Kor. J. Appl. Microbiol. Biotech.
Vol. 18, No. 1, 6-11 (1990)

Hydrogen Evolution from Biomass-Derived Carbohydrates by *Clostridia*

Bae, Moo* and Hye-Joo Yi

Department of Biology, College of Natural Science, Ewha Womans' University, Seoul 120-750, Korea

Clostridia 에 의한 Biomass 구성당으로부터의 수소생성

배 무* · 이혜주

이화여자대학교 자연과학대학 생물학과

Hydrogen evolution from biomass-derived carbohydrates by some *Clostridia* and optimal culture conditions for hydrogen evolution were investigated. Among the organisms tested, *Clostridium butyricum* was efficient hydrogen producer with starch, xylan, pectin, cellobiose and xylose. In batch fermentation of *Cl. butyricum*, optimal conditions for hydrogen evolution were achieved at pH 7.0-8.5, 10-50 mM phosphate, and 2% (w/v) glucose. Total amount of molecular hydrogen evolved by the organism slightly increased at the presence of acetate (< 150 mM) or butyrate (< 20 mM) in the initial fermentation medium. Especially, in case of more than the above concentration of butyrate, growth and hydrogen evolution were dramatically inhibited. In the conditions which were described here, 70 mmole of molecular hydrogen per mg of DCW was produced with 1% (w/v) glucose by the organism.

Interest in molecular hydrogen as ideal fuel source has been hauling the study of biological process for the hydrogen production from non-fossil renewable energy resources. Until recently, extensive studies have been devoted to a number of photosynthetic organisms or strict anaerobes capable of producing hydrogen gas (1-4). In recent years, anaerobes which were able to evolve hydrogen especially from biomass are being an object of study in regard of reuse waste resources including wastewater (5-7). Mixed culture with cooperative species or immobilized cell system to evolve hydrogen from biomass components has also been conducted (8-13). However, it remains to be solved how the other cultural metabolites of *Clostridium* are controlled to diminish and converted to molecular hydrogen.

Materials and Methods

Key words: Hydrogen, *Clostridia*, biomass-derived carbohydrates

*Corresponding author

Microorganisms

The microorganisms used in the study were *Clostridium acetobutylicum* ATCC824 (NCIB 8052), *Cl. butyricum* NCIB 9576, *Cl. pasteurianum* ATCC 6013 (NCIB 9486), *Cl. thermocellum* ATCC 27405, which were provided by KIST. The cultures incubated for 4-5 days were kept as a spore suspension in 10 mM potassium phosphate (pH 7.0) at 4 °C .

Media and growth conditions

The culture medium (PYG) contained the following components per liter of distilled water (*l*): K_2HPO_4 0.9g, KH_2PO_4 0.9g, $MgSO_4 \cdot 7H_2O$ 0.09g, $CaCl_2 \cdot 2H_2O$ 0.09g, NaCl 0.9g. $(NH_4)_2SO_4$ 0.9g, peptone 10g, yeast extract 5g, cysteine-HCl 0.5g, $Na_2CO_3 \cdot 10H_2O$ 4g, biotin 10 μg, *p*-aminobenzoic acid 100 μg, resazurin 100 μg, glucose (or carbon source) 10g. Carbohydrates (mono- and disaccharide) tested as substrate were seperately sterilized by autoclaving (120°C, 20 min), and then added to the medium at a concentration of 10 g/*l*. When the effects of phosphate and glucose were investigated, the

modified Ormerod's (14) medium (pH 8.0) was used as fermentation medium. The anaerobic culture technique of Hungate (15) or Bryant (16) and the serum bottle modification method (17) were used in the study. The organisms were grown in 50 m*l* serum bottles containing 10 m*l* of pre-reduced medium. After the pH of the medium was adjusted (7.0), the medium was sterilized as dispensed into serum bottles. And then serum bottles were sealed with butyl rubber stopper, the gas phase was replaced with argon by flushing, and culture media were autoclaved for 20 min at 120°C. Spore suspension was transferred to the prepared medium and incubated at 60°C (*Cl. thermocellum*) or 37°C (the other strains) without shaking for 24 hrs. The seed culture was inoculated at 5% (v/v) to the fermentation medium, and incubated at the same conditions above described.

Measuring of the cell growth

Cell growth was followed by measuring the absorbance at 660 nm with Spectronic 501 (Milton Roy Co.) spectrophotometer. Dry Cell Weight (DCW) estimated was relative value to optical density. Growth of xylan-growth culture was estimated by measuring the microbial protein; difference of protein content between the culture and cell free medium was estimated for the microbial protein. Protein content was determined by Lowry method (25) using bovine serum albumin as standard.

The quantitative determination of hydrogen

Gas sample in the gas-tight serum bottle was measured by gas chromatography (Shimadzu GC-9A) under the following conditions.

Column material	Molecular sieve 5A
Temperature	80°C
Detector	Thermal Conductivity Detector
Temperature	120°C
Injector temperature	100°C
Carrier gas	Argon
Flow rate	40 m*l*/min
Sample size	100 μl

Determination of reducing sugars

Reducing sugars were determined with dinitrosalicylic acid method of Miller *et al.* (18). The amounts of total carbohydrates were determined with the anthrone-sulfuric acid reagent (19, 20). For some particulate substrates, due to sedimentation with the cells, the whole culture was used in the sugar determination.

Fermentation products

Excluding molecular hydrogen, solvents (ethanol, acetone, butanol) and organic acids (acetate, butyrate) were determined by gas chromatography. The fermentation was continually monitored for pH, growth, hydrogen evolution, soluble products and substrate concentration. Samples were centrifuged for 5 min at 12,000 rpm to remove cells, and the supernatant was used for analysis after being acidified with 5 N HCl (0.125 m*l*/m*l* of sample), injected (21, 22), and analyzed by following conditions of gas-chromatography (Shimazu GC-9A).

Column material	Porapak Q
Temperature	200°C
Detector	Flame Ionized Detector
Injector temperature	200°C
Carrier gas	Nitrogen
Flow rate	50 m*l*/min
Internal standard material	*l*-propanol
Sample size	2 μl

Results and Discussion

Hydrogen evolution from glucose by *Clostridia*

Fermentation pattern of *Clostridia* with glucose has been extensively studied. Additionally, hydrogen evolution was also studied in some *Clostridia* (27). Fig. 1 shows the time course of hydrogen evolution by several *Clostridia* under anaerobic condition. *Cl. pasteurianum* has the higher hydrogen productivity from glucose as a electron donor.

Hydrogen evolution from various carbohydrates as components of biomass

Biomass, naturally occurring biopolymer, is composed of mainly pentose and hexose in the form of cellulose, xylan, pectin, and starch. These principal components were fermented in various types by *Clostridia*. Using these various substrates, *Clostridia* were capable of evolving molecular hydrogen in addition of other fermentation products. Hydrogen

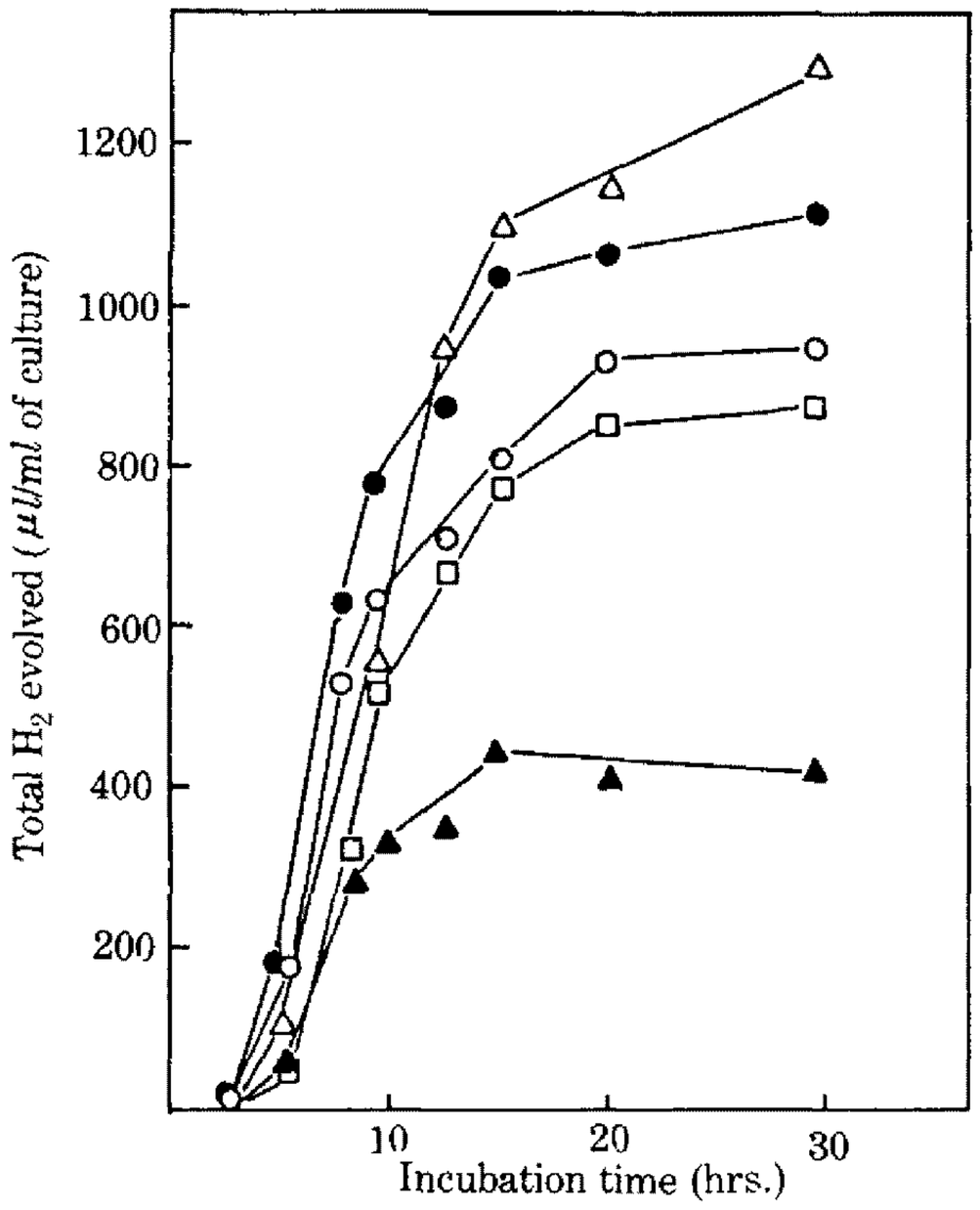

Fig. 1. Time course of hydrogen evolution by *Clostridia* with glucose.

○-○: *Cl. acetobutylicum* ATCC 824
●-●: *Cl. butyricum* NCIB 9576
△-△: *Cl. pasteurianum* ATCC 6013
▲-▲: *Cl. thermocellum* ATCC 27405
□-□: Isolate No. 7
Incubation temp.: ATCC 27405 ..... 60°C
The other strains ..... 37°C

evolution from various electron donors by *Cl. acetobutylicum, Cl. butyricum, Cl. pasteurianum* was presented in Table 1. The relatively large amounts of hydrogen were produced from various substrates by *Cl. butyricum* NCIB 9576 among them. Thus, this organism, capable of producing hydrogen at maximum rate within 24 hrs. from various substrates, was selected for the further study. It was also demonstrated that this organism was able to dissimilate various components of naturally abundunt biomass such as xylose, xylan, starch and cellobiose to evolve hydrogen gas, acetate and butyrate (Table 2, Fig. 2). Fig. 2 presents the patterns of metabolites formation from glucose, including hydrogen evolution rate, soluble products (acetate, butyrate) and pH value in batch culture fermentation by *Cl. butyricum* NCIB 9576.

Culture conditions on hydrogen evolution by *Cl. butyricum* NCIB 9576

Table 1. Effect of electron donors as biomass-derived carbohydrates on hydrogen evolution by *Clostridia*.

Electron donors (1%)	H_2 evolution (μl/hr/mg of DCW)		
	Cl. acetobutylicum	*Cl. butyricum*	*Cl. pasteurianum*
Ribose	14.9	78.1	57.5
Xylose	32.8	69.5	61.4
Glucose	25.8	49.8	52.6
Fructose	39.7	57.7	33.6
Galactose	11.7	29.3	ND
Lactose	30.7	51.0	50.8
Maltose	7.6	12.2	69.8
Sucrose	26.8	44.0	46.4
Cellobiose	24.1	41.8	57.6
Starch	38.1	72.3	49.1
Xylan	30.6	40.7	59.8
Pectin	10.7	44.8	16.8
Mannitol	56.7	34.7	ND
Sorbitol	26.8	17.2	ND
Glycerol	13.7	54.5	ND

ND: Not determined
*Incubation was continued until maximum hydrogen concentration was achieved (ca. 24 hrs: polymer used as electron donor; ca. 72 hrs.). When xylan and pectin were used as electron donor, growth was determined by protein analysis.

Table 2. Total fermentation products formed by *Clostridium butyricum* NCIB 9576 on the medium containing cellobiose, xylose, starch, and xylan.

Substrate (1%)	Substrates consumed (%)	Final pH	Total H_2 evolved (μl/mg of DCW)	Soluble products (acids)	
				Acetate	Butyrate (g/l)
Glucose	98	5.7	0.82	0.49	0.65
Xylose	93	5.6	0.95	0.36	0.81
Cellobiose	94	5.9	1.36	0.40	0.58
Starch	97	5.6	0.96	0.47	0.94
Xylan	41	5.9	0.52	0.20	0.54

Cells were precultured on the medium containing the same substrates. When xylan was tested as substrate, preculture was performed on the xylose containing medium.

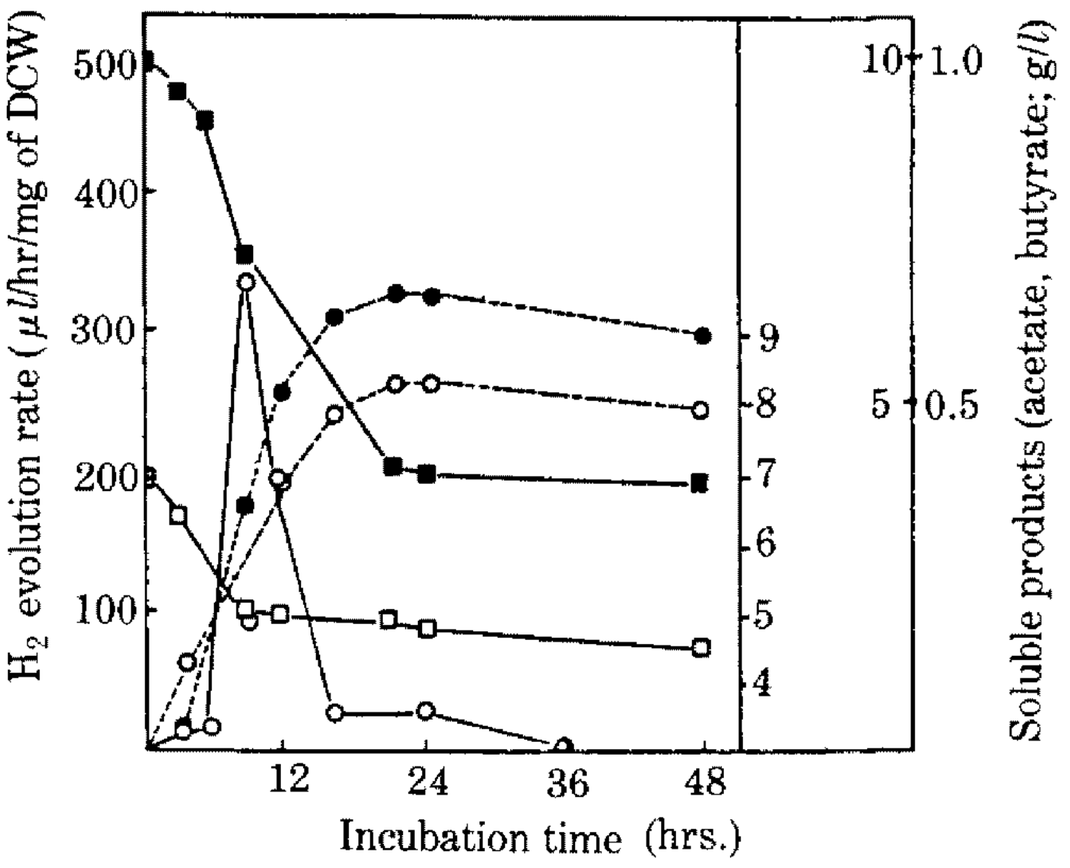

Fig. 2. Metabolites formation and pH change by *Cl. butyricum* NCIB 9576 during growth in modified PYG medium as batch fermentation medium containing 1% glucose (w/v).

○–○: Hydrogen evolution rate, ○···○: Acetic acid, ■–■: Residual glucose, □–□: pH, ●···●: Butyric acid

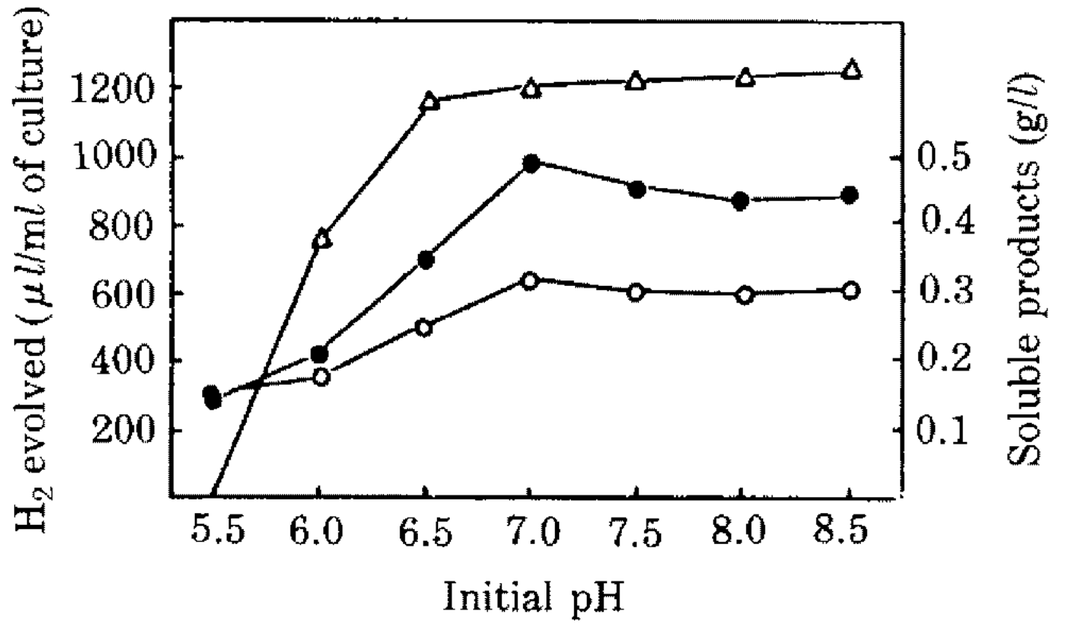

Fig. 3. Effect of the pH on hydrogen evolution and acid production by *Cl. butyricum* NCIB 9576.

Hydrogen and acids were analyzed after 20 and 48 hr incubation, respectively.

△–△: Hydrogen evolved, ●–●: Butyric acid, ○–○: Acetic acid

Quantities of fermentation products of *Clostridia* largely depends on pH value, growth rate and glucose concentration (27). Fig. 3 indicates effects of initial pH on the metabolites formation and its optimal pH range at 7 to 8.5. According to the observation of Yerushalme (26) on the cell metabolism of *Clostridia,* phosphate in media plays important roles on their metabolite formation of the culture. In this work, maximum hydrogen evolution as well as cell growth of the organism was attained at the concentration of 10 mM phosphate, as shown in Fig. 4 and phosphate

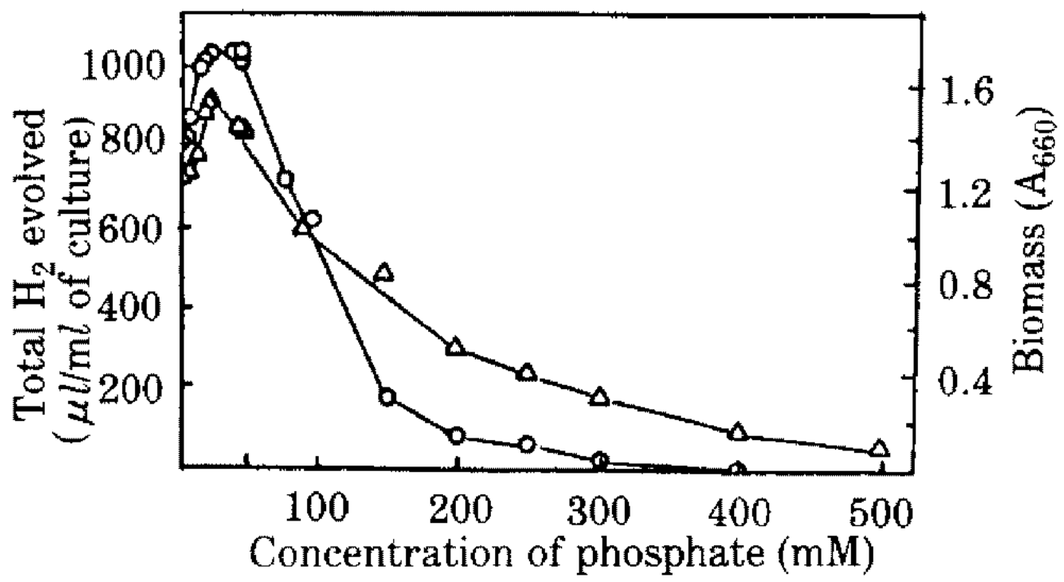

Fig. 4. Effect of phosphate concentration on hydrogen evolution by *Cl. butyricum* NCIB 9576.

Growth and hydrogen evolved were determined after 60 hr incubation. No growth was observed on the concentration of phosphate, 500 mM.

○–○: Total hydrogen evolved, △–△: biomass

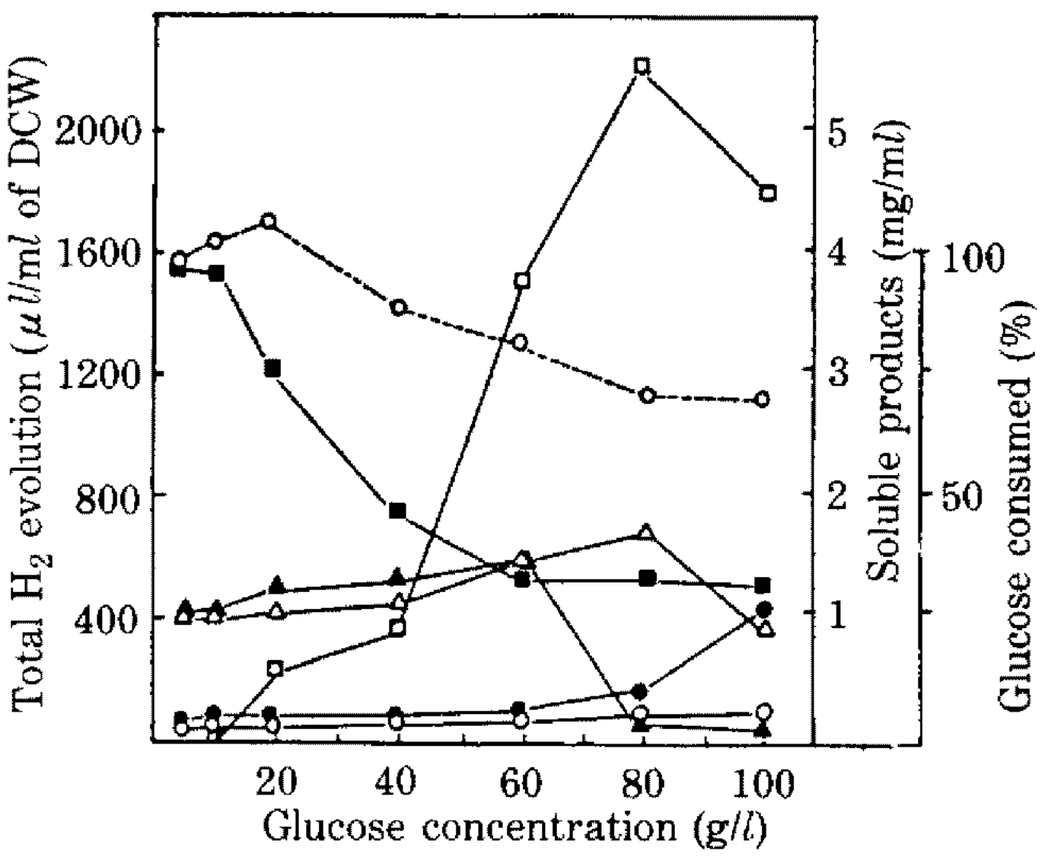

Fig. 5. Effect of glucose concentration on products formation.

Hydrogen gas, organic acids and solvents were determined after 72hr incubation.

○···○: Total hydrogen evolved, □–□: Butanol, △–△: Acetate, ■–■: Glucose consumed, ○–○: Ethanol, ●–●: Acetone, ▲–▲: Butyrate

Cl. butyricum was grown in modified Ormerod's medium. Dry Cell Weight (DCW) was determined by protein analysis.

at higher concentration was remarkably inhibitory on hydrogen evolution. The effect of glucose concentration for the formation of metabolites such as butanol, acetate, acetone and butyrate by the organism was shown in Fig. 5. This indicates that a good yield in hydrogen production was observed at 2% (w/v) of initial glucose in the medium. When fermented in media of more than 6% of sugar con-

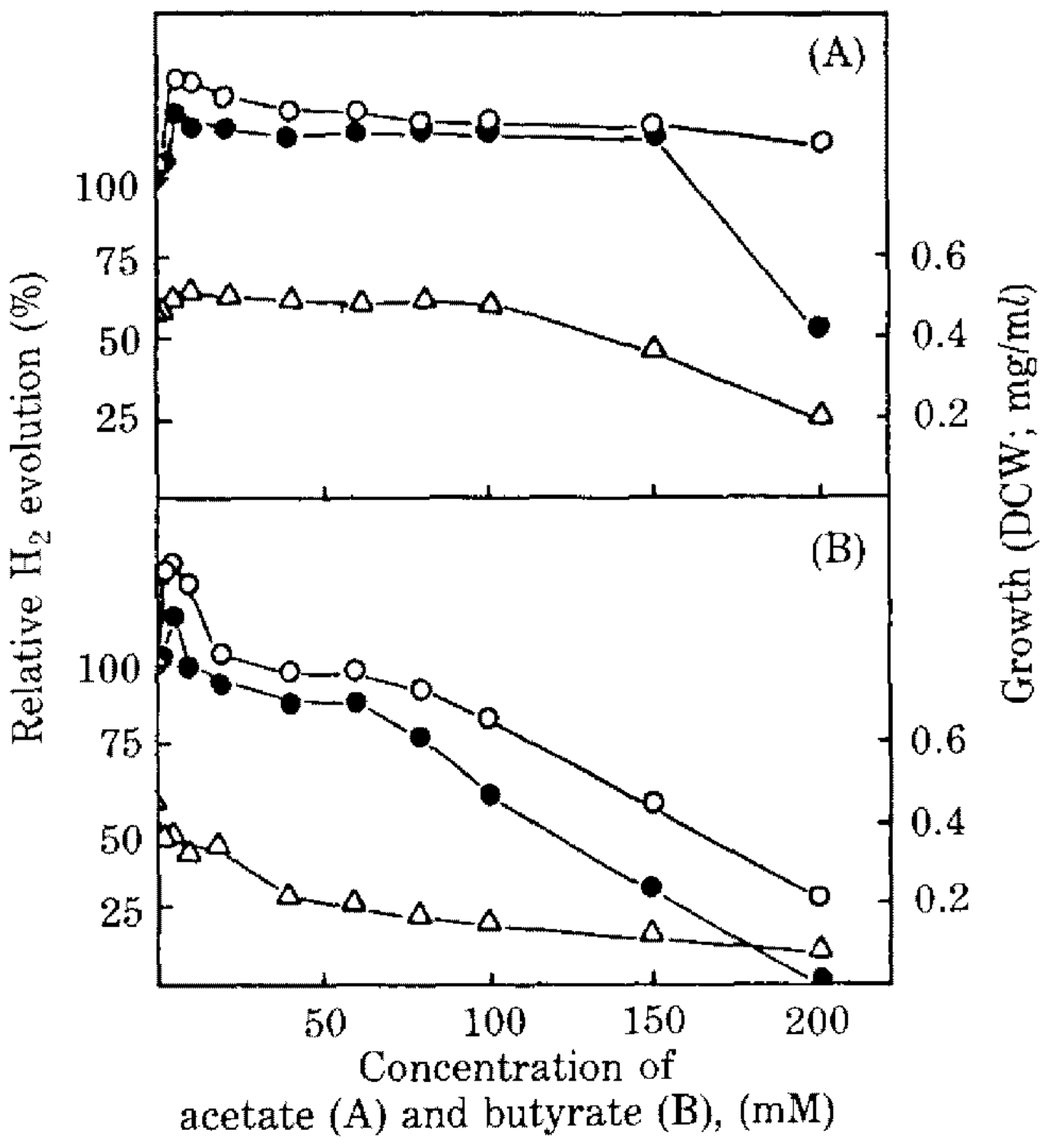

Fig. 6. Effect of acetate and butyrate on growth and hydrogen evolution of *Cl. butyricum* NCIB 9576 in defined medium with glucose.

●-●: Total hydrogen evolved (24 hrs. incubation)
○-○: Total hydrogen evolved (48 hrs. incubation)
△-△: Growth (DCW: 24 hrs. incubation)

tent, production of butanol by the organism increased significantly. This phenomena were partially illustrated with the despatch of solvent formation by low water activity of cellular micro-environment (27). The addition of acetate or butyrate to initial fermentation medium has slightly promoted cell growth and hydrogen evolution of this organism as shown in Fig. 6. However, if more than 150 mM acetate or more than 60 mM butyrate in initial fermentation medium was presented, it was remarkably inhibitory on both cell growth and hydrogen evolution. Thus, in order to enhance hydrogen evolution, it is a problem to be solved to remove or reduce these metabolites from the fermentation broth.

요 약

Clostridium 속 세균에서 biomass의 생물학적 전환에 의한 수소 생성에 관하여 조사하기 위해 4균종을 대상으로 biomass를 구성하는 당류 이용도 및 그로부터의 수소 생성을 검토하였다. 효율적인 수소 생성 세균은 *Cl. butyricum*과 *Cl. pasteurianum*으로 xylose, cellobiose를 포함한 그외 단당, 이당류에서 높은 수소 생성능을 나타내었으며, 특히 *Cl. butyricum*의 경우, starch, xylan 외에도 pectin의 이용성이 비교적 우수한 것으로 관찰되었다. 포도당(1%) 이용시 회분 발효를 통하여 *Cl. butyricum*이 생성하는 유기산(acetate와 butyrate)의 molar ratio(A/B)는 0.7-0.8을 나타내었다. *Cl. butyricum*의 회분 발효시 수소 생성에 영향을 미치는 배양조건을 검토하기 위해 pH, phosphate의 농도, glucose의 농도 및 배양 초기 대사산물(acetate, butyrate)의 영향을 관찰하였다. 그 결과 수소 발생에 최적인 pH는 7.0-8.5, phosphate 농도는 10-50 mM에서 최적을 나타내었으나 500 mM 이상에서는 증식 및 수소 발생이 완전히 저해됨을 나타내었다. 포도당은 2%(w/v)에서 최대 수소 발생량을 나타내었으며 6% 이상에서는 solvent, 특히 butanol 생성의 급격한 증가를 나타내었다. 배양 초기에 acetate와 butyrate가 소량 존재할 경우 수소 생성은 약간의 증가를 보여주나 acetate 150 mM 이상, butyrate 20 mM 이상의 농도에서는 급격한 증식 저해를 나타내었다. 따라서 *Cl. butyricum*이 합성 배지에서 증식하는 경우 위의 최적조건에서 배양함으로써 건체량당 약 70 mmole의 수소를 1%(w/v)의 포도당으로부터 생산할 수 있었다.

Acknowledgement

This work was supported by a research grant (1988) from the Korea Science and Engineering Foundation to M. Bae.

References

1. Gest, H.: *Bacterial. Rev.*, **18,** 43 (1954).
2. Gray, C.T. and H. Gest: *Science,* **9,** 186 (1965).
3. Zeikus, J.G.: *Ann. Rev. Microbiol.*, **34,** 423 (1980).
4. Kondratieva, E.N. and I.N. Gogotov: *Adv. Biochem. Eng. Biotechnol.*, **28,** 139 (1983).
5. Sawada, H. and P.L. Rogers: *J. Ferment. Technol.*, **55,** 297 (1977).
6. Sawada, H. and P.L. Rogers: *J. Ferment. Technol.*, **55,** 311 (1977).
7. Winkler, M.: Biological treatment of waste-water, Ellis Horwood. (1981).
8. Matsunaga, T., I. Karube and S. Suzuki: *Biotechnol. Bioeng.*, **22,** 2607 (1980).
9. Brosseau, J.D. and J.E. Zajic: *Adv. Biotechnol.*, **2,** 281 (1981).

10. Odom, J.M. and J.D. Wall: *Appl. Environ. Microbiol.*, **45**, 1300 (1983).
11. Miyake, J., X.Y. Mao and S. Kawamura: *J. Ferment. Technol.*, **62**, 531 (1984).
12. Ahring, B.K. and P. Westermann: *Appl. Environ. Microbiol.*, **53**, 434 (1987).
13. Miyamoto, K., S. Ohta, Y. Nawa, Y. Mori and Y. Miura: *Agric. Biol. Chem.*, **51**, 1319 (1987).
14. Ormerod, K.S., J.G. Ormerod and G. Howard: *Arch. Biochem. Biophy.*, **94**, 449 (1961).
15. Hungate, R.E.: Methods in Microbiology, A.P., **3**, 117 (1969).
16. Bryant, M.P.: *Am. J. Clin. Nut.*, **25**, 1324 (1972).
17. Miller, T.L. and M.J. Wolin: *Appl. Microbiol.*, **27**, 985 (1974).
18. Miller, G.L.: *Anal. Chem.*, **31**, 426 (1959).
19. Viles, F.J.Jr. and L. Silverman: *Anal. Chem.*, **21**, 950 (1949).
20. Kohler, L.H.: *Anal. Chem.*, **24**, 1576 (1952).
21. Palo, V. and H. Ilkova: *J. Chromatog.*, **53**, 363 (1970).
22. Bricknell, K.S. and S.M. Finegold: *Anal. Biochem.*, **51**, 23 (1973).
23. Ennis, B.M. and C.T. Marshall: *Biotechnol. Lett.*, **8**(10), 725 (1986).
24. Harris, J. and J.G. Morris: *Biotechol. Lett.*, **8**(12), 889 (1986).
25. Lowry, O.H. and R.J. Morris: *Biotechnol. Lett.*, **8**(12), 889 (1986); Randall: *J. Biol. Chem.*, **183**, 265 (1951).
26. Yerushalmi, L. and B. Volesky: *Appl. Microbiol. Biotechnol.*, **25**, 513 (1987).
27. Rogers, P.: *Adv. Appl. Microbiol.*, **30**, 1 (1986).

(Received October 12, 1989)