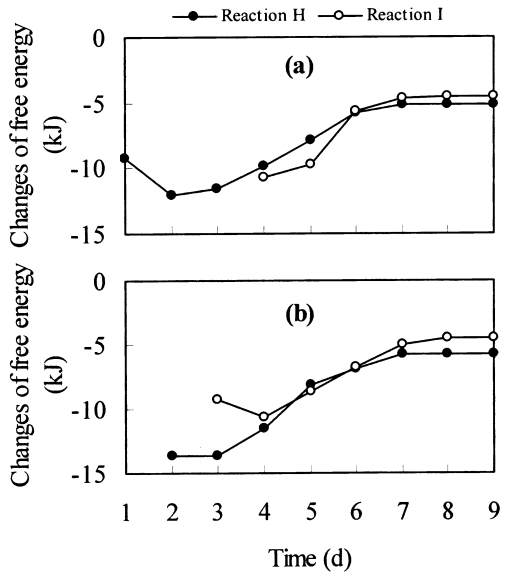

Figure 5. Changes of Gibbs free energy in *i*-butyrate and *i*-valerate-forming reactions in batches receiving (a) 4 g COD/L of substrate, and (b) 20 g COD/L of substrate.

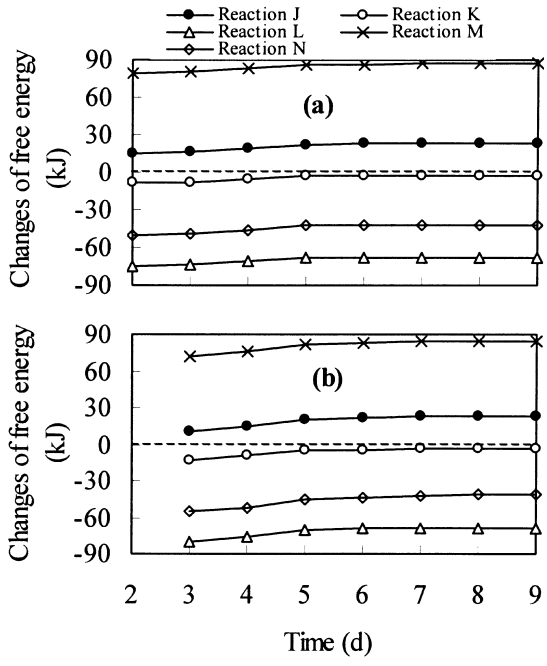

Figure 6. Changes of Gibbs free energy in propanol-forming reactions in batches receiving (a) 4 g COD/L of substrate, and (b) 20 g COD/L of substrate.

hydrogen, and carbon dioxide as in reaction (F). Therefore, the formation of caproate was dependent of P_{H2}.

Production of *i*-Butyrate and *i*-Valerate

As illustrated in Figures 1c and 3c, both *i*-butyrate and *i*-valerate were present with significant levels as lactose was the sole carbon source. Their formation was coincident with the decrease of their corresponding straighted-chain VFA, suggesting that *i*-butyrate and *i*-valerate were produced through respective isomerization of butyrate and valerate. *i*-Butyrate and *i*-valerate in this study might be produced by the following reactions:

$$\text{Reaction (H) butyrate} \rightarrow \textit{i}\text{-butyrate}, \Delta G^{o} = -1.98 \text{ kJ}$$

$$\text{Reaction (I) valerate} \rightarrow \textit{i}\text{-valerate}, \Delta G^{o} = -2.02 \text{ kJ}$$

Figure 5a and 5b, respectively, illustrates that the ΔG values for reactions (H) and (I) during the batches receiving 4 and 20 g COD/L of lactose. The ΔG values for the two reactions were consistently negative, confirming that the formation of *i*-butyrate and *i*-valerate were very likely from their isomerization as in reactions (H) and (I).

The ΔG values for reaction (H) were -5.7 and -5.8 kJ/mol when *i*-butyrate reaching plateau on day 6 for 4 g COD/L and day 7 for 20 g COD/l, respectively. This indicates that the minimum amount of Gibbs free energy needed to sustain isomerization of butyrate was approximately 5.7–5.8 kJ/mol. For the reaction (I), the ΔG values were -4.6 and -4.5 kJ/mol when *i*-valerate reaching plateau on day 7 for 4 g COD/L and on day 8 for 20 g COD/L, respectively, suggesting that the minimum amount of Gibbs free energy needed to sustain isomerization of butyrate was approximately 4.5–4.6 kJ/mol.

Production of Propanol

Propanol was present at a significant level in all the batches. Five possible reactions by which propanol could be formed, and their associated ΔG° are listed in Table V. These seem to be the most reasonable pathways for propanol formation, although many other reactions could have been written. The ΔG values for the five reactions were calculated using measured concentrations or partial

Table V. Propanol-forming reactions and the changes of standard Gibbs free energy.

Reactions	ΔG° (kJ)
J. $CH_3CH_2COO^- + 2H_2 + H^+ \rightarrow CH_3(CH_2)_2OH + H_2O$	−12.04
K. $CH_3CH_2OH + CH_3CH_2COO^- \rightarrow CH_3(CH_2)_2OH + CH_3COO^-$	−2.38
L. $CH_3CH_2OH + CO_2 + 3H_2 \rightarrow CH_3(CH_2)_2OH + 2H_2O$	−74.04
M. $2CH_3COO^- + H_2 + 2H^+ \rightarrow CH_3(CH_2)_2OH + CO_2 + H_2O$	11.21
N. $CH_3COO^- + 5H_2 + H^+ + CO_2 \rightarrow CH_3(CH_2)_2OH + 3H_2O$	−83.70

Table VI. Butanol-forming reactions and the changes of standard Gibbs free energy.

Reactions	ΔG° (kJ)
O. $CH_3(CH_2)_2COO^- + 2H_2 + H^+ \rightarrow CH_3(CH_2)_3OH + H_2O$	−16.52
P. $CH_3CH_2OH + CH_3(CH_2)_2COO^- \rightarrow CH_3(CH_2)_3OH + CH_3COO^-$	−6.87
Q. $CH_3CH_2OH + 2CO_2 + 6H_2 \rightarrow CH_3(CH_2)_3OH + 4H_2O$	−150.09
R. $CH_3CH_2COO^- + 5H_2 + H^+ + CO_2 \rightarrow CH_3(CH_2)_3OH + 3H_2O$	−88.07
S. $2CH_3COO^- + 4H_2 \rightarrow CH_3(CH_2)_3OH + 3H_2O$	−144.46

pressures of reactants and products. Figure 6a and 6b illustrates only the period of time in which propanol was actually detected.

The ΔG values of reactions (J) and (M) were consistently positive, indicating that they should not be involved in the formation of propanol. Therefore, only reactions (K), (L), and (N) were thermodynamically possible. However, since ethanol concentration was still slightly increasing while propanol was produced, reactions (K) and (L), which coupled with the consumption of ethanol were not unlikely involved in the formation of propanol. Throughout the experiment the ΔG values of reaction (N) were lower than −41 kJ/mol, suggesting that propanol was most likely produced directly from acetate, hydrogen, and carbon dioxide as in reaction (N).

The ΔG values for reaction (N) were −42.0 and −41.8 kJ/mol when propanol reached plateau on day 7 for 4 g COD/L and day 8 for 20 g COD/L, respectively, suggesting that the minimum amount of Gibbs free energy needed to sustain production of propanol through reaction (N) was approximately 41.8–42.0 kJ/mol.

Production of Butanol

Butanol could be produced by the reactions listed in Table VI when their ΔG values are negative. The ΔG values for the five reactions were calculated using measured concentrations or partial pressures of reactants and products. Figure 7 illustrates only the period of time in which butanol was actually detected.

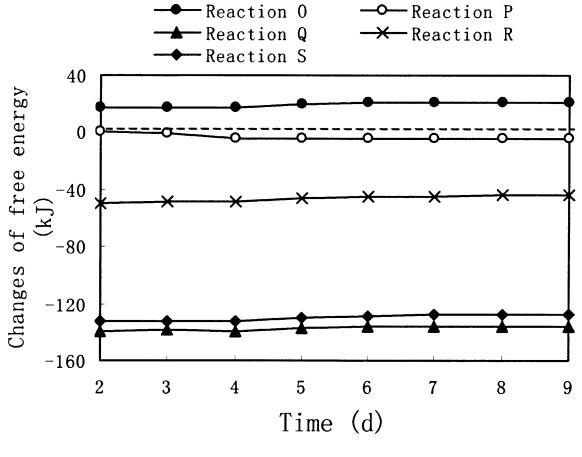

Figure 7. Changes of Gibbs free energy in butanol-forming reactions in the batch receiving 20 g COD/L of substrate.

The ΔG values of reaction (O) were consistently positive, suggesting that it should not be involved in the formation of propanol. Since the ΔG values of reaction (P) were positive as butanol was actually produced, reaction (P) was not involved in the formation of butanol. Hence, only reactions (Q), (R), and (S) were thermodynamically possible. However, minor production of butanol from midstrength wastewater (even undetectable in the batches receiving 2 and 4 g COD/L) did not agree with the above assumption as P_{H2} was in similar ranges for both mid- and high-strength wastewaters. Furthermore, butanol reached its plateau much earlier than propanol, and its concentration did not increase when the concentrations of butyrate and hydrogen were still decreasing. Therefore, formation of butanol was likely controlled more by substrate level or population dynamics than by thermodynamics.

DISCUSSION

HMW-VFA, propanol, and butanol were formed from the acidogenesis of lactose. These products may result from reactions that provide pathways for consuming hydrogen, carbon dioxide, and lower-molecular-weight VFA (LMW-VFA). Patterns of formation and dissipation of these products, and the conditions under which they appeared may be indicative of the reactions by which they were formed and the microorganisms responsible.

The formation of HMW-VFA, e.g., valerate and caproate, by reductive back-reactions was evidenced by their rising concentrations when the back-reaction became energetically favorable. Similar phenomenon about HMW-VFA formation has been observed in other systems and under different conditions. Smith and McCarty (1989a) observed the formation of valerate and caproate in perturbed CSTRs receiving ethanol- and propionate.

Thermodynamic analysis on the formation patterns of valerate indicates that reaction (C) was potentially responsible for its formation. This reaction requires hydrogen as the electron donor and consumes propionate and carbon dioxide. This result is in agreement with the observation that valerate formation was strongly dependent on the P_{H2} (Smith and McCarty, 1989a).

Caproate could be formed by two possible ways (Fang and Jia, 1999). One way using hydrogen as electron donor, caproate was formed only when hydrogen pressure was

greater than $10^{-3.3}$ atm, while caproate could also be formed independent of hydrogen according to reaction (D) listed in Table III. Because in this study hydrogen pressure was consistently greater than 0.43 atm, caproate was thus probably formed by the two ways. However, the reaction energetic analysis suggests that the formation of caproate was dependent on P_{H2}. These results are consistent with the findings of Smith and McCarty (1989b).

i-Butyrate and *i*-valerate are produced from the fermentation of branched amino acids (McInerney, 1988), and are usual intermediates undetectable or present in very low levels in a steady-state methanogenic reactor degrading carbohydrates. However, this study indicates that *i*-butyrate and *i*-valerate could be produced through respective isomerization of butyrate and valerate. Lovley and Klug (1982) first observed the isomerization of butyrate when methanogenic bacteria were inhibited. Tholosan et al. (1988), by using ^{13}C-labeled butyrate as a sole substrate, demonstrated that isomerization between butyrate and *i*-butyrate was due to the migration of the carboxyl group.

Propanol is not a normal product in steady-state operation of methanogenic reactors, and is not even detected in the acidogenesis of various substrates (Breure and van Andel, 1984; Cohen et al., 1979; Dinopoulou et al., 1988; Kissalita et al., 1989). However, under perturbation conditions, propanol is formed in propionate- or ethanol-fed methanogenic CSTRs. Propanol formation is attributed to a perturbation induced change in intracellular conditions within the bacteria producing it, resulting in a shift in the flow of electrons and a modification in overall catabolic stoichiometry (Smith and McCarty, 1989a). Wu and Hickey (1996) also observed the production of propanol in the anaerobic ethanol oxidation.

As illustrated in Figures 1d and 3d, the butanol formation was strongly associated with substrate concentration. Jones and Woods (1986) reported VFA were the main products for the acidification of low-strength wastewaters, but butanol was for high-strength wastewaters. Many acidogenes, such as *Clostridium acetobutylicum* (Bahl et al., 1982), *Clostridium butyricum* (Andel et al., 1985), *Clostridium cellobioparum* (Chung, 1976), *Clostridium fallax* (Ueki et al., 1991), and *Clostridium pasteurianum* (Dabrock et al., 1992), and mixed acidogenic cultures (Fang and Yu, 2001), produce hydrogen, acetate, butyrate at low substrate concentrations and shift the metabolic pathways when the substrate concentration exceeds certain thresholds to produce butanol, and in some cases, acetone. In this study, acetone was not detected.

In the acidogenesis of carbohydrates, as the concentration of the LMW-VFA becomes sufficiently high, they result in a reduction of the pH gradient across the membrane and the total inhibition of all metabolic functions in the cell (Gottschalk, 1986). The shift from VFA production to butanol production in *Clostridium acetobutylicum* and related species is an adaptive response of the cell to inhibitory effects produced by LMW-VFA (Jones and Woods, 1986). The shift appears to be able to act as a detoxification mechanism, which allows the cell to avoid the inhibitory effects that would occur when LMW-VFA reach toxic levels. The addition of 600 mg/L acetate or butyrate to batch cultures of *Clostridium acetobutylicum* resulted in a rapid production of butanol (Gottschalk, 1986). It has been reported that butanol production did not initiate until acetate or butyrate reached a level of 0.4 to 0.6 g/L (Jones and Woods, 1986). This provides the explanation for the high production of butanol at the high-strength wastewaters and not at the low- or mid-strength wastewaters.

In summary, a complexity to the overall acidogenic system was founded with the formation of HMW-VFA, propanol, and butanol from acidogenesis of lactose, and the thermodynamic analysis by using Gibbs free energy calculations was appropriate for elucidating the different acidogenic patterns and mechanisms. Furthermore, this study suggests the relative roles and importance of thermodynamic and kinetic constraints in impacting end-product formation. Therefore, thermodynamic analysis might be used as a useful means for controlling the end-product formation. However, if the dominant microorganisms can form the HMW-VFA, propanol and butanol, the question remains as to what microorganisms and enzymes can form them. Perhaps, these facts indicate that biosynthetic enzymes can perform the reductive back-reactions under certain conditions. This warrants further investigations.

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