

# Effects of Prechilling and Sequential Washing on Enumeration of Microorganisms from Refuse

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**Techniques were evaluated for formation of a liquid inoculum from shredded municipal refuse, including chilling the refuse at 4°C prior to blending and multiple washing and blending cycles. The average count of cellulolytic bacteria from six different detachment treatments was  $5.1 \times 10^4$  cells per g (dry weight) of refuse with a range of  $0.7 \times 10^4$  to  $12.7 \times 10^4$  cells per g (dry weight). The liquid obtained from blending the refuse in phosphate buffer followed by hand squeezing was the selected detachment procedure. The inoculum formation procedure was validated by the addition of ruminal cellulolytic bacteria to refuse and recovery of the cellulolytic bacteria by most-probable-number enumerations. The ratio of measured to expected cell counts among tests in which different volumes of ruminal fluid were added to refuse ranged from 2.7 to 14.4. There was no evidence of anaerobic cellulolytic fungi in a refuse sample.**

A total of 95% of the solid waste generated in the United States is disposed of in landfills (6a). After burial, refuse undergoes biological decomposition to produce methane. Methane production from refuse in sanitary landfills represents a large but underutilized source of energy. In 1985, there were 75 facilities for the recovery of landfill gas either in operation or under construction (5). However, many potential projects to recover landfill gas are not developed because methane yields and production rates are difficult to predict and are typically 1 to 50% of the values calculated from refuse biodegradability data. Numerous researchers (2, 6, 11, 15, 26) have evaluated methods for enhancement of methane production from refuse, and others have studied microbiological characteristics (10, 12–14, 27–29, 31a). The present understanding of the microbiology of refuse decomposition is not adequate to provide increased methane yields in sanitary landfills. A study was initiated to measure changes in chemical composition and population development of key trophic groups of bacteria involved in refuse conversion to methane (hydrolytic, acetogenic, and methanogenic) to develop an updated characterization of refuse decomposition. Procedures for enumeration of hydrolytic, hydrogen-producing acetogenic, and methanogenic bacteria in refuse have not been published and were needed to study the refuse ecosystem. Experiments to develop techniques for enumeration of microorganisms in refuse are reported here.

Microorganisms in the refuse ecosystem may be attached to fibrous material such as cellulose or inert solids, or they may exist in the liquid phase closely associated with the solid phase. A repeatable procedure to bring as many of these organisms as possible into the liquid phase was needed. Donnelly and Scarpino (10) formed an inoculum from refuse by blending 20-g samples in a Waring blender for 15 s with phosphate buffer.

Tween 80, Triton X-100, and methylcellulose (0.01%) were found to be equally effective at releasing cellulolytic bacteria from cattle waste digester solids (31).

The problem of cell attachment to cellulosic substrates has been recognized by others studying the ruminal habitat, and their work is applicable to the refuse ecosystem (7, 8, 19–23, 30). Minato and Suto (20–23) studied attachment of ruminal hydrolytic bacteria to starch granules and cellulose powder. The three detachment treatments developed by Minato and Suto included (i) chilling to 4°C, (ii) addition of methylcellulose, and (iii) incubation at 38°C. Each treatment eluted a certain fraction of the attached bacteria, but no one technique was useful for complete detachment. Tween 80 was found to cause some cell lysis.

Dehority and Grubb (8) showed that chilling whole ruminal contents at 0°C for up to 8 h increased counts by 35%. Addition of Tween 80 to chilled ruminal contents further increased cell counts. Leedle and Hespell (19) demonstrated that blending for 1 min increased cell counts by 26 to 38% compared with no blending. Blending did not increase enumeration efficiency (viable count/total count).

Senshu et al. (30) found that repeated washing of a ruminal fluid sample in fresh mineral solution increased the total bacterial count with each washing up to 10 washings. Craig et al. (7) showed that inocula formed by repeated washing of ruminal particulate matter or chilling of ruminal contents prior to repeated washing increased *in vitro* fermentation rates of casein by factors of 2 and 2.27, respectively.

The use of a phosphate buffer-detergent mixture has been reported to prevent cell aggregation (17). However, cells may lyse upon exposure to even low detergent concentrations (17, 20–23). Thus, the use of detergents for the detachment of microorganisms was not evaluated in this study. Separation techniques developed by others (7, 8, 19), including blending, chilling, and multiple washing, were evaluated here.

The presence in the landfill ecosystem of anaerobic fungi which could degrade recalcitrant lignocellulosic substrates could be important in that polymer hydrolysis limits the rate of refuse decomposition to methane after consumption of carboxylic acids (3). Anaerobic fungi have been found in the rumen (1, 4, 24) and have been reported to attack and degrade lignified plant tissues resistant to ruminal bacteria (1).

The objective of this report is to present the results of

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experiments performed to evaluate and validate techniques for the detachment and enumeration of microorganisms from the refuse ecosystem. After selection of an inoculum formation technique, it was validated by the addition of ruminal cellulolytic bacteria to refuse and recovery of the bacteria by the selected inoculum formation technique and most-probable-number (MPN) enumerations. The presence of anaerobic cellulolytic fungi in refuse in an active state of methane production was also evaluated.

### MATERIALS AND METHODS

**Materials.** Shredded domestic refuse as described elsewhere (3) was used for all experimental work. Prior to use in the experiments described here, the refuse was incubated at 41°C in 2-liter Nalgene containers for several months. The refuse was saturated with distilled water to induce leachate formation. Leachate was neutralized to pH 7 with sodium carbonate and recycled daily to enhance methane production. Refuse actively producing methane was used for all experiments.

**Media and enumeration techniques.** The media and enumeration techniques for the cellulolytic and hemicellulolytic bacteria as well as the inoculum dilution solution are described elsewhere (3). Three-tube MPN tests were used for enumerations. MPNs and 95% confidence intervals were either determined from the table of deMan (9) or calculated as described by Parnow (25).

**Inoculum formation.** In several of the tests described below, a hand-squeezed extract formed from refuse blended in anaerobic phosphate buffer (23.7 mM, pH 7.2) was used as the inoculum. Phosphate buffer was autoclaved for 1 h and then cooled while being sparged with nitrogen. When specified, refuse was blended in a Waring blender (model CB-6, 20,000 rpm free running) flushed with sterile nitrogen. All equipment was autoclaved prior to use, and the refuse was blended and handled under nitrogen. In preliminary work, it was demonstrated that the use of hands (covered by disposable gloves) for formation of a hand-squeezed extract did not introduce either hemicellulolytic or methanogenic bacteria to the inoculum. The hemicellulolytic bacteria were the most numerous trophic group enumerated in the population development study (3). Thus, the failure to contaminate the inoculum with hemicellulolytic bacteria was taken as evidence that contamination with other trophic groups was not of concern.

**Experiment to evaluate cell detachment techniques.** Six procedures were evaluated for cell detachment. In the first procedure, termed one hand squeezing, an inoculum was formed by adding 1 liter of phosphate buffer to 200 g of wet refuse, giving a moisture content of 95% (wt/wt). At this moisture content, there was ample free liquid for blending. The refuse was then blended for 90 s. The free liquid released by hand squeezing of the refuse (L1) was collected in a nitrogen-purged flask and used as the inoculum. The solids remaining from this process were returned to the blender, and 430 ml of fresh buffer was added. The solids were blended for 60 s, and the hand-squeezed extract (L2) was collected. This was repeated two more times, and extracts L1 to L4 were combined for use as an inoculum to compare one hand squeezing with four hand squeezings. A third inoculum containing both the liquid and particulate fractions of the refuse was formed by sampling the blended refuse prior to the first hand squeezing. The inoculum volume for the single and multiple hand squeezing methods was measured by syringe, while a wide-bore pipette was used for the particulate-plus-liquid method.

An additional three inocula were formed by first chilling the refuse for 6 h and then forming one hand squeezing, four hand squeezings, and particulate-plus-liquid inocula as described above.

**Enumeration of cellulolytic and hemicellulolytic bacteria in triplicate.** After selection of an inoculum formation procedure, cellulolytic and hemicellulolytic bacteria were enumerated in triplicate to measure the repeatability of the MPN test. Phosphate buffer (1 liter) was added to 200 g of wet refuse (giving a moisture content of 95%) and blended for 90 s. The inoculum was prepared by hand squeezing the blended refuse. Three distinct dilution series, termed A, B, and C, were prepared from the inoculum.

**Recovery of ruminal cellulolytic bacteria added to refuse.** The objectives of this experiment were to use the MPN technique for a quantitative experiment, to demonstrate that the extraction technique was not toxic to ruminal cellulolytic bacteria, to determine whether attachment of ruminal cellulolytic bacteria to refuse particulate material would reduce recovery of the added ruminal bacteria, and to determine whether there is anything in refuse which is toxic to ruminal cellulolytic bacteria. Known volumes of ruminal fluid were added to measured amounts of refuse, and the cellulolytic bacteria in refuse, refuse plus ruminal fluid, and ruminal fluid were enumerated by the MPN technique. Unlike the previous two experiments, the phosphate buffer used for formation of the liquid extract was reduced with 0.05% cysteine hydrochloride to minimize the potential for oxidation of the ruminal fluid.

Ruminal fluid was sampled from a fistulated hay-fed cow 3 h after feeding and immediately transported to the laboratory in a preheated thermos. Once in the laboratory, the ruminal fluid was filtered through two layers of cheesecloth. The liquid passing through the cheesecloth after hand squeezing was used as the source of ruminal cellulolytic bacteria.

A refuse extract was formed by adding 1 liter of phosphate buffer to 200 g of wet refuse (giving a moisture content of 95%) and blending for 90 s. After blending, a hand-squeezed extract was formed, and 50 ml of this extract was used to enumerate the refuse cellulolytic bacteria. An additional 49 ml of this extract was spiked with 1 ml of ruminal fluid to test a low-level spike in the absence of refuse particulate matter. The refuse and the remainder of the liquid refuse extract were then returned to the blender. A 20-ml sample of ruminal fluid, equivalent to 2% of the remaining refuse extract in the blender, was added to 50 ml of phosphate buffer to help distribute the ruminal fluid. This ruminal fluid-plus-phosphate buffer solution was then added to the refuse and blended for 30 s, after which a hand-squeezed extract was formed. This inoculum tested the addition of a low-level spike of ruminal fluid to refuse particulate matter. The solids and the remaining liquid refuse extract were again returned to the blender, and 500 ml of ruminal fluid was added to the remaining refuse extract to constitute a final concentration of 33% ruminal fluid. This solution was blended for 30 s, after which a hand-squeezed extract was formed for the inoculum to test a high-level spike of ruminal fluid into refuse particulate matter. Finally, 100 ml of ruminal fluid was blended with 100 ml of phosphate buffer for 15 s to form the inoculum for enumeration of ruminal fluid cellulolytic bacteria.

Sterile clarified ruminal fluid was included in the 10<sup>-1</sup> dilution bottle (10%, vol/vol) for all inocula except the ruminal fluid cellulolytic bacteria enumeration to control for the presence of a variable amount of ruminal fluid carried over from the inoculum in each test.

**Determination of presence of anaerobic cellulolytic fungi in**

TABLE 1. Evaluation of methods for cell detachment and inoculum formation

Description	Cellulolytic population (10 <sup>4</sup> cells/g [dry wt])	95% Confidence interval <sup>a</sup>
One hand squeezing	7.0	1.7–32
Four hand squeezings	1.7	0.5–7.2
Liquids plus particulate	1.0	0.3–4.6
One hand squeezing, chilled	7.3	3.7–38.0
Four hand squeezings, chilled	0.7	0.2–3.7
Liquids plus particulate, chilled	12.7	3.7–50.3

<sup>a</sup> Confidence intervals are given in the same order of magnitude as the population data.

**refuse.** The objective of this experiment was to determine whether anaerobic cellulolytic fungi were present in a refuse sample. The medium was similar to the cellulose medium referred to above with the addition of 0.5% agar. Whatman no. 1 filter paper strips were used as the substrate so that if degradation was observed it would be possible to remove the filter paper strip, stain it, and inspect it microscopically for the presence of fungi (32). The inoculum (5 ml) included both the solid and liquid fractions of a blended refuse sample since fungi can only grow when attached to a solid substrate. All inoculations were performed with a wide-bore pipette to include solids.

Three series of tubes were inoculated to evaluate the potential for cellulolytic fungal activity including (i) control tubes containing the medium and filter paper strips, (ii) tubes containing antibiotics (streptomycin sulfate at 130 U/ml and penicillin [Pen K] at 2,000 U/ml) to prevent the growth of bacteria, and (iii) tubes containing antibiotics as above and cycloheximide (0.05 mg/ml) to prevent the growth of bacteria and fungi. Three tubes were inoculated at 10<sup>-1</sup>, 10<sup>-2</sup>, and 10<sup>-3</sup> dilutions. Antibiotic and fungicide concentrations were as described by Windham and Akin (32). They were purchased from Sigma Chemical Co. (St. Louis, Mo.) and filter sterilized. The presence of fungi would have been suspected if there was filter paper decomposition in the control tubes and the tubes containing antibiotics but not in the tubes containing antibiotics plus cycloheximide.

## RESULTS AND DISCUSSION

**Cell detachment.** The data in Table 1 do not suggest that any one cell detachment technique was most effective. Consideration of the three pairs of tests comparing extraction of chilled and nonchilled refuse shows that there was no consistent and significant increase in cell count associated with refuse chilling. The difference in the MPN between the one hand squeezing of nonchilled refuse and the liquid-plus-particulate inoculum from chilled refuse is insignificant ( $P = 95\%$ ). Thus, blending the refuse followed by formation of a hand-squeezed extract was selected for use in the experiment on population development. The failure to detect significant differences among the treatments is explained by the small (100 to 230%) differences observed by others relative to the precision of the MPN technique.

Selection of the one-hand-squeezing method for inoculum formation was based on tests with cellulolytic bacteria, the trophic group which was thought most likely to attach to particulate material. It was assumed that an extraction technique acceptable for the cellulolytic bacteria would be

TABLE 2. Results of cellulolytic and hemicellulolytic MPN determinations in triplicate<sup>a</sup>

Test	Result code <sup>b</sup>	Three-tube MPN		95% Confidence interval <sup>d</sup>
		Dilution <sup>c</sup>	MPN (cells/ml)	
<b>Cellulose</b>				
C-A	3-1-0 <sup>e</sup>	10 <sup>-5</sup> –10 <sup>-7</sup>	4 × 10 <sup>5</sup>	2–21
C-B	3-0-1	10 <sup>-5</sup> –10 <sup>-7</sup>	4 × 10 <sup>5</sup>	1–18
C-C	2-2-1 <sup>f</sup>	10 <sup>-4</sup> –10 <sup>-6</sup>	2.75 × 10 <sup>4</sup>	1.0–7.5
<b>Hemicellulose</b>				
H-A	2-0-1 <sup>g</sup>	10 <sup>-7</sup> –10 <sup>-9</sup>	1.4 × 10 <sup>7</sup>	0.5–4.8
H-B	3-0-0	10 <sup>-7</sup> –10 <sup>-9</sup>	2.3 × 10 <sup>7</sup>	0.7–12.9
H-C	3-0-0	10 <sup>-7</sup> –10 <sup>-9</sup>	2.3 × 10 <sup>7</sup>	0.7–12.9

<sup>a</sup> Three separate dilution series of the inoculum were formed for tests A, B, and C, respectively.

<sup>b</sup> The number of positive tubes at each dilution (see footnote c) used for calculation of the MPN. For example, in test C-A there were three positive tubes in the 10<sup>-5</sup> dilution, one in the 10<sup>-6</sup> dilution, and none in the 10<sup>-7</sup> dilution.

<sup>c</sup> The range of dilutions used for the result codes.

<sup>d</sup> Confidence intervals are given in the same order of magnitude as the MPN results.

<sup>e</sup> In test C-A, there were only two positive tubes at the 10<sup>-4</sup> dilution.

<sup>f</sup> In test C-C, there were only two positive tubes at the 10<sup>-1</sup> dilution and the result code would be expected in 1% of all cases (9).

<sup>g</sup> Result code expected in 4% of all cases (9).

acceptable for the hemicellulolytic bacteria, acetogens, and methanogens which also participate in refuse decomposition.

The selected inoculum formation technique may not detach all attached cells. However, trends between refuse samples and ratios between trophic groups are of more significance than absolute cell counts. Thus, as long as the inoculum formation procedure is constant, a constant fraction of the population should be detached each time.

**Enumeration of cellulolytic and hemicellulolytic bacteria in triplicate.** Results of an MPN determination in triplicate for cellulolytic bacteria are presented in Table 2 (designated as tests C-A, C-B, and C-C). There is just over 1 order of magnitude difference in the results of test C-C compared with those of tests C-A and C-B. Given this repeatability, we decided to perform the cellulolytic MPN determination in duplicate for the population development study.

Data for the hemicellulolytic MPN determination in triplicate are also reported in Table 2. There were no significant differences between the populations measured for three tests designated as H-A, H-B, and H-C.

**Recovery of ruminal fluid cellulolytic bacteria from refuse.** The results on the addition of cellulolytic bacteria from ruminal fluid to refuse are presented in Table 3. The difference in counts between the low-level spike additions with and without exposure to refuse particulate matter is not significant ( $P = 95\%$ ). Thus, there was no effect resulting from the attachment of ruminal fluid cellulolytic bacteria to refuse. The measured MPNs for all spike addition tests were close to the expected MPNs based on the volume of ruminal fluid added. The ratio of the measured to expected MPN (Table 3) was calculated from the MPN of ruminal fluid, although this number was no more precise than the other data. Because this ratio was always greater than 1, there is no evidence to suggest a toxic effect on ruminal cellulolytic bacteria owing to either the extraction technique or the chemical composition of refuse.

The cellulolytic MPN for ruminal fluid, 8 × 10<sup>6</sup> cells per ml, is consistent with the counts of 1 × 10<sup>7</sup> to 1 × 10<sup>9</sup> cells per ml reported elsewhere (16, 18) given the precision of the

TABLE 3. Recovery of cellulolytic bacteria from ruminal fluid added to a refuse sample

Sample	Measured MPN <sup>a</sup>	Final concn of ruminal fluid in refuse (%)	Expected count <sup>b</sup>	Measured count/expected count	Result code	95% Confidence interval <sup>c</sup>
Refuse	$1.4 \times 10^4$				2-0-1	0.5-4.8
Refuse + low-level spike	$9 \times 10^5$	2	$1.6 \times 10^5$	5.6	3-2-0	3-39
Refuse + high-level spike	$7 \times 10^6$	33	$2.6 \times 10^6$	2.7	3-1-1	2-28
Refuse + low-level spike without exposure to refuse particulate matter	$2.3 \times 10^6$	2	$1.6 \times 10^5$	14.4	3-0-0	0.7-12.9
Ruminal fluid	$4 \times 10^6$	50 <sup>d</sup>	$8 \times 10^{6e}$		3-1-0	2-21

<sup>a</sup> Cells per milliliter of inoculum.

<sup>b</sup> The expected count, in cells per milliliter of inoculum, is based on the measured MPN of the ruminal fluid in test D and the dilution factors.

<sup>c</sup> The 95% confidence intervals are expressed in the same order of magnitude as the MPN.

<sup>d</sup> Final concentration of ruminal fluid in phosphate buffer. No refuse extract was added.

<sup>e</sup> The MPN of the ruminal fluid cellulolytic bacteria after correction for dilution. No refuse extract was added.

MPN technique and variation in experimental conditions used by others.

The quantitative recovery of cellulolytic bacteria in this experiment demonstrates that the procedure for inoculum formation and cell measurement is a valid method for the enumeration of cellulolytic bacteria. The applicability of this result to the other trophic groups which participate in refuse decomposition was assumed.

**Atypical observations on cellulolytic bacteria in the refuse ecosystem.** In preliminary work with cellulolytic bacteria, anomalies of the nature reported in Table 2 (footnotes *e* and *f*) and atypical results codes as reported for test C-C in Table 2 and refuse in Table 3 were common. Microscopic inspection of the  $10^{-1}$ ,  $10^{-2}$ , and  $10^{-3}$  dilutions of the inoculum used for the MPN determinations in triplicate (Table 2) did not reveal clumps or cells attached to fibrous material which could explain the atypical observations.

The results codes for all cellulolytic MPNs in which ruminal fluid was present (Table 3) were of the type expected in 96% of all tests (9). In each of these tests, the influence of the ruminal fluid cellulolytic bacteria eliminated the contribution of the refuse cellulolytic bacteria to the measured MPN. When only the refuse cellulolytic bacteria were measured, the results code was one which would be expected in only 4% of all tests. The data for the refuse cellulolytic bacteria suggest some unique property that caused anomalous MPN results. The absence of unusual codes in work with ruminal fluid suggests that the inoculation techniques were performed properly.

**Presence of anaerobic cellulolytic fungi in refuse.** There was complete disappearance of the filter paper in all control tubes for determination of fungi in refuse. There was no filter paper decomposition in any of the tubes containing antibiotics or antibiotics plus cycloheximide. Thus, there were no anaerobic cellulolytic fungi in the sample tested.

In summary, these results indicate that relatively simple techniques were sufficient for the enumeration of bacteria in decomposing shredded refuse. Furthermore, additional procedures used to detach particle-adherent ruminal bacteria were without effect on the enumeration of cellulolytic bacteria in the refuse ecosystem.

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