

Effects of Formate on Fermentative Hydrogen Production by *Enterobacter aerogenes*

Tatsuo Kurokawa, Shigeharu Tanisho

Department of Environment and Natural Sciences, Yokohama National University, Japan

Received: 31 October 2003 / Accepted: 31 October 2004 / Online publication: 19 April 2005

Abstract

This paper describes the effects of formate on fermentative hydrogen production by *Enterobacter aerogenes* by way of batch culture. When 20 mM formate was added to pH 6.3 and pH 5.8 *E. aerogenes* glucose cultures (formate culture) at the beginning of cultivation, hydrogen evolution through both glucose consumption and decomposition of the extrinsic formate occurred together, while hydrogen evolution occurred only through glucose consumption in the control cultures. The hydrogen evolution rates in the formate cultures were faster than in the control cultures, although cell growth and glucose consumption rates in the formate cultures were slower than the control cultures'. The decomposition rate of the extrinsic formate in the pH 5.8 formate culture was faster than in the pH 6.3 formate culture. The hydrogen yield from glucose in the pH 6.3 formate culture increased due to the increasing amount of the nicotinamide adenine dinucleotide for hydrogen production.

Key words: fermentative hydrogen production — formate — hydrogen evolution rate — hydrogen yield

Introduction

Enterobacter aerogenes is a facultative anaerobic bacterium that performs fermentative hydrogen production similar to *Escherichia coli*. In fermentative hydrogen production, improvement in the hydrogen yield and hydrogen evolution rate is de-

sired. Hydrogen yield of *E. aerogenes* wild type is about 1.0 around pH 6.0 in the 1% glucose culture (Tanisho et al., 1989; Converti and Perego, 2002). Recently it was reported that the hydrogen yield and hydrogen evolution rate were improved by mutants of *E. aerogenes* that are deficient in alcohol or acids production (Rachman et al., 1997, 1998; Nakashimada et al., 2002). The hydrogen evolution mechanism of *E. coli* is based on the decomposition of formate, which is a fermentative product from glucose consumption. *E. aerogenes* also produces formate from glucose and decomposes formate. Moreover, it was suggested that hydrogen could be evolved by the reoxidation of nicotinamide adenine dinucleotide (NADH), which is formed during glycolysis (Tanisho et al., 1989; Tanisho, 2001). This was proved by evolving hydrogen in cell-free extract of *E. aerogenes* when extrinsic NADH was reoxidized (Nakashimada et al., 2002). We became interested in the mechanism of hydrogen evolution and how formate added to a glucose culture would influence hydrogen production and metabolism.

One study on the formate hydrogenylase (FHL) pathway of *E. coli*, which decomposes formate into hydrogen and carbon dioxide, looked at the addition of formate to the glucose culture and concluded that the transport of formate to the inside of the *E. coli* cell increases internal formate concentration and induces synthesis of FHL (Rossmann et al., 1991). There is also a report describing hydrogen evolution by *E. coli* when an acid is added (Nandi et al., 2001). In that study, when thiosulfate, an end product from glucose consumption, was added to the glucose culture, hydrogen yield improved. The suggested reason was that the presence of thiosulfate suppressed thiosulfate production during *E. coli*'s metabolism of glucose, and the electrons from glucose used for production of hydrogen increased. For *E. aerogenes*, formate is an end product from its

Correspondence to: Tatsuo Kurokawa; E-mail: d01ta016@ynu.ac.jp; Ymozhokr7@aol.com

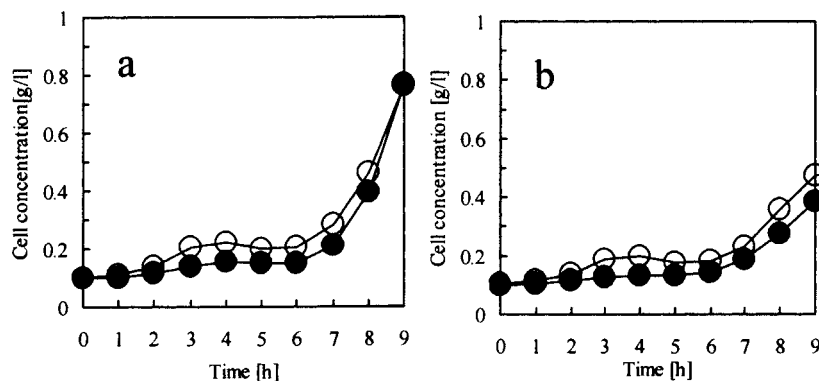


Fig. 1. Cell Concentrations at pH 6.3 (a) and at pH 5.8 (b) over time; O, control culture; ●, formate culture

metabolism of glucose, and by adding formate to the glucose culture, hydrogen yield may increase. Furthermore, a study found that hydrogen evolution by *E. coli* depends on culture pH: *E. coli* decomposes formate at pH 6.2, but not at pH 7.8 (Doeile, 1975). Hydrogen evolution by *E. aerogenes* also depends on culture pH, which is optimally around pH 6.0 (Tanisho et al., 1989). Our research involved experiments with glucose cultures at pH 6.3 and pH 5.8, with and without extrinsic formate, in which we analyzed yields of fermentative products from glucose and estimated formate synthesis and decomposition.

Materials and Methods

Organism and Culture Conditions. *E. aerogenes* st. E. 82005 was used, a species that has been inoculated periodically in our laboratory. A preculture was performed for 16 hours at 27°C in a culture medium consisting of 1.5% glucose, 0.5% peptone, 1.4% Na_2HPO_4 , 0.6% KH_2PO_4 , 0.2% $(\text{NH}_4)_2\text{SO}_4$, 0.1% citrate $2\text{H}_2\text{O}$, and 0.02% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, all measured in weight percent. After centrifuging at 8000 rpm, 60 ml of the incubated culture was inoculated into 2000 ml of glucose peptone culture. The culture contained 1.0% glucose, 0.5% peptone, 0.1% Na_2HPO_4 , 0.2% KH_2PO_4 , 0.2% $(\text{NH}_4)_2\text{SO}_4$, 0.1% citrate $2\text{H}_2\text{O}$, and 0.02% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in ion-exchanged water. Sodium formate was added to a concentration of 20 mM at the beginning of cultivation (formate culture). After replacing the gas phase of the reactor with nitrogen, the cultivation took place under anaerobic conditions at a temperature of 37°C in a 2.5-L stirred reactor by way of batch culture. The churning speed of the cultivation was at 120 rpm. A constant culture pH was maintained by a pH controller using 1 N NaOH and 1 N HCl. Two to three fermentations were repeated under the same conditions to obtain an average value.

Analysis. The hydrogen gas volume was measured by 1 N NaOH displacement in a liquid-gas exchange method. The nitrogen gas volume pushed out by carbon dioxide evolution under the initial operation condition was corrected by analyzing the carbon dioxide volume in the gas phase of the reactor. Gas chromatography (Shimadzu GC-3BT) was used to determine the gas volume and components. The quantitative analysis of a fermentation product and glucose was performed on a high-performance liquid chromatograph (655A HITACHI) equipped with a GL-C610H column (HITACHI Chemical). The column was operated at 40°C with 0.1% phosphoric acid as the carrier liquid at a flow rate of 1.0 ml/min. Cell weight was determined by comparing the optical density of cultures at 550 nm to a standard curve.

Results and Discussion

The cell concentrations in the formate cultures were lower than in the control cultures at both pH 6.3 and pH 5.8 (Figure 1), indicating that cell growth in the formate cultures was slower than in the control cultures. Cell growth is delayed by the presence of weak acid depending on the culture pH (Goodwin and Zeileus, 1987; Roe et al., 1998; Colin et al., 2001). The most suitable pH for cell mass productivity of *E. aerogenes* is around pH 7.0, and as the culture pH decreases below pH 7.0, the cell weight measured after cultivation also decreases (Tanisho et al., 1989). The delay of cell growth was reflected in the formate cultures by decreased glucose consumption (Figures 2, 3). Yet, despite decreased glucose consumption in the formate cultures, accumulated hydrogen volume was either larger (Figure 2) or similar to the control cultures' hydrogen volumes (Figure 3). This is attributed to the extrinsic formate decomposition in the formate cultures. In the formate cultures, the formate concentration remained either in a constant state at pH 6.3 (Figure 2,

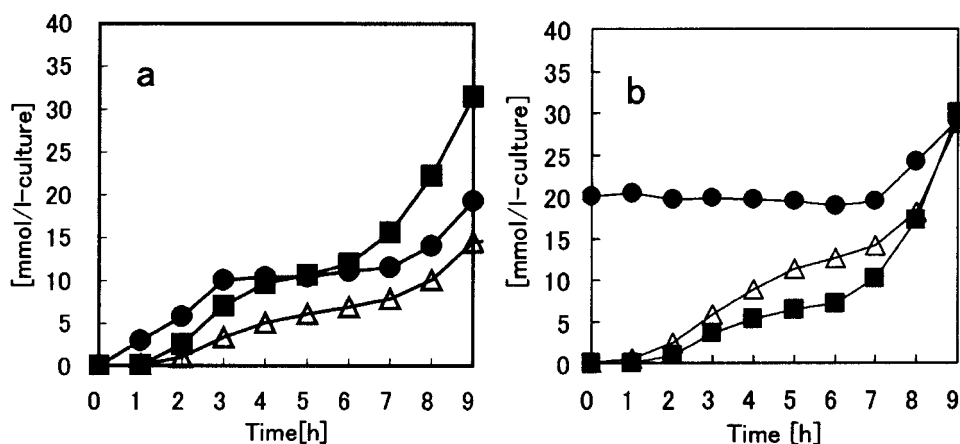


Fig. 2. Accumulated hydrogen volume (\square), amount of glucose consumed (\blacksquare) and formate concentration (\circ) in the pH 6.3 control culture (a) and the pH 6.3 formate culture (b) over time.

b) or decreased at pH 5.8 (Figure 3, b) with consumption of glucose, while formate concentrations in the control cultures increased through glucose consumption (Figures 2, a; 3, a). This indicates that the glucose consumption and the extrinsic formate decomposition occurred together in the formate cultures. So the accumulated hydrogen volume in the formate culture would be the sum of the hydrogen evolved from the glucose consumption plus the hydrogen evolved from decomposition of the extrinsic formate and represented by equation 1 shown below. Equation 2 represents the accumulated hydrogen volume in the control culture.

Accumulated Hydrogen Volume in Formate Culture = Hydrogen Volume Evolved from Glucose Consumption + Hydrogen Volume Evolved from Extrinsic Formate Decomposition (1)

Accumulated Hydrogen Volume in Control Culture = Hydrogen Volume Evolved from Glucose Consumption (2)

Equations 1 and 2 explain why the formate cultures, which had lower glucose consumption, still accumulated greater hydrogen volume than did the control culture at pH 6.3 (Figure 2) or hydrogen volume as great as the control culture at pH 5.8 (Figure 3); that is, hydrogen volume evolved from decomposition of the extrinsic formate.

The hydrogen evolution rates in the formate cultures were faster than in the control cultures, although the glucose consumption rates in the formate cultures, which reflected slower cell growth, were slower than in the control cultures both at pH 6.3 (Figure 4) and at pH 5.8 (Figure 5). This is attributed to the extrinsic formate decomposition in the formate cultures.

Comparing the two control cultures, the hydrogen evolution rate at pH 5.8 was faster than at pH 6.3, while the glucose consumption rate was almost the same (Figures 4, a; 5, a). This indicates that the hydrogen yield from glucose at pH 5.8 was higher than the yield at pH 6.3. Comparing the two formate cultures, the difference in glucose consumption rates was in clear contrast to the controls

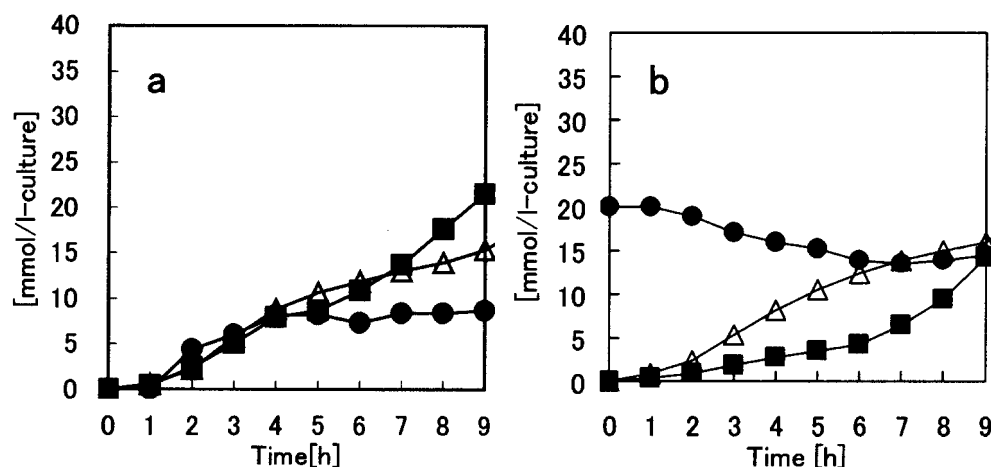


Fig. 3. Accumulated hydrogen volume (\triangle), amount of glucose consumed (\blacksquare) and formate concentration (\circ) in the pH 5.8 control culture (a) and the pH 5.8 formate culture (b) over time.

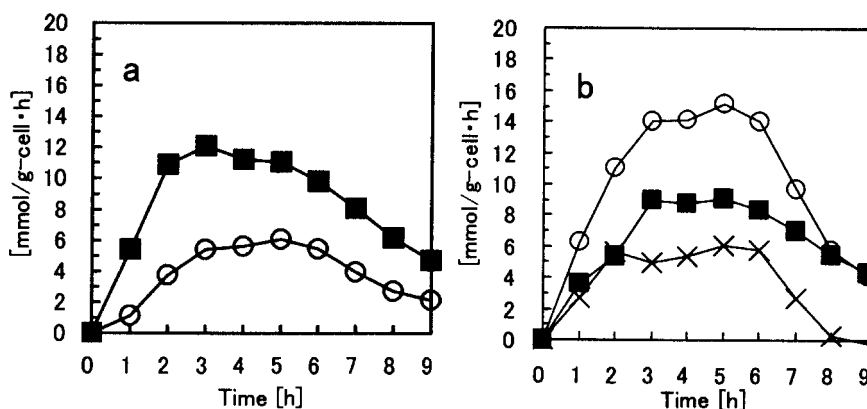


Fig. 4. Hydrogen evolution rate (\circ), glucose consumption rate (\blacksquare), and decomposition rate of the extrinsic formate (\times) calculated by Equation (6) in the pH 6.3 control culture (a) and the pH 6.3 formate culture (b) over time.

(Figures 4, b; 5, b), suggesting that cell growth was affected strongly by the additional formate. However, in spite of the difference in glucose consumption rates, hydrogen evolution rates were almost the same (Figures 4, b; 5, b) owing to the extrinsic formate decomposition rate. The hydrogen evolution rates are represented by equations 3 and 4, which are given by differentiating equations 1 and 2 with respect to time.

$$\begin{aligned} \text{Hydrogen Evolution Rate in Formate Culture} = \\ \text{Hydrogen Evolution Rate from Glucose Consumption} + \text{Decomposition Rate of Extrinsic Formate} \end{aligned} \quad (3)$$

$$\begin{aligned} \text{Hydrogen Evolution Rate in Control Culture} = \\ \text{Hydrogen Evolution Rate from Glucose Consumption} \end{aligned} \quad (4)$$

The hydrogen yields in the formate culture were 0.84 at pH 6.3 and 0.82 at pH 5.8, respectively, after 22 hours of cultivation the point where all of the 1% glucose was consumed (Table 1). When these yields are regarded as a constant 1.0 from the beginning of

cultivation, the approximation of equation 5 is derived as follows:

$$\begin{aligned} \text{Hydrogen Evolution Rate from Glucose Consumption in Formate Culture} = \\ \text{Glucose Consumption Rate in the Formate Culture} \end{aligned} \quad (5)$$

From equations 3 and 5, the extrinsic formate decomposition rate is represented as equation 6 shown below.

$$\begin{aligned} \text{Extrinsic Formate Decomposition Rate} = \\ \text{Hydrogen Evolution Rate in Formate Culture} - \text{Glucose Consumption Rate in the Formate Culture} \end{aligned} \quad (6)$$

By this approximation, the decomposition rate of the extrinsic formate in the pH 5.8 formate culture was faster than in the pH 6.3 formate culture (Figures 4, b; 5, b).

According to one study, synthesis of FHL in the *E. coli* cells, which decomposes formate, is enhanced in the presence of formate in the culture. The extrinsic formate, maintained as formic acid in culture through chemical equilibrium, is transported

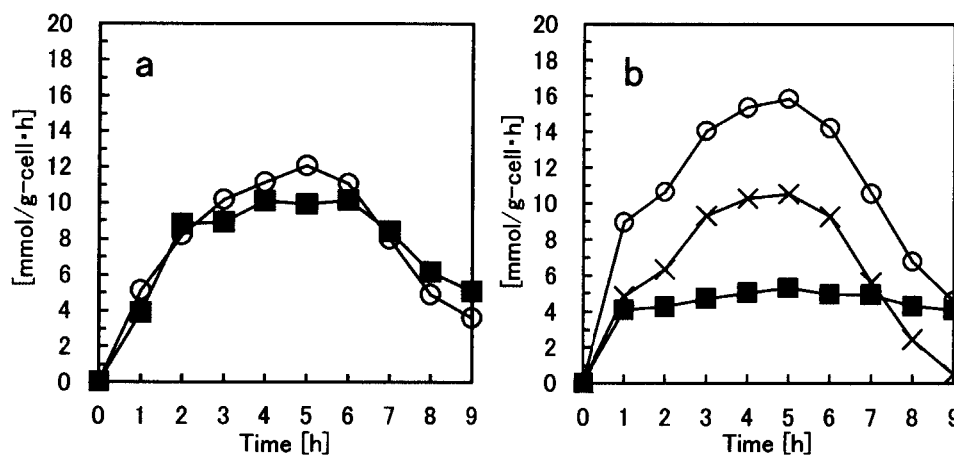


Fig. 5. Hydrogen evolution rate (\circ), glucose consumption rate (\blacksquare) and decomposition rate of the extrinsic formate (\times) calculated by Equation (6) in the pH 5.8 control culture (a) and the pH 5.8 formate culture (b) over time.

Table 1. Estimated Results of Formate Synthesis, Decomposition, and Hydrogen Yield from Glucose^a

Culture	Formate concentration	Formate synthesis ^b	Formate decomposition ^c	Hydrogen from NADH reoxidation ^d	Accumulated hydrogen volume	Hydrogen yield ^e
pH 6.3 control	34	67	33 (0.49)	7	40	0.72
pH 6.3 formate	42	70	48 (0.69)	18	66	0.84
pH 5.8 control	24	58	34 (0.59)	15	49	0.89
pH 5.8 formate	25	52	47 (0.90)	18	65	0.82

^aThe data are the average of duplicate analyses (mmol/L/culture; hydrogen yield, mmol/mmol glucose). Cultivation conditions: 10 g/L glucose, 37°C, churning at 120 rpm. All the values were determined at 22 hours of cultivation.

^bEquation 7: (product in parentheses indicates the amount produced from glucose): Amount of Formate Synthesis = (Acetate) + (Ethanol) + 2(Butyrate).

^cNumber in parentheses indicates ratio of formate decomposition to formate synthesis. Amount of formate decomposition was calculated by the following equations 8 and 9: Formate Concentration in Formate Culture = 20 + Amount of Formate Synthesis – Amount of Decomposition of Formate (8); Formate Concentration in Control Culture = Amount of Formate Synthesis – Amount of Decomposition of Formate (9).

^dHydrogen volume evolved through NADH reoxidation calculated by the following Equation 10: Accumulated Hydrogen Volume = Hydrogen Volume Evolved from NADH Reoxidation + Hydrogen Volume Evolved from Formate Decomposition.

^eEquation 11: Hydrogen Yield from Glucose in the Formate Culture = (Accumulated Hydrogen Volume – 20)/Amount of Glucose Consumed.

into the cell, where it dissociates back to formate and a proton increasing internal formate concentration and signaling the induction of FHL synthesis (Rossmann et al., 1991). This same study also suggested that internal formate concentration is higher and synthesis of FHL is enhanced more in the case of a lower culture pH because more formic acid is formed with a lower pH and transported into the cell. In our research, using 20 mM formate at the beginning of cultivation, the formic acid concentration was calculated by the Henderson-Hasselbalch equation shown below as 0.06 mM at pH 6.3 and 0.18 mM at pH 5.8.

$$\text{pH} = \text{pK}_a + \log \left[\frac{[\text{HCOO}^-]}{[\text{HCOOH}]} \right] \quad (\text{pK}_a = 3.75)$$

Because of the higher formic acid concentration in the pH 5.8 formate culture, synthesis of FHL was strongly induced, and as such the decomposition rate of the extrinsic formate was faster than in the pH 6.3 formate culture. In the case of the hydrogen production by immobilized cells of *E. coli* decomposing 1 M formate, formic acid concentration was calcu-

lated by the equation as 0.35 mM at pH 7.2 (Nandi and Sengupya, 1996).

After 22 hours of cultivation, both the pH 6.3 and pH 5.8 formate cultures had larger accumulated hydrogen volumes than the controls (Table 1). To determine why this was the case, we examined the fermentative products yields from glucose (Table 2). The ethanol and lactate yields were also lower than the controls; in the pH 6.3 formate culture, and the ethanol yield was lower than the controls; in the pH 5.8 formate culture, while the 2,3-butandiol and butyrate yields were similar to the controls; in both the pH 6.3 and pH 5.8 formate cultures (Table 2). This suggests that the extrinsic formate affects metabolism of glucose (Kirkpatrick et al., 2001). Metabolites that are produced when NADH reoxidation decreases, such as ethanol, lactate, or 2,3-butandiol, the amount of NADH used during hydrogen production increases, leading to an improved hydrogen yield theoretically (Tanisho, 2001). Actually the mutants of *E. aerogenes*, which are

Table 2. Yields of Products from Glucose at 22 Hours of Cultivation's^a

Culture	Yields of products from glucose (mmol/mmol glucose)					Balance (mmol/mmol)	
	Lactate	Acetate	2,3 Butandiol	Ethanol	Butyrate	Pyruvate ^b	NADH ^c
pH 6.3 control	0.63	0.36	0.07	0.20	0.33	1.00	0.97
pH 6.3 formate	0.49	0.40	0.05	0.15	0.36	0.93	1.10
pH 5.8 control	1.06	0.24	0.04	0.20	0.31	1.11	1.34
pH 5.8 formate	1.11	0.21	0.03	0.09	0.32	1.05	1.30

^aThe data are the average of duplicate analyses. Cultivation conditions: 10 g/L glucose, 37°C, churning at 120 rpm.

^{b,c}Values were calculated by the following equations 12 and 13, respectively: (product in parentheses indicates the amount produced from glucose)

Pyruvate balance = (Amount of Fermentative Products Except Formate Formed from Pyruvate)/(Amount of Pyruvate Produced from Glucose During Glycolysis) = [(Lactate) + (Ethanol) + 2(2,3 Butandiol) + (Acetate) + 2(Butyrate)]/2(Amount of Glucose Consumed) (12)

NADH Balance = (Amount of Fermentative Products and Hydrogen Produced when NADH Reoxidizes)/Amount of NADH Produced from Glucose During Glycolysis = [(Lactate) + 2(Ethanol) + (2,3 Butandiol) + 2(Butyrate) + (Hydrogen from NADH Reoxidation)]/2(Amount of Glucose Consumed) (13).

deficient in alcohol or acids production, improve the hydrogen yield (Rachman et al., 1997, 1998; Nakashimada et al., 2002). Analyzing the fermentative products yields in the formate cultures allowed the formate synthesis and decomposition and the hydrogen yields from glucose to be estimated (Table 1).

The estimation was performed as follows. Formate is synthesized through the following reaction:



The amount of formate synthesis is equal to the amount of acetyl–coenzyme A. Since acetate, ethanol, and butyrate are synthesized from glucose through acetyl–coenzyme A, the amount of formate synthesis is evaluated by the sum of acetate, ethanol, and butyrate produced from glucose (Table 1, equation 7). Butyrate was also produced by *E. aerogenes* on the continuous hydrogen production (Tanisho and Ishimata, 1994). The amount of formate decomposition was calculated from the amount of formate synthesis and formate concentration by equations 8 and 9 shown in Table 1. Although the amount of formate synthesis in the pH 5.8 formate culture was lower than in the pH 6.3 formate culture, the amount of formate decomposition was equal (Table 1). So the ratio of formate decomposition to formate synthesis in the pH 5.8 formate culture was higher than in the pH 6.3 formate culture (Table 1). This shows that formate decomposition proceeded actively at pH 5.8, and this is consistent with the faster decomposition rate of extrinsic formate at pH 5.8 (Figures 4, b; 5, b).

Because the amount of formate decomposition in the formate cultures exceeded 20 mmol/L culture (Table 1), all of the extrinsic formate was decomposed. The accumulated hydrogen volume both in the control cultures and the formate cultures exceeded each of the amounts of formate decomposition (Table 1). This indicates that hydrogen evolution occurred not only by the formate decomposition, but also by the NADH reoxidation. The hydrogen volume evolved from NADH reoxidation evaluated by the equation 10 increased in the formate cultures (Table 1). However, the amount of formate synthesis and the hydrogen yield in the pH 5.8 formate culture were lower than the control culture (Table 1), most likely due to the extrinsic formate, end product, suppressing formate production from glucose through a negative feedback control. We concluded that formate, an end product, when added to the pH 6.3 glucose culture, increased the hydrogen yield by increasing the

amount of the NADH in the cell utilized for hydrogen production.

Acknowledgments

We thank Dr. Tatsuki Wakayama (National Institute of Advanced Industrial Science and Technology) for his helpful discussions and kind advice.

References

1. Colin T, Bories A, Lavigne C, Moulin G (2001) Effects of acetate and butyrate during glycerol fermentation by *Clostridium butyricum*. *Curr Microbiol* 43, 238–243
2. Converti A, Perego P (2002) Use of carbon and energy balances in the study of the anaerobic metabolism of *Enterobacter aerogenes* at variable starting glucose concentrations. *Appl Microbiol Biotechnol* 59, 303–309
3. Doelle HW (1975) *Bacterial Metabolism*. 2nd ed. (New York NY.: Academic Press) 593–595
4. Goodwin S, Zeikus GJ (1987) Physiological adaptations of anaerobic bacteria to low pH: metabolic control of proton motive force in *Sarcina ventriculi*. *J Bacteriol* 169, 2150–2157
5. Kirkpatrick C, Maurer ML, Oyelakin EN, Yoncheva NY, Maurer R, Slonczewski LJ (2001) Acetate and formate stress: opposite responses in the proteome of *Escherichia coli*. *J Bacteriol* 183, 6466–6477
6. Nakashimada Y, Rachman MA, Kakizono T, Nishio N (2002) Hydrogen production of *Enterobacter aerogenes* altered by extracellular and intracellular redox states. *Int J Hydrogen Energy* 27, 1399–1405
7. Nandi R, Sengupta S (1996) Involvement of anaerobic reductases in the spontaneous lysis of formate by immobilized cells of *Escherichia coli*. *Enzyme Microbiol* 19, 20–25
8. Nandi R, Dey S, Sengupta S (2001) Thiousulphate improves yield of hydrogen production from glucose by the immobilized formate hydrogenylase system of *Escherichia coli*. *Biotechnol Bio* 754, 492–494
9. Rachman MA, Furutani Y, Nakashimada Y, Kakizono T, Nishio N (1997) Enhanced hydrogen production in altered mixed acid fermentation of glucose by *Enterobacter aerogenes*. *J Ferment Bioeng* 4, 358–363
10. Rachman MA, Furutani Y, Nakashimada Y, Kakizono T, Nishio N (1998) Hydrogen production with high yield and high evolution rate by self-flocculated cells of *Enterobacter aerogenes* in a packed-bed reactor. *Appl Microbiol Biotechnol* 494, 450–454
11. Roe JA, McIlaggan D, Davidson L, O'Byrne C, Booth RI (1998) Perturbation of anion balance during inhibition of growth of *Escherichia coli* by weak acids. *J Bacteriol* 180, 767–772
12. Rossmann R, Sawers G, Bock A (1991) Mechanism of regulation of formate-hydrogenylase pathway by oxy-

- gen, nitrate, and pH: definition of the formate regulon. *Mol Microbiol* 511, 2807–2814
13. Tanisho S (2001) A scheme for developing the yield of hydrogen by fermentation. *Biohydrogen* 2, 131–140
 14. Tanisho S, Ishiwata Y (1994) Continuous hydrogen production from molasses by the bacterium *Enterobacter aerogenes*. *Int J Hydrogen Energy* 1910, 807–812
 15. Tanisho S, Kamiya N, Wakao N (1989) Hydrogen evolution of *Enterobacter aerogenes* depending on culture pH: mechanism of hydrogen evolution from NADH by means of membrane-bound hydrogenase. *Biochim Biophys Acta* 9731, 1–6