[J. Ferment. Technol., Vol. 60, No. 6, p. 509–515, 1982]

Hydrogen and Methane Fermentation of Rice Straw and Kitchen Leftovers

Susumu Oi, Shinji Tamura, Katsumi Nakai*, Toshio Tanaka, and Makoto Taniguchi

*Faculty of Science, Osaka City University, Sugimoto-cho, Sumiyoshi-ku, Osaka 558, Japan; *Research Center, Osaka Gas Co. Ltd., Torishima, Konohana-ku, Osaka, Japan*

Sequential fermentations for hydrogen and methane were carried out at 37°C with a mash containing rice straw obtained from horse feces and kitchen leftovers as substrates by the sequential addition of fresh horse feces and acclimated methane sludge. The efficiency of the fermentations was improved by pretreatment of the substrates with 0.16% NaOH at 123°C for 20 min and with 0.18% cellulolytic and hemicellulolytic enzymes at 30°C at pH 4.5 for 3 days. This treatment liberated 12% of sugar materials from the substrates and doubled the volume of gas evolved in the fermentations. The volume of hydrogen evolved in 4 days from the substrates supplemented with 100 mg of urea by the microbes in 2 g of fresh horse feces was 338 ml, representing 13.8% of the total volume of gas evolved. The volume of methane evolved in the following 12 days on addition of 15 ml of acclimated sludge was 604 ml. The volume percentage of methane in the gas was 58% at 5 days, increasing to 82% at 12 days. The COD of the supernatant of the mash decreased from the initial 8,400 to 528 ppm at the end of the fermentation. The amount of carbon in the gas evolved was calculated to represent 38.3% of the carbon contained in the two substrates. A small amount of acetic acid was accumulated in the mash during the hydrogen fermentation, while large amounts of volatile acids were accumulated in the mash during the methane fermentation.

Mannitol, sorbitol, lactate and gluconate were good substrates for hydrogen evolution by fresh horse feces; butyric and valeric acids were accumulated in the mash.

Hydrogen and methane have attracted attention as clean fuel resources. All methanogenic bacteria can utilize hydrogen as a sole source of reducing power for methanogenesis and for cell carbon synthesis. Therefore, a multi-bacteria system including hydrogen-producing bacteria is needed for continuous methane production from organic materials.[1] The products of hydrolysis by cellulolytic, lipolytic and proteolytic bacteria, such as sugars, and fatty and amino acids, are converted to hydrogen, carbon dioxide, and formic and acetic acids by hydrogen-producing bacteria. Finally, anaerobic methanogenic bacteria produce methane from acetic and formic acids or hydrogen and carbon dioxide.[2,3]

Hydrogen evolution by hydrogenase is a major route though which *Clostridia* cells dispose of the excess electrons produced by the oxidative breakdown of carbohydrates. In 1962, Rohrback *et al.* reported the production of hydrogen from glucose by *Cl. butyricum*.[4] Recently, it has been reported that hydrogen was continuously produced from glucose by immobilized cells of hydrogen-producing bacteria, and that hydrogen evolution continued for at least 20 days.[5]

In a previous paper, some of us reported that the efficiency of a methane fermentation was greatly improved by pretreatment of bagasse and coffee grounds with dioxane or dioxolane followed by incubation with cellulolytic and hemicellulolytic enzymes.[6,7]

The present paper describes sequential fermentations for hydrogen and methane of rice straw and kitchen leftovers treated with alkali, and cellulolytic and hemicellulolytic enzymes.

Materials and Methods

Horse feces, kitchen leftovers and rice straw obtained from horse feces Horse feces were collected within 3–4 h after defecation from a specified horse in the stable of the riding club at Osaka City University. The composition of kitchen leftovers is shown in Table 1. Rice straw employed in the study was obtained from fresh horse feces by washing the feces with tap water in a gauze bag for 4 days at room temperature, and then the straw left in the bag was dried in a stream of air at 60°C, which gave 22.6 g of rice straw from 112.5 g of feces. This straw had slightly higher carbon and hydrogen contents and lower nitrogen and ash contents than native rice straw. The compositions and chemical analyses of horse feces, kitchen leftovers and rice straw are shown in Table 2.

Alkali and enzymatic treatments Rice straw and kitchen leftovers were treated with NaOH at various concentrations at 123°C for 20 min. Treatments with cellulolytic and hemicellulolytic enzymes were carried out with 0.18%, 0.50% or 1.0% of commercial enzyme preparations at pH 4.5 and 30°C for 72 h.

Methane bacteria and their acclimatization The sludge used was obtained from blow mud found in an old lotus-root field in a suburb of Osaka City. The mud, which contained gas of which 78% was methane, was precultured in a suspension of 3.0% soybean seed coat at 37°C.

Fermentation methods Hydrogen fermentation was carried out in a 100-ml syringe containing 10 ml of a mash of 200 mg of substrate and 1 g of fresh horse feces, or a 200-ml syringe containing 45 ml of a mash of 0.5 g of rice straw from horse feces and 1.78 g of kitchen leftovers and 2 g of fresh horse feces at 37°C for 3 or 4 days. Methane fermentation was carried out at 37°C for 12 days in a 200-ml syringe containing 15 ml of the acclimated sludge and 45 ml of the suspension obtained after hydrogen fermentation of the mash of 0.5 g of rice straw and 1.78 g of kitchen leftovers.

Table 1. Composition of kitchen leftovers.

Contents	g/*l*
Cabbage leaf	100
Orange peel	100
Potato	10
Soy bean (dried seed)	10
Green tea grounds	35
Fish bones	20
Edible seaweed (dried)	25
Wheat flour	30

Mixed with 1 *l* of tap water in a mixer.

Table 2. Chemical compositions of horse feces, kitchen leftovers and rice straw.

	Horse feces[a]	Kitchen leftovers	Rice straw[b]
Total sugar content[c]	26.9 %	50.5 %	35.6 %
Crude protein	1.05	1.30	4.20
Ash	1.80	0.30	18.3
Water	75.0	92.8	7.0
Carbon	43.2	43.2	36.6
Nitrogen	0.67	2.88	0.82
Hydrogen	5.72	6.38	5.00

[a] The content of rice straw in horse feces was estimated to be 80%.
[b] Removed from horse feces as described in *Materials and Methods*.
[c] Glucose equivalent for the fraction dissolved by 72% H_2SO_4 in 48 h at 25°C, and then boiled for 2 h after diluting the acid to 3% with water.

Analyses Reducing sugar was determined by the Schaffer-Somogyi method.[8] After treatment with NaOH or cellulolytic and hemicellulolytic enzymes, the sugar materials in the supernatant of the reaction mixture were determined by the phenol sulfuric acid method.[9] Degree of dissolution was calculated from these data as the percentage of sugar materials liberated from the substrates. Total nitrogen content was determined by the micro-Kjeldahl method. Ash was determined by weighing the residue after incineration. Chemical oxygen demand was assayed by the method described in JIS K0101.[10]

The volume of gas evolved was measured with a gas burette using saturated sodium chloride solution to equilibrate the pressure. Gas composition and volatile fatty acids in the mash were determined as described previously.[6]

Chemicals "Cellulosin AC" (Ueda Chem. Co. Ltd., Osaka) and cellulase "Onozuka" R-10 (Kinki Yakulto Co. Ltd., Osaka) were the cellulolytic and hemicellulolytic enzymes used to examine the hydrolysis of rice straw from horse feces and kitchen leftovers. All other chemicals were of commercial grade.

Results

Dissolution of rice straw from horse feces by treatment with alkali and cellulolytic or hemicellulolytic enzymes The dissolution of rice straw from horse feces rose to 27.5% with the increase in concentration of alkali added to the reaction

mixture, as shown in Table 3. With alkali followed by enzymatic treatment, dissolution after 72 h was 15.2% with 1.0 %Cellulosin AC and 27.4% with 1.0% cellulase "Onozuka" R-10, as shown in Table 4.

Hydrogen evolution from saccharides, polyalcohols and organic acids by anaerobic microbes in fresh horse feces Hydrogen evolution from saccharides, polyalcohols and organic acids was examined by addition of fresh horse feces as the source of anaerobic microbes and incubation at pH 7.0 and 37°C for 3 days. The results are listed in Table 5. Two hundred mg of substrate was added to the mash, because about the same amount of sugars was liberated from the rice straw and kitchen leftovers in the methane fermentation after treatment with alkali and cellulolytic or hemicellulolytic enzymes. The volumes of gas evolved from mannitol and lactate were, respectively, 80 ml and 85 ml, the highest for the substrates tested. The gas evolved from saccharides other than arabinose contained 13–25% hydrogen, and that from polyalcohols contained between 23% and 40% hydrogen. The gas evolved from lactate had the highest content of hydrogen, 49%. During the 3-day incubations, the pH of the mash fell from 7.0 to 4.0. No evolution of methane was observed, probably because the pH of the mash was unsuitable for the growth of the methanogenic bacteria in the native horse feces. The main volatile fatty acids accumulated were acetic acid from saccharides, and butyric

Table 3. Dissolution of rice straw by alkali treatment.

NaOH (%)	Dissolution[a] (%)
None	1.7
0.16	6.0
0.16	2.25[b]
0.32	10.2
0.80	15.1
1.60	20.0
3.20	22.8
4.00	27.5

Rice straw was treated with alkali at 123°C for 20 min, and then total sugar content was determined by the phenol sulfuric acid method.

[a] Percent of dissolution $= \frac{\text{total sugars formed}}{\text{weight of rice straw}} \times 100$

[b] A mixture of 1 g of rice straw and 1.78 g of kitchen leftovers was treated with 0.16% NaOH.

Table 4. Dissolution of rice straw by successive treatment with alkali and cellulolytic or hemicellulolytic enzymes.

Treatment	Addition (%)	Dissolution (%)[a] Incubation time (h)			
		0	24	48	72
None	—	1.7	1.7	1.7	1.7
NaOH[b]	0.16	6.0	6.0	6.6	6.6
Cellulosin AC[c]	0.18	6.0	9.9	11.0	12.0
	0.5	6.0	11.0	12.4	14.9
	1.0	6.0	11.1	12.6	15.2
Cellulase "Onozuka" R-10[c]	0.5	6.0	17.7	21.5	25.0
	1.0	6.0	21.5	24.7	27.4

[a] The percentage of sugar materials found in the supernatant after treatment to the total weight of rice straw (200 mg).

[b] Treated with 0.16% NaOH at 123°C for 20 min, cooled to room temperature, and then incubated at 30°C.

[c] After treatment with 0.16% NaOH at 123°C for 20 min, 45 ml of the reaction mixture was cooled and adjusted to pH 4.5 with 6 N HCl, then enzyme was added, and the mixture was incubated at 30°C. The amounts of enzyme added are expressed as percentages of the total weight of rice straw in the mixture.

and valeric acids from polyalcohols and gluconate.

Hydrogen and methane fermentation of rice straw and kitchen leftovers Sequential fermentations of rice straw from horse feces and kitchen leftovers were investigated with anaerobic microbes in fresh horse feces and methanogens in the acclimated sludge over a period of 16 days. Sugar materials solubilized by pretreatment of these materials with 0.16% NaOH at 123°C for 20 min amounted to 2.25%, and those released by the enzymatic hydrolysis (0.18% Cellulosin AC) at pH 4.5 and 30°C for 3 days amounted to 12%, as shown in Tables 3 and 4. Alkali treatment with 0.16% NaOH was chosen for the sequential fermentations, because higher concentrations on neutralization with HCl gave concentrations of sodium chloride that were inhibitory to the fermentation. Cellulosin AC was chosen instead of Cellulase "Onozuka" R-10 for enzymatic pretreatment, because although it gave a smaller degree of hydrolysis, the amounts of hydrogen and methane formed by the fermentation were greater. Its concentration was limited to 0.18% based on economic considerations of application of the pretreatment to a larger scale fermentation.

Hydrogen fermentation was first carried out by the addition of 2.0 g of fresh horse feces (0.5 g dry weight) and incubation for 4 days at pH 7.0 in the presence of the additional nitrogen source of 100 mg urea. As shown in Table 6, the hydrogen content of the gas evolved was 23% at 2 days, and the total volume of gas evolved was 338 ml after 4 days.

After the 4-day hydrogen fermentation, the pH of the mash was adjusted to 7.0 by the

Table 5. Gas evolution and composition of volatile fatty acids in the mash containing saccharides, polyalcohols and organic acids by anaerobic microbes in horse feces.

Substrate added	Gas evolved[a] (ml)	Gas composition		Volatile acids[b] (mg/ml)				
		H_2 (%)	CO_2 (%)	AA	PA	IBA	BA	VA[c]
Saccharides								
Glucose	10	25	75	0.34	0.07	N[d]	0.05	N
Maltose	6	13	87	0.63	0.15	N	0.08	N
Galactose	10	17	83	0.85	0.12	N	N	N
Arabinose	6	42	58	0.02	0.02	N	0.02	N
Lactose	10	15	85	0.77	0.11	N	0.04	N
Rhamnose	20	16	84	0.76	0.78	0.02	0.58	0.47
Polyalcohols								
Inositol	10	0	100	4.54	3.26	N	0.21	N
Sorbitol	50	40	60	0.15	0.16	N	1.61	0.06
Mannitol	80	34	66	0.17	0.22	N	1.45	0.84
Dulcitol	28	23	77	0.18	0.17	0.02	0.86	0.11
Glycerol	25	36	64	0.40	0.02	N	1.06	0.24
Organic acids								
Lactate	85	49	51	0.21	0.32	N	0.23	0.32
Gluconate	45	24	76	0.34	0.17	N	0.99	0.84

[a] Ten ml of a mash of 1 g of horse feces and 200 mg of substrate was incubated in a 100-ml syringe at 37°C for 3 days.
[b] After 3 days incubation, the mash was acidified, and volatile acids were extracted into ethyl ether and analyzed by gas chromatography.
[c] AA, acetic acid; PA, propionic acid; IBA, isobutyric acid; BA, butyric acid; VA, valeric acid.
[d] N, negligible.

Table 6. Hydrogen and methane fermentations of rice straw and kitchen leftovers by anaerobic microbes in horse feces and methanogens in acclimated sludge, and compositions of the supernatant and volatile fatty acids in the mash.

Incubation (days)	pH	Gas evolved, total vol. (ml/days)	(ml)	Gas composition (%) H_2	CH_4	CO_2	Volatile acids (mg/ml) AA	PA	IBA	BA	VA[e]	Nonvolatile materials[d] (mg)	Organic substances[d] (mg)	COD (ppm)	Total sugar[d] (mg)	Nitrogen[d] (mg)
H_2-fermentation[a]																
Start	7.0						N	N	N	N	N	1198	915	8400	203	51.6
2	5.0	65	130	23	N[c]	77	0.047	N	N	N	N	—	—	—	—	—
4	3.5	104	338	8	N	92	0.032	N	N	N	N	1090	839	5400	49	21.6
CH_4-fermentation[b]																
Start	7.2						0.024	N	N	N	N	819	631	4060	36.8	16.2
2	6.8	58	116	23	N	77	0.311	0.539	0.011	0.473	0.055	—	—	—	—	—
5	7.0	30	206	N	53	47	0.478	0.642	0.023	0.706	0.280	—	—	—	—	—
8	7.0	50	356	N	62	38	0.217	0.550	0.019	0.288	0.250	—	—	—	—	—
12	7.0	62	604	N	82	18	0.079	0.703	0.087	0.016	0.003	678	427	528	4.4	2.4

[a] Hydrogen fermentation: A 45-ml mash of 0.5 g of rice straw from horse feces and 1.78 g of kitchen leftovers was treated with 0.16% NaOH at 123°C for 20 min, then the pH was adjusted to 4.5 with 6N HCl, and 5 mg of Cellulosin AC (0.18%) was added, and then the mash was incubated for 3 days.
The pH of the mash was then adjusted to 7.0 with 5N NaOH, 2.0 g of fresh horse feces (0.5 g dry weight) was added, and the hydrogen fermentation was carried out at 37°C for 4 days after the addition of 100 mg urea as a nitrogen source.

[b] Methane fermentation: After the 4-day hydrogen fermentation, the pH of the mash was adjusted to 7.0 with 5N NaOH, and 15 ml of the acclimated methane sludge was added to the mash. The methane fermentation was carried out at 37°C for 12 days.

[c] N, negligible.

[d] Per 60 ml of mash.

[e] AA, acetic acid; PA, propionic acid; IBA, isobutyric acid; BA, butyric acid; VA, valeric acid.

addition of 5 N NaOH in small portions, and methane fermentation was initiated by the addition of 15 ml of the acclimated sludge. The methane content of the gas evolved was 53% at 5 days, increasing to 82% at 12 days, as shown in Table 6. The volume of gas evolved in the following 12 days on addition of 15 ml of acclimated sludge was 604 ml.

The total volume of gas evolved was 942 ml in the sequential hydrogen and methane fermentations covering 16 days; the volume percentage of methane in the gas was 36.6%, and those of carbon dioxide and hydrogen were 55.7% and 7.7%, respectively. The compositions of the supernatant of the mash before and after the fermentation are summarized in Table 6. The COD of the supernatant of the mash decreased from the initial 8,400 to 5,400 ppm after 4 days of hydrogen fermentation and to 528 ppm at the end of the methane fermentation. Organic substances in the supernatant of the mash decreased from 915 mg in total at first to 427 mg at the end of methane fermentation. Total sugar contents and the amounts of nitrogen in the supernatant of the mash decreased from 203 mg to 4.4 mg and from 51.6 mg to 2.4 mg at the end of the fermentation, respectively.

In the experiment for comparison of the gas evolution from the substrates pretreated with alkali, and cellulolytic and hemicellulolytic enzymes with the results for the substrates without both pretreatments, 170 ml of gas containing 23% of hydrogen in volume percentage was evolved in 4 days, and 263 ml of gas containing 63% of methane in volume percentage was evolved in 12 days in sequential fermentation. When the substrates were pretreated with both alkali and enzyme, the efficiency of the fermentation was improved about 2-fold in terms of gas evolution.

A small amount of acetic acid was accumulated in the mash in the hydrogen fermentation, while larger amounts of acetic, propionic, butyric and valeric acids were accumulated in the methane fermentation. After 5 days of methane fermentation, the amounts of acids accumulated were maximal, and thereafter decreased gradually. Propionic and isobutyric acid accumulated again in the mash after 8 days of incubation, as shown in Table 6.

Discussion

Clostridium thermocellum was examined for the ability to ferment various sugars by monitoring hydrogen and carbon dioxide formation.[11] Recently, it has been shown that hydrogen was continuously produced from glucose by immobilized whole cells of *Clostrium butyricum* for at least 20 days.[5] In the case of fresh horse feces, hydrogen was evolved for 4 days in the mash, and 1.6 mmol hydrogen was evolved from 2.78 g of substrates consisting of 1.0 g of rice straw from horse feces and 1.78 g of kitchen leftovers. The immobilized 0.1 g of whole cells of *Cl. butyricum* produced 60 μmol of hydrogen from 18 mg glucose on 18 h incubation at 37°C. With fresh horse feces, 1.6 mmol of hydrogen was evolved from about 1.25 g (6.94 mmol) of sugar materials contained in the substrates. Thus a 69-fold larger amount of sugar materials was employed with the fresh horse feces and a 26.6-fold larger amount of hydrogen was evolved than with 0.1 g of wet cells of *Cl. butyricum* from the substrates in 3 days. The anaerobic microbes in horse feces could utilize various sugars and polyalcohols. Mannitol, lactate and gluconate were specially good substrates for hydrogen evolution, as shown in Table 5. With mannitol, 1.2 mmol of hydrogen was evolved from 1.1 mmol of substrate on 3 days incubation with 1 g of the fresh horse feces. If 0.1 g of anaerobic microbes was contained in the fresh horse feces, the ability of hydrogen evolution by horse feces would be in the same range as that of the native cells of *Cl. butyricum*. About 25% of the horses stabled at Osaka City University evacuated feces which could be used for the test of hydrogen evolution.

Twelve % of sugar materials was solubilized from rice straw from horse feces and kitchen leftovers by the sequential treatment with alkali and enzyme, i.e., 144 mg of sugar materials were liberated. The degree of

dissolution found in the experiments was of the same degree as that of other materials reported previously.[6,7] That of bagasse, for example, was 14%, and that of coffee grounds was 10%. If sugars are added to the fermentation mash in larger amounts than 200 mg, it might be expected that larger amounts of hydrogen would be evolved than that reported here. However, it is generally recognized that hydrogen and methane fermentations are governed by a balance among the metabolites produced by anaerobic microbes.

The fermentative processes of hydrogen and methane were well separated by the sequential addition of the fresh horse feces and the acclimated methane sludge. The total volume of gas evolved in the sequential fermentations was 1.5 times larger than when fresh horse feces and the acclimated sludge were added simultaneously. Also, the gas evolution per g of substrates was approximately double that when alkali and enzyme pretreatment was omitted.

The total volume of gas evolved was 942 ml in the sequential hydrogen and methane fermentations covering 16 days; the percentage of methane in the gas was 36.6% and that of carbon dioxide was 55.7%. The amounts of methane and carbon dioxide in the gas evolved were 15.4 mmol and 23.4 mmol, respectively. The total weight of carbon in the methane and carbon dioxide evolved was calculated to be 0.46 g. On the other hand, the total weight of carbon in the rice straw and kitchen leftovers used in the fermentation was calculated to be 1.20 g from the data shown in Table 2. Thus the conversion rate of carbon in the substrates to gaseous compounds was calculated to be 38.3%. The gas evolved per g of substrate from bagasse and coffee grounds amounted, respectively, to 200 ml, of which 68% was methane, and 335 ml, of which 58% was methane.[6,7] In the case of rice straw and kitchen leftovers, the gas evolved in the sequential fermentations amounted to 942 ml, of which 36.6% was methane, but this was with use of 1.0 g of rice straw and 1.78 g of kitchen leftovers. Thus the amount of methane formed was, respectively, slightly or fairly smaller than that from bagasse and coffee grounds.

Acknowledgement

This study was supported in part by a grant (No. 56045111) from the Ministry of Education, Science and Culture of Japan.

References

1) Pfeffer, J. T.: *Process Biochem.*, **13**, 8 (1978).
2) Zeikus, J. B.: *Bacteriol. Rev.*, **41**, 514 (1977).
3) Mah, R. A., Ward, D. M., Baresi, L., Glass, T. L.: *Ann. Rev. Microbiol.*, **31**, 309 (1977).
4) Rohrback, G. H., Scott, W. R., Canfield, J. H.: *Proceedings of the 16th Annual Power Sources Conference*, p. 18 (1962).
5) Karube, I., Matsunaga, T., Tsuru, S., Suzuki, S.: *Biochim. Biophys. Acta*, **444**, 338 (1976).
6) Oi, S., Yamanaka, H., Yamamoto, T.: *J. Ferment. Technol.*, **58**, 367 (1980).
7) Oi, S., Tanaka, T., Yamamoto, T.: *Agric. Biol. Chem.*, **45**, 871 (1981).
8) Somogyi, M.: *J. Biol. Chem.*, **195**, 19 (1952).
9) Dubois, M., Gillers, K. A., Hamilton, J. K., Rebers, F. A., Smith, F.: *Anal. Chem.*, **28**, 350 (1950).
10) JIS Handbook, Testing Method for Industrial Water, JIS K0101, Nippon Kikaku Kyokai, p. 447 (1966).
11) Ng, T. K., Weimer, P. J., Zeikus, J. G.: *Arch. Microbiol.*, **114**, 1 (1977).

(Received May 14, 1982)