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Medium for the Enumeration and Isolation of Bacteria from a Swine Waste Digester

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A habitat-simulating medium was developed for the enumeration and isolation of bacteria from a swine waste digester. A roll tube medium with growth factors for strict anaerobes from previously studied anaerobic ecosystems was used to evaluate the effects of deletion, addition, or level of digester fluid, digester fluid treated with acid or base, rumen fluid, fecal extract, anaerobic pit extract, tissue extract, carbohydrates, peptones, short-chain fatty acids, minerals, vitamins, N and P sources, reducing and solidifying agents, buffers, and gases on colony counts. Decreasing the agar concentration from 2.5 to 1.0% increased the counts twofold. Blending increased the counts 1.7-fold. With a medium (174) containing digester fluid, peptones, minerals, cysteine, sodium carbonate, and agar, colony counts were 60% of the microscopic count and improved yields 2.5 to 20 times those obtained with media previously used for digesters or developed for other anaerobic ecosystems. Colony counts continued to increase for up to 4 weeks of incubation. Medium 174 permits the enumeration of total, methanogenic, and, with deletion of reducing agent, aerotolerant bacteria. The results suggest that the predominant bacteria grow slowly and have requirements different from those of bacteria from other ecosystems.

The development of the Hungate technique (23) for the maintenance of anaerobic conditions and the utilization of a habitat-simulating medium with rumen fluid (8, 10, 23) permitted the enumeration and isolation of the predominant carbohydrate-fermenting rumen organisms. After the nutritional requirements for many of the predominant rumen organisms were determined, Caldwell and Bryant (11) developed a more defined medium (medium 10) in which rumen fluid was replaced by Trypticase, yeast extract, hemin, and volatile fatty acids. The recovery of bacteria (viable count per total microscopic count) with all of the above media has been 10 to 30% with animals on a forage diet and higher with those on grain diets (8, 24).

The media developed for the rumen have been adapted for use in other anaerobic ecosystems, including the ground squirrel and chicken cecum (4, 34, 36), mouse gut (19, 40), and human and swine feces (16, 31, 35). The recovery of bacteria from these systems has ranged from 5 to 90%. The best recoveries have been obtained from human feces, in which the bacteria are apparently less fastidious than those of other ecosystems (16).

Similar media also have been used in studies of anaerobic digesters. Mah and Sussman (28) recovered approximately 1 to 10% of the bacteria from a domestic digester with a medium containing glucose, digester fluid (DF), minerals, reducing agent, and buffer. The addition of glucose did not improve the recovery of bacteria over that in media without glucose nor was there a difference between medium 10 and media with DF or rumen fluid. Kirsch (27) recovered 8.6% of the bacteria in medium 10 with 16% DF (medium 11). Toerien and Siebert (43) used a medium with high amounts of organic material, including starch (2%), dextrin (1.4%), peptones (0.6%), and beef extract (0.2%); however, the recovery was not reported. Hobson and Shaw (21) replaced rumen fluid with DF in a medium developed for the rumen (medium 2) (20) for the enumeration and isolation of bacteria from a swine waste digester. Again, the recovery was not reported. Initial studies in our laboratory with a swine waste digester indicated that the recovery was 15% with a medium similar to medium 11 (J. R. Fischer, E. L. Iannotti, J. H. Porter, and A. Garcia, Am. Soc. Agric. Eng., Paper MC-77-604, 1977). Deletion of the sugars increased the viable count.

This paper reports on the development of media that permit the enumeration and isolation of bacteria from a swine waste digester.

MATERIALS AND METHODS

Digesters. The pilot-size digester used was a conical-bottom polyethylene tank with a working capacity of 450 liters (Fischer et al., Am. Soc. Agric. Eng., Paper MC-77-604, 1977). The contents were mixed by periodic gas recirculation and maintained at 35°C by internal heating coils. The loading rate was 4 g of volatile solids per liter of digester volume per day. The retention time was 15 days. Manure was obtained from finishing hogs on a 14% protein finishing diet. Fresh manure was collected from a concrete finishing floor every 7 to 10 days and refrigerated until use.

The digester was started with the contents of a 200-liter digester that had been operating for approximately 2 years. Methane production began immediately, and the digester operated stably during these studies (Fischer et al., Am. Soc. Agric. Eng., Paper MC-77-604, 1977). Stability was judged by consistent gas production, solids destruction, levels of volatile fatty acids, and pH.

Samples from a farm-size digester were used for studies of the enumeration of methanogenic bacteria. The design and operation of this farm-size digester have been previously described (J. R. Fischer, N. F. Meador, D. M. Sievers, C. D. Fulhage, and E. L. Iannotti, Am. Soc. Agric. Eng., Paper MC-77-4052, 1977). The digester has a volume of 140 m^3 and is designed to handle the waste from four confinement swine units at the University of Missouri. The waste is flushed into a settling basin, from which the settled solids are pumped to the digester. The average loading rate was 2.1 g of volatile solids/liter per day; the retention time was 11 days.

Anaerobic technique. Media preparation, dilution, and inoculation were done with the exclusion of oxygen by the Hungate technique (23), as modified by Bryant and Burkey (8). Samples for bacteriological analysis were collected after agitation but before loading. Thus, the samples represented that population present approximately 24 h after the last influent addition and a minimum of organisms in the swine waste. Separate samples were taken for each experiment. A sterile container was filled to capacity and immediately transported to the laboratory for processing. The sample was mixed by repeated inversion of the sample bottle. Twenty milliliters was transferred with a sterile, CO_2-filled, widemouthed chemical pipette to a sterile Waring blender, which was gassed with a heavy flow of CO_2. The blender contained 180 ml of sterile anaerobic dilution solution (8). Unless stated otherwise, the sample was blended for 1 min under CO_2. Ten milliliters of this dilution was immediately removed with a CO_2-filled pipette and transferred to 90 ml of dilution solution in a milk dilution bottle. This dilution (10^{-2}) was used for microscopic clump count and was further diluted in 10- and 100-fold steps for addition to media. Samples (0.2 or 0.5 ml) of the final dilution were used to inoculate each of five replicate roll tubes of molten medium. Inoculated tubes were placed immediately in a spinner (Bellco Glass, Inc.) modified so that a continuous stream of ice water was flushed over the tube. Cultures were incubated in an upright position at 35°C.

Extracts. DF was prepared by filtering the digester effluent through cheesecloth and freezing until use. At the time of use, the DF was clarified by centrifugation at 15,000 × *g* for 30 min. When the DF was filter sterilized, the clarified DF was passed successively through 8-, 0.45-, and 0.22-μm filters (Millipore Corp.).

The basic hydrolysate of DF was prepared by bringing 1 liter of the effluent to pH 13 with 50% NaOH and autoclaving it for 30 min (the pH after autoclaving was 9.8). The basic hydrolysate was neutralized with concentrated HCl and clarified by centrifugation (1,500 × *g*, 30 min).

An acid hydrolysate was prepared by bringing 1 liter of the effluent to pH 1 with HCl and autoclaving for 30 min. The final pH was 1.1. This hydrolysate was neutralized with 50% NaOH and clarified by centrifugation as above.

Fecal extract was obtained from swine manure used to load the digester. One volume of manure was added to 3 volumes of water and blended for 2 min. This mixture was autoclaved for 30 min, cooled, and clarified by centrifugation.

Pit extract was prepared by autoclaving for 30 min a mixture of sheep feces and liquid from an anaerobic pit. The autoclaved liquid was decanted and clarified by centrifugation as above. The sheep were fed a corn-cotton seed-based diet. The feces had been in the pit for many months, so the extract represented a long-term anaerobic digestion.

Rumen fluid was obtained from a fistulated cow on a forage diet. The fluid was filtered through four thicknesses of cheesecloth and clarified by centrifugation as above (10).

Media. Medium N (Fischer et al., Am. Soc. Agric. Eng., Paper MC-77-604, 1977) and medium 11 (27) with swine waste DF were used as base media. These media are modifications of medium 10 (11). In essence, medium 11 (Table 1) is medium 10 with 15% DF, and medium N is medium 11 with carbohydrates deleted. These media (Table 1) are slightly modified to fit the stock solutions in our laboratory. Medium 10 and medium 11 standardly contain 2% agar (11, 27). During initial studies, a swarm in many tubes made counts difficult after several weeks of incubation. Increasing the agar concentration from 2.0 to 2.5% eliminated this problem and did not significantly change the percent recoveries.

Unless stated otherwise, all of the ingredients except Na_2CO_3, cysteine-HCl, and $Na_2S \cdot 9H_2O$ were added before autoclaving. Each medium was adjusted to pH 6.8 and boiled under CO_2 in a round-bottom flask. The flask was stoppered, the stopper was wired on, and the medium was autoclaved at 15 lb/in^2 (1.05 kg/cm^2) for 20 min. After the autoclaved medium was cooled to 47°C, CO_2-equilibrated Na_2CO_3 was added, and the medium was tubed in 9-ml amounts into sterile rubber-stoppered tubes (18 by 150 mm) under CO_2.

Before use, the tubes were steamed for 15 min in a press to melt the agar, and 0.2 ml of a combined stock solution of cysteine-HCl and $Na_2S \cdot 9H_2O$ was added to each tube. The gas phase was changed to H_2-CO_2(1:1, vol/vol) at the time of inoculation, unless stated otherwise. For a 100% CO_2 or a 1:1 CO_2-H_2 gas phase, 0.4% Na_2CO_3 was added to the medium. For the 100% H_2 or N_2 gas phase, 0.8% Na_2CO_3 was added, and the medium was gassed with CO_2 until pH 7 was reached. The medium was then tubed under N_2 or H_2. For a 1:9 CO_2-N_2 gas phase, the medium was made with 0.8% sodium

TABLE 1. *Composition of medium 11 and medium 174*[a]

Component	% in medium[b]	
	Medium 11	Medium 174
Glucose	0.03	
Cellobiose	0.03	
Soluble starch	0.05	
Trypticase	0.2	0.2
Yeast extract	0.05	
Hemin	0.0001	
Resazurin	0.0001	0.0001
DF (vol/vol)	15.0	45
Minerals (vol/vol)[c]	3.75	3.75
Volatile fatty acids (vol/vol)[d]	0.45	
$Na_2S \cdot 9H_2O$	0.05	
Cysteine-HCl	0.05	0.05
Na_2CO_3	0.4	0.8
Agar	2.5	1.5

[a] The final pH was 6.9 in medium 11 and 7.4 in medium 174. The gas phase was 1:1 CO_2-H_2.

[b] Weight/volume unless otherwise indicated.

[c] K_2HPO_4 was prepared as one stock solution; and the other minerals were prepared as another. The final mineral concentrations in the media were: K_2HPO_4, 1.3×10^{-3} M; KH_2PO_4, 1.7×10^{-3} M; NaCl, 7.7×10^{-3} M; $(NH)_2SO_4$, 1.7×10^{-3} M; $MgSO_4 \cdot 7H_2O$, 3.7×10^{-4} M; $CaCl_2 \cdot 2H_2O$, 2.7×10^{-4} M.

[d] The volatile fatty acids were added as a common stock solution. The final concentrations in the media were: acetic, 1.3×10^{-2} M; propionic 4.0×10^{-3} M; butyric, 2.0×10^{-3} M, isobutyric, 4.1×10^{-4} M; *n*-valeric, isovaleric, and DL-2-methylbutyric, 4.1×10^{-4} M.

carbonate. No Na_2CO_3 or CO_2 gassing was used with the N_2 gas phase during the studies of buffer.

Granulated agar (Baltimore Biological Laboratory) was the solidifying agent in most studies. Medium N was also prepared with purified agar (Difco) and silica gel. The silica gel was prepared by adjusting the pH of $Na_2SiO_3 \cdot 9\ H_2O$ to pH 10 with cation-exchange resin (Bio-Rad Laboratories, AG 50 X 8) (42) and filter sterilizing (0.22-μm filter). At the time of use, the silicate solution was added to concentrated medium N. Phosphoric acid (5 N) was added to bring the medium to pH 7.0. The concentration of $Na_2SiO_3 \cdot 9\ H_2O$ ranged from 3.4 to 40.2%; Thatcher and Weaver (42) added 13.4%. The diluted sample was added to the tubes, and the tubes were placed in the spinner. A stream of water at 35°C was flushed over the tube to decrease gelling time.

Medium 2 (20, 21), medium 98-5 (10), and blood agar (22) were prepared as previously reported.

Dispersal of cells. For determination of the effect of blending, 20 ml of the swine waste was added to 180 ml of dilution solution in a round-bottom flask. This mixture was shaken vigorously for several minutes and then immediately added to a Waring blender previously gassed with CO_2. A good dispersion was obtained. One-milliliter samples were removed immediately after addition to the blender and after 10, 20, 30, 40, 50, 60, 90, 120, 180, and 240 s of homogenization and serially diluted. The final dilution was used to inoculate five tubes of medium N. Swine waste was also treated by (i) shaking in dilution solution, (ii) shaking in dilution solution with 10 μl of Tween 80 per liter (26), and (iii) homogenizing in a blender.

Microscopic clump and cultural counts. Microscopic clump counts were done with Gram stains of 0.01 ml of the 10^{-2} dilution spread evenly over 1 cm^2. Initially, clump counts were also counted with a Petroff-Hauser chamber with Formalin-fixed cells (30); the counts with the chamber were slightly higher and more variable. These problems were attributed to buoying of the cover slip (30). The microscopic counts with the Gram stains were averages of clump counts of three to five separate smears. Five fields from the edge to the middle were counted for each smear. During these counts, an average of 4,200 clumps was counted for each determination. The numbers of rods and cocci or coccobacilli were also enumerated. Only data from smears are given in this paper.

Initially, colonies were counted by using a stereoscopic microscope (×17) at weekly intervals for 4 weeks and then at weeks 7 and 10; later, colony counts were determined after 2, 4, and 7 weeks unless indicated otherwise. The data presented are for the 4-week count since there were no changes in significance after week 4.

Enumeration of methanogenic bacteria. Medium 174 broth (see Table 1) and the medium of Bryant et al. (9) with 30% rumen fluid were prepared in tubes fitted with serum tops and rubber stoppers (Bellco Glass, Inc.) (3). The reducing agent was added 2 h before use. The gas phase was 1:1 CO_2-H_2. Samples from 10^{-7}, 10^{-8}, and 10^{-9} dilutions were added to each of three tubes of both media directly through the stoppers. After 1 month of incubation at 35°C, methane was determined, and the methanogenic bacteria were quantitated by most probable number (1).

Enumeration of aerotolerant bacteria. Standard plate count medium was prepared according to standard methods (1). Medium 174 was prepared without carbonate, cysteine, or resazurin. The sample was treated as above, and 0.1-ml portions were spread evenly over the surface of eight plates of each medium and incubated aerobically at 35°C.

Isolation of organisms. Colonies were picked with a bent platinum-iridium wire or a Pasteur pipette (25) to broths or butts of agar medium. As many colonies as possible were picked from the same roll tube. The tubes were examined for growth daily with a high-intensity light.

Chemical determinations. Total solids and alkalinity were measured by standard methods (1). The components of the gas were quantified with a model 920 Varian Aerograph gas chromatograph equipped with a thermoconductivity detector and a silica gel column (30/60 mesh). The carrier gas was N_2.

Statistics. Percent recoveries with different media were calculated by dividing counts of the individual tubes by the average microscopic clump count × 100. Percent recoveries were analyzed by the least-significant-difference method and by Duncan's multiple range test (39).

RESULTS AND DISCUSSION

Microscopic clump counts and percent recovery. The average microscopic clump count in the pilot digester was 9.99 ± 1.91 (standard deviation) × 10^9/ml (range, 6.44 × 10^9 to 14.71 × 10^9/ml). The average clump count per gram of total solids (dry weight) was 2.88 × 10^{11}. The number of bacteria in the swine digester is an order of magnitude lower than that in feces (2, 31, 44) and the same as that in the domestic digester (27, 28). The rumen has values intermediate between those of feces and the digesters (8, 24). The predominant morphotype in the stained slides was consistently cocci (or coccobacilli). This group made up 73.9% (standard deviation = 0.62%; range, 72.8 to 75.1%) of the microscopic count.

The total viable counts were determined with medium 11 during six separate sampling periods. During these experiments, the microscopic counts were 7.96, 6.44, 7.91, 10.74, 10.03, and 10.70 × 10^9/ml. The viable counts varied proportionately, so that the percent recoveries (20.2, 20.5, 20.1, 23.8, 19.4, and 20.2; mean = 20.7) were quite consistent. In medium 11, 72% of the week 10 count was found after week 1; 87% was found after week 4, and 97% was found after week 7.

Crude extract. Table 2 shows the effect of various crude extracts in medium 11 on percent recovery of bacteria from a swine waste digester. The recovery was significantly higher in the medium with DF (20.2%) than in the medium without DF (16.0%). Previously, Mah and Sussman (28) found that DF was not necessary for the maximum viable count of bacteria in a sludge digester. However, Kirsch (27) isolated significantly higher numbers of bacteria when DF was included in media.

Kirsch (27) added a mixture of acid-hydrolyzed sewage and untreated DF to his medium. Data were not presented on the effect of treating the waste before its addition to the medium. In our studies (Table 2) there was no significant difference in recovery between media with DF and DF treated with base. Acid treatment reduced the recovery 10-fold. A mixture of acid- and base-treated DF also lowered the recovery significantly.

Replacement of DF with equal volumes of fecal extract, pit extract, or rumen fluid lowered the recovery of bacteria from the swine waste digester; however, the differences were not significant (Table 2). Recoveries with the rumen fluid were very low at 1 week but continued to increase throughout the counting period until the recovery was nearly equal to that in medium 11 at 10 weeks. Although no further observations were made, a plot of the counts indicated that further increases could be expected. Increasing the concentration of rumen fluid from 15 to 30% did not affect the recoveries or the steady increase. Deletion of volatile acids, hemin, Trypticase, and yeast extract, and concomitant replacement of DF with rumen fluid, significantly lowered the recovery.

TABLE 2. *Effect of various crude extracts in medium 11 on recovery of bacteria from a swine waste digester*

Extract	Concn (%, vol/vol)	Recovery[a] (%)	Statistical significance[b]
None		16.0	a
DF	15	20.2	b
DF			
Basic hydrolysate	15	20.9	b
Acid hydrolysate	15	2.0	c
DF			
Basic hydrolysate	7.5		
Acid hydrolysate	7.5	15.6	a
Fecal extract	15	17.7	ab
Pit extract	15	17.2	ab
Rumen fluid	15	17.8	ab
Rumen fluid	30	18.0	ab
Rumen fluid[c]	15	10.3	d

[a] Mean colony counts of five tubes, after 4 weeks of incubation, divided by microscopic clump count.

[b] Means not followed by the same letter are significantly different at the 5% level of probability.

[c] Trypticase, yeast extract, hemin, and volatile acids were deleted from this medium.

Toerien and Siebert (43) added beef extract to the medium for enumeration of bacteria from laboratory anaerobic digesters. Addition of beef extract (0.2%) to medium 11 did not increase the recovery. Addition of trace metals and vitamins also did not affect the recovery after 4 weeks of incubation, although the difference between weeks 1 and 4 was reduced.

Increasing the level of DF from 15 to 30, 45, or 75% did not significantly change the recovery in medium 11. The recoveries were 20.5, 21.5, 22.0, and 18.0%, respectively. The decrease in recovery with 75% DF may be attributed to the difficulty of detecting small colonies in this very dark medium.

Concentration of energy sources. Most media used to study anaerobic ecosystems have a low level of added energy sources (0.2%); however, levels >0.2% have been used in studies of anaerobic digestion (21, 43). For examination of the effect of different levels of possible energy sources on recovery of bacteria, the concentrations of carbohydrates and protein were increased in medium 11. Medium 11 contains 0.11% carbohydrates (0.03% glucose, 0.03% cellobiose, and 0.05% soluble starch) and 0.2%

Trypticase. Increasing the carbohydrates 10-fold or the protein 5-fold lowered the recovery from 20.1 to 16.2 or 17.4%, respectively, but these reductions were not significant. Increasing both the carbohydrates (1.1%) and the peptones (1.0%) significantly reduced the recovery to 10.9%. Colony counts in the media with high concentrations of carbohydrate and peptone did not continue to increase as they did in the medium with the low concentration of carbohydrates.

Deletions from medium 11. Deletions of carbohydrates (glucose, cellobiose, and soluble starch) from medium 11 significantly increased the recovery of bacteria. The mean recovery during three experiments was 20.0% for medium 11 and 24.3% for the medium without carbohydrates (medium N). The deletion of carbohydrates resulted in smaller colonies and poor recovery after week 1 of incubation; however, 97% of the week 10 count was found after week 7. Single deletion of Trypticase (21.0% recovery), yeast extract (19.8%), volatile fatty acids (19.3%), hemin (21.6%), or minerals (17.7%) did not significantly change the recoveries.

Deletion of carbohydrates from media used to isolate bacteria from a domestic anaerobic digester did not change the numbers of colonies found (28). Bryant and Robinson (10) found lower, but not significantly reduced, counts when carbohydrates were deleted from a medium for the rumen. Dehority and Grubb (14, 18), also working with the rumen, reported finding only 77% of the original number of colonies after carbohydrates were deleted.

Modification of medium N. Sweet E medium, used to culture isolated fecal organisms (22, 31), contains arabinose, cellobiose, fructose, maltose, starch, and pyruvate. This medium with the addition of mannitol and glycerol was compared with medium 11 and medium N. The mean recovery (20.6%) was not significantly different from that with medium 11 and was significantly lower than that with medium N; thus, the incorporation of a spectrum of compounds failed to improve the recovery.

The single addition of 0.03% soluble starch (23.5% recovery), cellobiose (24.6%), xylose (22.5%), fructose (22.0%), mannitol (23.3%), inulin (22.5%), sucrose (21.1%), glycerol (24.8%), glycerol phosphate (24.9%), ethanol (22.4%), methionine (21.6%), formate (21.4%), acetate (25.6%), propionate (25.6%), citrate (23.8%), malate (22.6%), fumarate (22.9%), succinate (22.8%), urea (26.0%), or nitrate (22.2%) to medium N (24.8%) did not significantly change the recovery. The recovery in media with soluble starch or cellobiose did continue to increase so that in each medium recovery was 27% of the microscopic count after 7 weeks. This recovery was not significantly different from the 7-week recovery for medium N.

The single addition of maltose (14.5%), glucose (18.5%), arabinose (20.4%), lactate (18.5%), and pyruvate (6.3%) significantly reduced the recovery. Interestingly, our preliminary data indicate that glucose, arabinose, and xylose are major sugars found in swine waste and are degraded during the digestion. Two of these (glucose and arabinose) reduce the recovery.

Deletion of Trypticase, yeast extract, volatile acids, hemin, and minerals did not significantly affect the recovery of organisms with medium 11. Deleting Trypticase, yeast extract, volatile acids, or hemin in medium N similarly did not affect the recoveries. The means of duplicate experiments were 24.5, 27.4, 24.5, and 25.3%, respectively. Deletion of Trypticase resulted in very small colonies. These colonies were at the limits of visibility. Only the experience of previous counts made it possible to distinguish such forms from debris in the medium. Because Trypticase increases colony size, it is an important component in the medium.

Deletion of minerals significantly reduced the recovery. Increasing the mineral concentration 3-fold or 10-fold (11.25 or 37.50 ml of the stock solution shown in Table 1 per 100 ml of medium) did not significantly change the percent recovery; however, increasing the concentrations of the minerals did decrease the time for development of the colonies. With the higher concentrations, 92% of the 7-week count was found after 2 weeks.

Increasing the concentration of Trypticase 2.5- or 5.0-fold, yeast extract 5- or 10-fold, volatile acids 5- or 10-fold, or hemin 2.5- or 10-fold did not significantly alter the recoveries (25.1, 21.4, 25.6, 23.6, 21.1, 22.1, 24.8, and 26.7%, respectively).

Replacement of Trypticase with Thiotone, Phytone, Casamino Acids, malt extract, or Gelysate did not significantly change the recovery. The mean recoveries in duplicate experiments were 24.3, 22.3, 27.6, 25.9, 21.9, and 23.6%, respectively.

Buffer and buffer concentration. Deletion of the buffer from medium N significantly reduced the recovery (Table 3). Replacement of the Na_2CO_3 with a phosphate buffer also significantly reduced the recovery. The phosphate buffer (pH 6.9) was added to the medium at a concentration of 0.4% (0.03 M) under N_2. Sodium carbonate in medium N is 0.4% (0.038 M). The difference in counts with the phosphate and carbonate buffer might have been due to the

TABLE 3. *Effect of buffer and buffer concentration in medium N on recovery of bacteria from a swine waste digester*

Buffer	Concn (%, wt/vol)	Recovery (%)			Statistical significance[b]
		Expt[a]		Mean	
		1	2		
None[c]	0	8.5	5.8	7.2	a
Phosphate	0.4	1.8		1.8	b
Na_2CO_3	0.4	25.0	25.2	25.1	c
Na_2CO_3	1.2	33.4	30.0	31.7	d
Na_2CO_3	2.0	16.5	24.1	20.3	e

[a] Mean colony counts of five tubes, after 4 weeks of incubation, divided by microscopic count.

[b] Means not followed by the same letter are significantly different at the 5% level of probability.

[c] Medium N.

differences in the gas phase, since many anaerobic bacteria require CO_2 for growth.

Increasing the Na_2CO_3 in the medium from 0.4 to 1.2% significantly increased the recovery (from 25.1 to 31.7%). A further increase to 2% reduced the recovery to 20.3%. The intermediate level of Na_2CO_3 (1.2%) is six times higher than that normally used with a 1:1 CO_2-H_2 gas phase. The medium that gave the best recoveries approximated the alkalinity of the digester. Bryant and Robinson (10) found that 0.1% Na_2CO_3 was inferior to 0.2% with a 50% CO_2 gas phase in studies with rumen organisms; increasing the concentration to 0.3% did not change the counts.

The results in this study could have been due to the final buffering capacity or ionic strength. No attempt was made to distinguish between these possibilities.

Type and concentration of reducing agent. The combined reducing agent cysteine-sodium sulfide was replaced with cysteine, sodium sulfide, sodium thioglycolate, ascorbic acid, or Cleland's reagent (13) in medium N. Inclusion of sodium sulfide, sodium thioglycolate, ascorbic acid, or Cleland's reagent significantly reduced the percent recovery of bacteria from the swine waste digester (Table 4). There was no significant difference between the mean recovery with cysteine and that with cysteine-sulfide, although cysteine gave significantly higher recoveries during one experiment (the third).

Deleting the reducing agent from the medium significantly reduced the recovery from 22.5 to 13.7% (means of duplicate experiments). Increasing the cysteine to 0.15 or 0.25% did not significantly alter the recovery (24.2 and 21.3%, respectively); however, a further increase (to 0.5%) reduced the recovery from 25.5 to 16.8%. Bryant and Robinson (10) found that doubling the concentration of cysteine-sulfide significantly reduced the counts, but holding the sulfide constant and increasing the cysteine had no effect on recovery of rumen bacteria. Similar results obtained in this study suggest that the higher sulfide concentrations were toxic.

Concentration of DF. Increasing the levels of DF in medium 11 did not change the recovery of bacteria from the swine waste digester. Increasing the level of DF in medium N increased the recovery (Table 5). Forty-five percent DF gave significantly higher recoveries than 15%. Recoveries were consistently higher with 75% DF than with 45%, but the difference was not significant. The medium with 75% DF was very dark and cloudy, making counting and picking of colonies extremely difficult. These results are very similar to those of rumen studies in which counts were significantly higher with 40% rumen fluid than with 20% but not 60% rumen fluid (10). Changes in levels of crude extract did not affect recoveries from sludge or swine feces (28, 35).

The loss of heat-labile growth factors in the DF was tested by filter sterilizing the clarified DF through a 0.2-μm-pore size membrane filter. Replacement of 15% DF with 15% filter-sterilized DF significantly increased the recovery in three separate experiments. The mean recovery with 15% filter-sterilized DF (30.1%) was equal to the mean with 45% heat-sterilized DF (31.2%).

Minimal media. Individual deletions of

TABLE 4. *Effect of reducing agent in medium N on recovery of bacteria from a swine waste digester*

Reducing agent[a]	Expt	Recovery (%)[b]	Mean	Statistical significance[c]
Cysteine	1	23.6	26.3	a
	2	26.7		
	3	29.9		
	4	24.8		
Cysteine-sodium sulfide[d]	1	24.8	24.7	a
	2	24.9		
	3	23.8		
	4	25.3		
Sodium sulfide	1	2.0	2.0	b
Sodium thioglycolate	1	16.4	16.4	c
Ascorbic acid	1	16.3	16.3	c
Cleland's reagent	1	17.5	17.5	c

[a] Reducing agents were added to medium N at 0.05% (wt/vol) after deletion of cysteine-sodium sulfide.

[b] Mean colony counts of five tubes, after 4 weeks of incubation, divided by microscopic clump count.

[c] Means not followed by the same letter are significantly different at the 5% level of probability.

[d] Medium N.

TABLE 5. *Effect of concentration of DF in medium N on recovery of bacteria from a swine waste digester*

DF concn (%, vol/vol)	Recovery (%)				Statistical significance[b]
	Expt[a]			Mean	
	1	2	3		
15[c] A[d]	22.0	25.0	25.2	24.1	a
15 F	33.3	28.3	28.6	30.1	b
45 A	27.2	33.9	32.4	31.2	b
75 A	29.5	36.0	34.3	33.0	b

[a] Mean colony counts of five tubes, after 4 weeks of incubation, divided by microscopic clump count.

[b] Means not followed by the same letter are significantly different at the 5% level of probability.

[c] Medium N.

[d] A, Sterilized in autoclave at 15 lb/in^2 (1.05 kg/cm^2) for 20 min; F, sterilized by passage through a 0.22-μm filter.

Trypticase, yeast extract, volatile acids, and hemin did not affect the recovery of bacteria from the digester. However, deletion of all of these factors at one time was not examined, and the above results suggest that a medium with only minerals, reducing agent, buffer, and DF would culture the digester organisms.

Simultaneous deletion of Trypticase, yeast extract, volatile acids, and hemin from medium N did not affect the percent recovery (23.8% compared to 22.6%). Further deletion of minerals significantly reduced the recovery from 22.6 to 16.4%. Increasing the concentration of the DF from 15 to 85% in a medium with minerals, reducing agent, and Na_2CO_3 did not change the recovery (21.8%) as might have been expected; in medium N, increasing the level of DF had increased the recovery. Deletion of minerals from the medium with 15% DF, reducing agents, and buffer significantly reduced the recovery, whereas deletion of minerals from a similar medium with 85% DF did not (20.3% recovery). Deletion of all components except the DF and agar significantly reduced the recovery to 14.2 and 14.9%, respectively, in media with 85 and 100% DF. DF alone is not sufficient for adequate recovery of bacteria from a swine waste digester; reducing agent and Na_2CO_3 must be added.

The colonies in these minimal media were very small, similar to those in medium N without Trypticase. When the levels of DF were increased, counting became very difficult. Because of this difficulty and because other media gave significantly higher recoveries, no further work was done with the minimal media.

Gas phase. Medium N with a 1:1 CO_2-H_2 gas phase gave significantly higher recovery (24.8%) than medium N with 100% CO_2 (14.9%), 100% N_2 (20.2%), 1:9 CO_2-N_2 (18.6%), or 100% hydrogen (18.1%). No attempt was made to adjust either the ionic strength or the buffering capacity of the media. The results could be a reflection of the changes in these components rather than the gases. There was no difference in colony count with different gas mixtures containing CO_2 with samples from the rumen or swine feces (10, 35). Salanitro et al. (35) did find a reduction in anaerobic count when N_2 was substituted for the CO_2 gas phase.

Dispersal of cells. Blending increased the recovery of bacteria significantly, from 13.3 to 22.7%, a 1.7-fold increase over the recovery with shaking. The recovery did not continue to increase after blending for 10 to 30 s, and it did not decrease after blending for 4 min. However, there was much variation in the counts of samples taken after the shorter blending times, indicating that dispersion was not complete. Therefore, the standard blending time of 1 min was adequate. In another experiment, swine waste was treated by (i) shaking in dilution solution, (ii) shaking in dilution solution with Tween 80, and (iii) blending. The respective recoveries in medium N were 16.5, 19.3, and 24.4%. Recoveries were higher with the Tween 80 treatment than with shaking but significantly lower than with blending.

Agar concentration. Figure 1 shows the effect of agar concentration in medium N on recovery of bacteria from a swine waste digester. Decreasing the agar concentration from 2.5 to 1% dramatically increased the recovery from

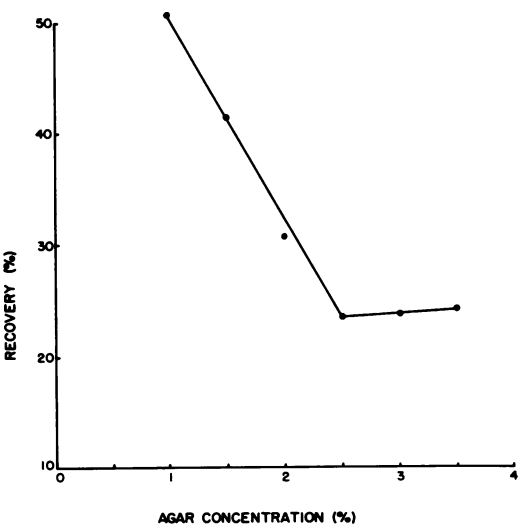

FIG. 1. *Effect of agar concentration in medium N on the recovery of bacteria from a swine waste digester.*

23.8 to 49.6%. These results were repeated as a unit three times and at individual points several times. Because of the slippage in the roll tubes with 1% agar, 1.5% agar is the lowest percentage that can be used effectively. The increased recovery was due to smaller colonies that took 3 to 4 weeks before they could be seen. These were not found in the tubes with high concentrations of agar. In the medium with 1.5% agar, only 76% of the 4-week counts were seen by week 2, whereas 91% were seen by 2 weeks in the medium with 3.5% agar.

Use of different lots of agar did not result in differences in recovery. Purified agar in medium N gave results almost identical to those with regular agar. Medium N was also prepared with silica gel (42). Increasing the concentration of silica gel decreased the colony count; however, the average count with silica gel was approximately one-third that with agar. The decrease in colony counts with increasing concentrations of silica gel could have been due to an effect in common with agar; however, phosphoric acid is added in increasing concentrations to bring about gelling of the silica, and a medium with phosphate buffer was shown previously to decrease counts.

Composite medium. During the above work, it was found that in medium N, Trypticase was needed for increased colony size, that increasing the minerals decreased the time for colony development, and that use of 1.2% sodium carbonate, 45% DF, filter-sterilized DF, and reduced agar increased the percent recovery. However, the effect all these modifications in one medium was not known.

So that fewer combinations of medium components need be tested, the assumption was made that the best medium contained Trypticase (0.2%), resazurin (0.0001%), filter-sterilized DF (45%), minerals (11.25%), cysteine (0.05%), agar (1.5%), and Na_2CO_3 (0.8%). Use of 0.8% Na_2CO_3 in combination with 45% DF would result in approximately the same buffering capacity as that in the digester. The changes in percent recovery of bacteria with modifications of this medium are given in Table 6. This medium and all modifications of it gave recoveries significantly greater than that in medium N (23.7%). In contrast to the results with medium N, adding the DF before autoclaving significantly increased the recovery (40.4%) over that with filter-sterilized DF (34.1%). The addition of 45% rather than 15% DF may have increased the level of heat-labile substance(s), whereas filter sterilization removed required particulate matter. Perhaps the addition of nonclarified DF would further increase the recovery. Bryant and Robinson (10), unlike Grubb and Dehority (18), found no difference in clarified and nonclarified rumen fluid for the recovery of rumen bacteria. The addition of nonclarified DF would obscure many of the small colonies.

There was little difference in recovery with 0.8% (40.4%) and 1.2% Na_2CO_3 (38.3%). Increasing the agar concentration from 1.5 to 2.0% significantly reduced the recovery from 40.4 to 33.6%. Decreasing the minerals from 11.25 to 3.75% (vol/vol) significantly increased the recovery in media with the DF either filter or heat sterilized.

The composition of the best medium (medium 174) is given in Table 1. The 60.2% recovery in this medium is significantly greater than those in all other media tested. This recovery is good for an anaerobic system. Recoveries of 50 to 90% of the microscopic count have been obtained with animal and human feces (16, 31, 35). The

TABLE 6. *Comparison of media incorporating factors previously found to increase recoveries of bacteria from a swine waste digester*[a]

DF (vol/vol)	Concn (%)			Recovery (%)				Statistical significance[d]
	Minerals[b] (vol/vol)	Sodium carbonate (wt/vol)	Agar (wt/vol)	Expt[c] 1	Expt[c] 2	Expt[c] 3	Mean	
45 A[e]	11.25	0.8	1.5	37.3	44.8	39.3	40.4	a
45 F	11.25	0.8	1.5	30.1	38.3	33.8	34.1	b
45 A	11.25	1.2	1.5	36.9	38.9	39.2	38.3	a
45 A	11.25	0.8	2.0	37.6	38.0	25.2	33.6	b
45 F	3.75	0.8	1.5	41.5	43.4	39.9	41.6	a
45 A	3.75	0.8	1.5	62.8	59.5	58.3	60.2	c
15 A	3.75	0.4	2.5	22.0	24.1	24.9	23.7	d

[a] All media also contained Trypticase (0.2%), resazurin (0.0001%), and cysteine-HCl (0.05%).
[b] See Table 1.
[c] Mean colony counts of five tubes, after 4 weeks of incubation, divided by microscopic clump count.
[d] Means not followed by the same letter are significantly different at the 5% level of probability.
[e] A, Sterilized in autoclave at 15 lb/in^2 (1.05 kg/cm^2) for 20 min; F, sterilized by passage through a 0.22-μm filter.

higher recoveries may be due to the nonfastidious nature of fecal organisms (16). The recoveries of bacteria from chicken (5, 6, 34, 36) and mouse cecum (2) were 20 to 46%. Recoveries have been 10 to 30% with ruminants on complex diets; higher recoveries have been observed with ruminants on high-grain diets (8, 24). Less than 10% of the bacteria seen in domestic sewage digesters have been cultured (27, 28).

The recoveries in medium 174 might be even higher if the agar concentration could be reduced or the agar replaced with a solidifying agent that does not reduce the recoveries. The full range of concentrations of agar was not examined in medium 174; however, if it is assumed that the slopes (Fig. 1) of the lines in a plot of agar concentration versus recoveries are the same for medium N and medium 174, a line drawn through 60% recovery at a 1.5% agar concentration and extrapolated back to 0% agar would reach the Y axis at approximately 100% recovery. The composite media in Table 6 demonstrated the same increase in recovery with decreasing agar concentration as medium N (Fig. 1).

Comparison of medium 174 with the other anaerobic media. Figure 2 compares the rates of colony development and the recoveries in medium 174 with those in other anaerobic media. Medium 174 gave significantly greater recoveries of bacteria from our swine waste digester than all other media. This medium recovered 2.5 times as many organisms as the next best medium, medium N, which was previously used to monitor the numbers of bacteria in a swine waste digester (Fischer et al., Am. Soc. Agric. Eng., Paper MC-77-604, 1977). Medium 2, which also has been used in studies of swine waste digestion (21), gave less than 20% of the recovery of medium 174. Medium 2 contains high levels of proteins, lactate, glucose, and maltose. High concentrations of nutrients and the latter compounds have been shown to suppress colony formation and are probably the reasons for the low yields in medium 2. Medium 11 has been used for enumeration and isolation of bacteria from a domestic waste anaerobic digester (27). Medium 10 and medium 98-5 (rumen media) gave similar results. As in medium 11 with rumen fluid (Table 2), the recoveries in medium 98-5 continued to increase throughout the counting period. Although medium 10 and medium 98-5 have been useful for a variety of anaerobic ecosystems (see above), they are not adequate for the swine waste digester. Blood agar has been utilized for isolation of anaerobic bacteria from feces and pathological processes (12, 17, 29, 41, 44), but it supports growth of less than 3% of the organisms in the swine waste digester.

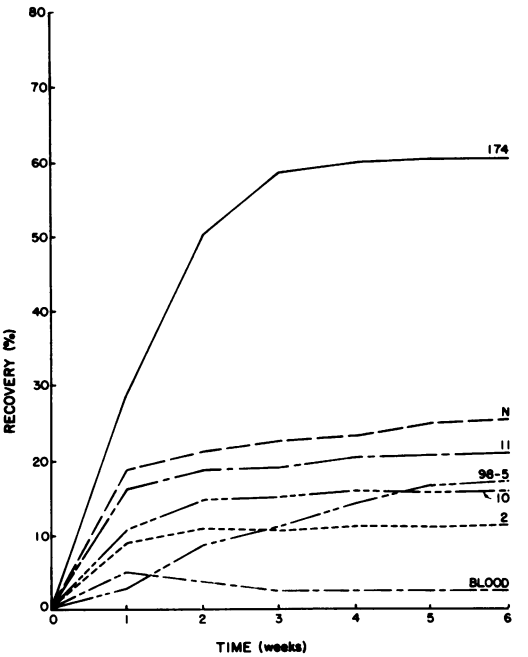

FIG. 2. *Recoveries of bacteria from a swine waste digester with different anaerobic media (174, N, 11, 98-5, 10, 2, and blood agar). The data are the average weekly recoveries from three separate sampling periods.*

Recoveries in medium 174 continued to increase for 4 weeks and then leveled off. Whereas 98% of the 4-week counts were obtained at 3 weeks in the three experiments contributing data for Fig. 2, during monitoring of the model digester and the farm digester (Fischer et al., Am. Soc. Agric. Eng., Paper MC-77-4052, 1977) we found that counts fluctuated more at 3 weeks than at 4 weeks and that during some sampling periods less than 98% of the 4-week counts were found at 3 weeks. Therefore, 4 weeks of incubation is recommended.

The results shown in Fig. 2 indicate that the improvements in recovery are due to slower-growing organisms not found in other media. Except with medium 98-5, there was a relationship between the recovery and the time of maximal colony formation. The long time for development of visible colonies appears to be characteristic of digesters. Mah and Sussman (28) found a 10-fold increase in the number of colonies between weeks 1 and 4. Kirsch (27) found a 60% increase between weeks 1 and 2; longer incubation times were not reported. Most colonies are seen in less than 1 week with fecal and cecal samples (16, 34, 35). The time for development of colonies is slightly longer with rumen samples (10).

Medium 174 is a highly buffered medium with relatively low levels of nutrients. The final pH in the roll tubes after 4 or more weeks of incubation remains near 7.4. The ability to maintain high pH or low levels of microbial end products, e.g., organic acids, is probably one of the most important characteristics of this medium. The media (Fig. 2) with lower recoveries had lower final pH values. Medium N and medium 2 had final pH values near neutrality and below 6.0, respectively. The levels of acids were not measured. If many digester organisms are sensitive to low pH or organic acids, the rapid growth of a few would reduce the pH and increase the levels of end products, reducing the percent recovery. This would explain the lower recoveries with carbohydrates, high levels of nutrients, lower buffer capacity, or readily utilizable substrates such as glucose, arabinose, maltose, lactate, or pyruvate.

Isolation of bacteria with medium 174. Because of the apparent growth inhibition by agar, colonies were picked with a bent needle to medium 174 broths. Growth was detected in only 25% of the tubes after 4 weeks of examination for turbidity or by phase microscopy. The extent of growth in most tubes in which bacteria were detected was very low. The use of a bent Pasteur pipette with slight suction and rinsing with broth (25) did not improve recovery. Reducing the incubation time of the roll tubes from 4 to 3 weeks also did not improve recovery.

Finally, picking the colonies with a bent needle to a slant of 1% agar greatly increased the recovery, with 92% of 150 transfers resulting in detectable growth. Growth was detected by examination of the agar butt under high-intensity light and comparing the inoculated tubes with tubes into which the agar between colonies had been picked. None of the latter tubes showed growth. The average time for first detectable growth was approximately 5 days; some cultures took longer than 3 weeks. More than 50% of the positive tubes had marginal growth. These could be subcultured. The success with the slants was attributed to the confinement of the growth to a small area where it could be detected and to greater viability (7). Changing the gas phase from 1:1 CO_2-H_2 to 100% CO_2 lowered the recovery to 85%.

Enumeration of methane bacteria. Methane was found in roll tubes of the highest dilutions of media 11, N, and 174. Thus, these media supported the growth of methane producers. There was no methane in media with a low final pH.

Medium 174, without agar, was compared with a rumen fluid-based medium (9) for the ability to culture methanogenic bacteria from a farm-size swine waste digester. Rumen fluid media have been used in the enumeration and isolation of rumen, fecal, and anaerobic digester methanogens (15, 32, 33, 37, 38). Table 7 gives the most probable numbers of methanogenic bacteria in four samples of digester effluent. Medium 174 was significantly better than the rumen fluid medium for the enumeration of methanogenic bacteria.

Total population counts were not determined at the same time as the above samples for methane organisms. Because of fluctuations in the farm system, comparisons with previous samples for total numbers of organisms would not have been accurate. However, a single determination indicated that about 50% of the farm digester bacteria were methanogenic. The model digester had from 1 to 5% methanogens (Fischer et al., Am. Soc. Agric. Eng., Paper MC-77-604, 1977) based on determinations made with medium N and rumen fluid medium; medium 174 had not been developed at that time.

Enumeration of aerotolerant bacteria. Medium 174 was compared with standard plate count medium (1) for enumeration of aerotolerant bacteria in the model swine waste digester. There was no significant difference between the two media except that plate count medium required only 48 h of incubation for development of colonies. The mean of eight plates was 4.71×10^6 for medium 174 and 4.98×10^6 for plate count medium. Medium 174 required about 1 week for maximum counts. Plate count medium is the medium of choice.

The mean of three samples for aerotolerant (plate count medium) and anaerobic (medium 174) organisms was 3.72×10^6 and 7.33×10^9, respectively. The aerotolerant population was only 0.05% of the total population. The farm

TABLE 7. *Enumeration of methane bacteria from a swine waste digester in medium 174 and in a rumen fluid-based medium*

Expt	Total no. ($\times 10^6$)/ml[a]	
	Medium 174	Rumen fluid medium[b]
1	460	4
2	1,100	4
3	460	240
4	1,100	240
Mean[c]	780	120

[a] Most-probable-number determination from presence of methane in sets of three culture tubes after 4 weeks of incubation.

[b] Bryant et al. (9).

[c] The means are significantly different at the 5% level of probability.

digester population contained 6% aerotolerant organisms (Fischer et al., Am. Soc. Agric. Eng., Paper MC-77-4052, 1977). Hobson and Shaw (21) reported that 79% of their isolates grew in the presence of air. These isolates were obtained with medium 2. If the recoveries with medium 2 in their system were as low as those in our system, this high percentage of aerotolerant organisms may be due to the failure to grow many of the anaerobic organisms.

Conclusions. Medium 174 permits the enumeration and isolation of a sizeable portion of the bacterial population found in the swine waste digester. The high recoveries with this medium increase the possibility that future studies with pure cultures of the isolates actually reflect the mixed population in the digester. More research should be done with other types of anaerobic digesters, but similarities in the digesters suggest that media similar to medium 174 might improve the low recoveries of 1 to 10% reported to date (27, 28).

The failure of media developed for other ecosystems to culture the majority of bacteria in the swine waste digester and the modifications necessary to increase the recoveries indicate that the bacteria from the swine waste digester have different requirements than the bacteria from the other ecosystems. Preliminary studies with pure cultures confirm this concept.

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