Detection of Hydrogen Gas-Producing Anaerobes in Refuse-Derived Fuel (RDF) Pellets

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Recently, we reported that refuse-derived fuel (RDF) pellets contain a relatively high number of viable bacterial cells and that these bacteria generate heat and hydrogen gas during fermentation under wet conditions. In this study we analyzed bacterial cell numbers of RDF samples manufactured with different concentrations of calcium hydroxide, which is usually added to waste materials for the prevention of rotting of food wastes and the acceleration of drying of solid wastes, and determined the amount of hydrogen gas produced by them under wet conditions. Furthermore, we analyzed microflora of the RDF samples before and during fermentation by denaturing gradient gel electrophoresis of 16S rDNA followed by sequencing. We found that the RDF samples contained various kinds of clostridia capable of producing hydrogen gas.

Key words: refuse-derived fuel (RDF); hydrogen gas; microflora; denaturing gradient gel electrophoresis (DGGE)

The refuse-derived fuel (RDF) system includes processes that remove noncombustible materials such as iron materials, glass, grit, and dehydrate and form combustible municipal solid waste into small pellets, which allows easy transportation, storage, and combustible stability. Furthermore, efficient use of RDF as an energy resource is expected to contribute to a recycle-based society. RDF used for a power plant covers a wide range of waste materials, which have been processed to fulfill guidelines, or regulatory or industry specifications, mainly to achieve a high calorific value. Calcium hydroxide, CaO, is usually added to waste materials to prevent rotting of food wastes and to accelerate the drying of solid wastes.1 RDF is thought to have a number of merits, e.g., low burden on the environment, low construction cost, good storage ability, stable calorific power, easy handling, and good transportability.2 In Japan, therefore, five power plants exclusively for RDF and dozens of RDF manufacturing plants are now operating.

Unfortunately, despite the good storage ability of RDF, an explosion occurred at a silo containing RDF of the Mie RDF Power Station in Tado Village, Mie Prefecture, Japan in 2003. Although the true cause of the explosion at the silo has not been made clear, we have found that RDF samples contain a relatively high number of viable bacterial cells, and that these bacteria generate heat and a large amount of hydrogen gas during fermentation under wet conditions in our recent studies.3) To our knowledge, there are few reports describing microorganisms related to RDF.4) To operate the RDF system more safely, we believe that extensive studies are necessary on the microbiology of RDF. In this paper, we describe hydrogen gas production from RDF pellets containing different concentrations of calcium hydroxide and microflora during fermentation in wet conditions.

Materials and Methods

Preparation of RDF pellets. All RDF pellet samples were prepared at the Kahada–Okuise RDF manufacturing plant in Mie Prefecture. The manufacturing process was briefly as follows: Garbage, including paper and plastics, carried into an open pit was cut out, mixed well, and dried in a rotary drum with hot air at 350°C. Then separation of metals was done electromagnetically and appropriate amounts of powdered calcium hydroxide were added to the dried garbage. RDF pellets were formed by an extruder under high pressure.

Counting of viable cell numbers. Viable cell numbers in the RDF samples were counted as follows: Several pellets (10 g) of each sample were well suspended in 90 ml of sterile physiological saline solution and homogenized with a Universal Homogenizer (Nihon Seiki Seisakusho, Tokyo) at 10,000 rpm for 20 sec. The resulting suspension was serially diluted with sterile physiological saline solution. Viable counts of bacteria were made by the usual plate-counting technique. To count the sum of aerobic and facultative anaerobic bacteria, SCD broth (Wako Pure Chemical Industries, Osaka, Japan) containing 1.5% agar was used, and the plates were incubated at 37°C. To count the sum of

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Abbreviations: DGGE, denaturing gradient gel electrophoresis; RDF, refuse-derived fuel
facultative and absolutely anaerobic bacteria, SCD broth supplemented with 0.5% yeast extract, 0.5% soluble starch, 0.03% cysteine–HCl, and resazurin (1 µg/ml) was used, and the plates were incubated at 37 °C under an anaerobic atmosphere using a BBL GasPak anaerobic jar system (Becton Dickinson, Sparks, MD.). The number of facultative anaerobic bacterial cells was found by cultivating the replica plates from the anaerobic cultures under aerobic conditions. The number of aerobic bacteria was estimated from the difference between the total cell numbers under the aerobic condition and those of facultative anaerobes, and the number of strictly anaerobic bacteria was calculated from the difference between the total cell numbers under the anaerobic condition and those of facultative anaerobes.

Fermentation of RDF samples. The production of fermentation gas from RDF was measured as follows: RDF pellets of RDF2 (100 g) were suspended in 250 ml sterile deionized water, placed in a glass bottle (500 ml volume) with a butyl rubber stopper, and incubated at 37 °C. Fermentation gas evolved was collected by the water displacement method with graduated cylinders filled with water of pH 3 or less, as described previously.\(^4\) The composition of the fermentation gas was analyzed by gas chromatography, using Gas Chromatograph GC-323 online with the EZChrom Elite Chromatography Data System (GL Science, Tokyo), and the amount of hydrogen gas evolved was calculated based on hydrogen gas standard curves.

Denaturing gradient gel electrophoresis (DGGE) of 16S rDNA. Extraction of total DNA was done from 5-g samples by the benzyl chloride method according to Ueno et al.\(^5\) Crude DNA obtained was further purified with a QIAamp DNA Stool Mini kit (Qiagen, Tokyo). From the total DNAs as templates, 16S rDNAs were amplified with a combination of two primers, 357F-GC, 5′-CGCCCGCCGCCGCCGCCGCGGGCGGGCGGGG- GCACGGGGGGCTACGGGGGGCCAGCG-3′ and 517R, 5′-ATTACCCGCGCTGCTGG-3′.

DGGE analysis was carried out using a Dcode system (Bio-Rad, CA) according to the standard protocol. In brief, amplified DNA samples were applied directly onto 6–12% (w/v) polyacrylamide gels in 0.5 × TAE (20 mM Tris–acetate [pH 7.4], 10 mM acetate, 0.5 mM Na₂EDTA) with a denaturing gradient ranging from 20% to 60%. One hundred percent of denaturant corresponded to 7 M urea and 40% (v/v) formamide. Electrophoresis was carried out at a constant voltage of 200 V at 61 °C for 5 h. The gel was stained with SYBR Green I (Invitrogen, CA).

Results and Discussion

Viable cell numbers in RDF samples containing different concentrations of calcium hydroxide

In the RDF manufacturing process, calcium hydroxide or CaO is usually added to waste materials to prevent rotting of food wastes and to accelerate the drying of solid wastes before they are extruded into pellets.\(^1\) We investigated the effect of the addition of calcium hydroxide to RDF pellets on viable cell numbers in them. Three RDF samples containing 0, 2, or 4% calcium hydroxide were prepared in the Kahada RDF plant, Mie, Japan from house garbage.

When the RDF samples containing 0, 2, or 4% calcium hydroxide were suspended in sterile distilled water, the pH values of the suspensions were 7.4, 9.6, and 12.5 respectively, allowing us to expect that the addition of calcium hydroxide alkalizes garbage and lead to bacteriostatic and/or bacteriocidal actions. As shown in Table 1, although the presence of calcium hydroxide reduced the viable cell numbers in the RDF samples as expected, its effect was not strong enough completely to inactivate bacteria in RDF pellets. That is, the viable cell number was reduced only to one tenth even in the presence of 4% calcium hydroxide as compared with the RDF sample without calcium hydroxide.

Hydrogen gas evolution from RDF samples containing different concentrations of calcium hydroxide

Although it is unlikely that microorganisms ferment the waste materials in normal RDF preparations that include a moisture content of less than 10%, microorganisms in RDF pellets become active immediately if the drying of the pellets is not sufficient during the manufacturing process, or if RDF pellets are moistened for unexpected reasons. In our previous paper, we showed that microorganisms present in the RDF samples fermented them to generate heat and hydrogen gas.\(^3\) In this study, we measured hydrogen gas production from RDF samples containing different concentrations of calcium hydroxide in wet conditions. As shown in Fig. 1, when 100 g of RDF sample without calcium hydroxide was incubated in wet conditions, it began to produce fermentation gas after about 18 h of incubation, and fermentation gas production stopped after 48 h. The total volume of fermentation gas evolved from 100 g of the sample was about 3.5-liter. Analysis of the fermentation gas by gas chromatography indicated that about 65% of it was hydrogen gas and the residual 35% was carbon dioxide during the whole fermentation period. When the RDF sample manufactured with 2% calcium hydroxide was incubated under the same conditions, a

<table>
<thead>
<tr>
<th>Ca(OH)₂ Concentration</th>
<th>pH</th>
<th>Aerobes (cfu/g)</th>
<th>Anaerobes (cfu/g)</th>
<th>Facultative anaerobes (cfu/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0%</td>
<td>7.41</td>
<td>9.6 × 10⁵</td>
<td>1.0 × 10⁵</td>
<td>7.6 × 10⁵</td>
</tr>
<tr>
<td>2%</td>
<td>9.62</td>
<td>2.6 × 10⁵</td>
<td>2.0 × 10⁴</td>
<td>1.5 × 10⁵</td>
</tr>
<tr>
<td>4%</td>
<td>12.52</td>
<td>5.9 × 10⁴</td>
<td>&lt;10⁴</td>
<td>5.0 × 10⁴</td>
</tr>
</tbody>
</table>
similar hydrogen gas production curve was obtained although the total amount of fermentation gas evolved was a little low (about 2.7-liter) as compared with the RDF sample without calcium hydroxide. On the other hand, when the RDF sample containing 4% calcium hydroxide was used, the initiation of fermentation gas production was retarded. That is, the fermentation gas production started after 36 h of incubation and continued for more than 36 h. The total gas (2-liter) evolved from this sample was lower than from the other two samples. The hydrogen gas content was about 65% in the fermentation gas. Although the initial pH of the suspension of the RDF sample containing 4% calcium hydroxide was higher than 12, the pH value of the suspension decreased to about 8.0 when fermentation finished. These observations suggest that the addition of calcium hydroxide to raw garbage lowered the viable cell number in the RDF pellets, but did not abolish hydrogen gas production in wet conditions, whereas the initiation of hydrogen gas production was retarded.

**DGGE analysis of microflora in RDF suspensions**

Although the presence of a large number of microorganisms in the RDF pellets was shown in recent studies, there was no information about microflora. Therefore, we analyzed microfloras of the RDF samples with or without calcium hydroxide after incubation in wet conditions by DGGE of PCR-amplified 16S rDNAs followed by sequencing of the separated DNA fragments.

As shown in Fig. 2, the untreated RDF samples containing different concentrations of calcium hydroxide showed similar but not identical patterns by DGGE analysis. Since calcium hydroxide powder is added to dried garbage before extrusion molding, the addition of this alkaline salt might not have affected the microflora of the RDF samples revealed by DGGE. If so, the difference in these DGGE patterns can be explained as follows: the microfloras in raw garbage used for manufacturing RDF pellets were originally different since the garbage was not exhaustively mixed in the large-scale plant. Alternatively, the efficiency of DNA extraction might be affected by the presence of calcium hydroxide.

When the respective RDF samples were moistened and incubated under anaerobic conditions, the microfloras in them changed drastically after incubation, judging by the DGGE patterns (Fig. 2). Microfloras in the RDF samples with 2% calcium hydroxide and without it became very similar to each other after incubation. By contrast, microflora in the RDF sample containing 4% calcium hydroxide was found to be different from those in the others despite their relatively similar microfloras before incubation in wet conditions, suggesting that alkaline pH affected the growth of some bacteria and finally changed the microflora under the alkaline pH condition.

**Identification of bacteria present in the RDF samples**

After DGGE of 16S rDNA amplified from DNAs of various RDF samples, representative DNA bands were recovered from the gels, and DNA sequences of the respective bands were determined (Table 2). DNA sequences from band nos. 1 to 5 were consistent with those from band nos. 12 to 16 respectively, confirming the finding by DGGE that microfloras in the RDF samples with (2%) or without calcium hydroxide became similar after incubation in wet conditions.
conditions. The amplified 16S rDNA fragments were not long enough, about 200 base pairs in length, to identify these bacteria at the species level. In Table 2, the names of bacteria, of which 16S rDNA sequences were in best agreement with the determined sequences, are shown with sequence identities. It is plausible that most of the identified bacteria belong to *Clostridium* species, since the RDF samples were incubated under anaerobic conditions. On the other hand, some facultative anaerobes, mainly *Paenibacillus* species, were identified in the RDF sample containing 4% calcium hydroxide. It is possible that these bacteria grew first, decreasing the pH of the suspension to a range that permits ordinary anaerobes to grow. Detection of *Bacillus* and *Paenibacillus* species, which are classified as strictly aerobic or facultative anaerobic bacteria, in RDF samples as shown by DGGE analysis was consistent with the observation that aerobic, anaerobic, and facultative anaerobic bacteria were detected by counting of viable cell numbers. In general, microbial hydrogen gas production is closely related to some acid and solvent productions, e.g., from 1 mol glucose, 2 mol carbon dioxide and 4 mol hydrogen gas are produced with acetic acid; 2 mol carbon dioxide and 2 mol hydrogen gas with butyric acid; and 2.5 mol carbon dioxide and 2 mol hydrogen gas with acetone and butanol, according to the following equations:

\[
\text{C}_6\text{H}_{12}\text{O}_6 + 2\text{H}_2\text{O} \rightarrow 2\text{CH}_3\text{COOH} + 2\text{CO}_2 + 4\text{H}_2
\]

\[
\text{C}_6\text{H}_{12}\text{O}_6 \rightarrow \text{CH}_3\text{CH}_2\text{CH}_2\text{COOH} + 2\text{CO}_2 + 2\text{H}_2
\]

\[
2\text{C}_6\text{H}_{12}\text{O}_6 \rightarrow \text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{OH} + 3\text{CH}_3\text{COOH} + 5\text{CO}_2 + 4\text{H}_2
\]

In ethanol and lactic acid fermentation, no hydrogen gas is produced from glucose. In this experiment, *Clostridium butyricum*, *Clostridium perfringens*, and *Clostridium acetobutylicum* were found in the RDF samples with (2%) or without calcium hydroxide. *C. butyricum* and *C. acetobutylicum* are well known by butyrate and acetone-butanol fermentation respectively.\(^{10}\) *C. perfringens* produces butyric acid, acetic acid, lactic acid, and ethanol.\(^{10}\) Therefore, it is no wonder that a large amount of hydrogen gas evolved from the RDF samples in wet conditions. As described above, 3.5-liter of total gas evolved from 100 g of the RDF pellets, meaning that about 0.1 mol of hydrogen gas was recovered from them, since hydrogen gas accounted for 65% of the total gas. The RDF samples (100 g) used in this study contained about 0.12 g/g of BOD (data not shown). This BOD value corresponded to about 0.06 mol of glucose equivalent in 100 g of the RDF pellets, suggesting that hydrogen gas production efficiency is relatively high, although we do not know the amount of carbohydrates easily metabolized by the microflora in the RDF pellets.

From the viewpoint of production of hydrogen gas as a clean energy resource, several papers have described efficient hydrogen gas production from biomass materials by natural microflora.\(^{11–13}\) Among them, however, only few papers deal with analysis of microflora responsible for hydrogen gas production. Ueno et al.\(^{11}\) established an anaerobic microflora, enriched from sludge compost at 60°C, capable of producing hydrogen gas from cellulose powder. Since this microflora was
thermophilic, it contained thermophilic and/or cellulolytic bacteria such as *Clostridium thermocellum*, *Thermoanaerobacterium thermosaccharolyticum*, and *Clostridium cellulosi*. A mesophilic anaerobic hydrogen gas-productive microflora was constructed from anaerobic high-solids digester after 45 min of heating at 100°C to inhibit/deactivate hydrogenotrophic microorganisms such as methanogens. It is likely that spore-forming bacteria preferentially survive severe heat-shock treatment, but the microflora was not investigated. Taking into consideration our results on bacteria in the RDF samples, heat treatment of any microfloras appears to be an efficient method to enrich spore-forming and hydrogen gas producing bacteria in the microfloras.

In conclusion, RDF samples contained a large number of spore-forming bacteria such as *Clostridium* species, which survived a hot-air drying process. The bacteria were capable of producing hydrogen gas from organic materials in the RDF samples in wet conditions. It is quite possible that the explosion accident at a silo containing RDF of the Mie RDF Power Station was caused by inflammation of hydrogen gas produced by anaerobic bacteria, since water was poured into the silo to extinguish the fire. The addition of calcium hydroxide in RDF pellets appears to have a minor effect on cell numbers in the RDF samples and the risk of hydrogen gas production in wet conditions.

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**References**