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Interactions Among Cellulolytic Bacteria from an Anaerobic Digester

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Abstract. High cellulolytic activity of particular strains did not cause dominance of one, or a few, species of fiber-digesting bacteria in a cattle-waste anaerobic digester. The population contained a large number of species and varieties with different cellulolytic and fiber-digesting activities. Although mixed cultures of some of these bacteria showed no intereffects, with others, cellulolysis was less or in some cases greater than that shown by individual components of the cultures. The interactions were probably related to effects on growth of the bacteria rather than on activities of components of the cellulase enzyme complex, and culture filtrates of two of the more numerous cellulolytic species of *Clostridium* affected growth of other cellulolytic bacteria. The inhibitory factor(s) appeared to be of bacteriocin type, but the stimulatory factor(s) was unknown. It was suggested that these interactions are localized or short-lived in the digester, and so the population remains in a "dynamic" steady state.

Some inhibitions of growth of rumen cellulolytic bacteria were caused by the digester bacteria, but it was suggested that factors other than these inhibitions are responsible for the absence of rumen bacteria from anaerobic digesters.

Introduction

An anaerobic digester is to some extent a "natural" microbial habitat in that the bacterial population is not artificially selected and the system is subject to inoculation and contamination from the surrounding environment. In this way it is similar to the rumen, water courses, muds, and soils. It is also similar to these systems in that the feedstock is not pure chemical compounds but a heterogeneous mixture of materials, and it involves not one reaction, as in an industrial fermentor, but a number of linked reactions carried out by a heterogeneous population of bacteria, which form groups taking part in different steps in the overall process. On the other hand, the digester is an "artificial" microbial habitat in that the bacteria are contained in a temperature-controlled, stirred-tank, continuous culture of defined retention time. However, although some major parameters of the digester operation are controlled, the system as

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a whole is self-regulating in terms of pH and balance of bacteria in the initi and steady-state populations.

Although the overall reactions in anaerobic digsters have been well docu mented, comparatively little is known about the bacteria involved, except fc the methanogenic bacteria. Much work has been done on these in recent year but isolations have tended to be made from different digesters, and no overa investigation of the types of bacteria and population dynamics in a singl digester has been done.

In sewage-sludge and farm-animal excreta digesters, the first reaction in th series leading to biogas production is hydrolysis of solids. This, particularly i farm-waste digesters, is the rate-limiting reaction of the whole process. Th long retention times required (12 or 15–25 or 30 days) for optimum gas pro duction in such digesters are directly related to the slow degradation of th animal feed residues, which form much of the feedstock solids [8, 10].

These solids are largely lignified cellulolic-hemicellulosic particles, the resi dues of vegetable feedstuffs which have resisted breakdown by digestive trac enzymes and microorganisms in the farm animal. Some observations hav been made on cellulolytic and hemicellulolytic bacterial populations in pig waste digesters [13], but of more interest is the cattle-waste digester and it comparison to the rumen. Rumen bacteria are found in the cattle cecum anc feces and in the saliva and the air surrounding the animals, and they are readily transferred between confined or grazing cattle or wild ruminants. It might be expected, given these sources of inocula, that rumen bacteria would continue to play a part in fiber degradation and fermentation in the anaerobic digester population. On the other hand, rumen cellulolytic bacteria inoculated in large numbers into a digester treating pig waste and straw survived only transitorily [19], and rumen bacteria were not reported in studies on cellulolytic bacteria in pig- or cattle-waste digesters [13, 17]. In this paper, some observations on the fiber-digesting bacteria of a cattle-waste digester and their interactions are described.

Materials and Methods

The Digester

The digester was a 150 l, stainless-steel vessel, fed with diluted waste (ca. 7% total solids) from dairy cattle fed on silage and concentrates. Digester feed input was approximately every 20 min from a stirred feed tank, and the digester tank was continuously mechanically stirred. The temperature was maintained at 35°C by a water jacket. The digester had been previously used with poultry and fattening-cattle wastes [1, 11], and prior to the present experiments had been running for 247 weeks on the dairy-cattle waste. At the time of sampling, the digester was stabilized at a retention time of 21 days, with acetic acid at about 300 mg l^{-1} and the only residual acid, and biogas production of 62% CH_4.

Bacterial Isolations

Samples were taken at the same time on 11 days, not all consecutive, and roll-tube dilution cultures were made in anaerobic media containing either

straw treated with NaOH (1% w/v); or ground, acid-treated filter paper (0.6% w/v). Colonies from cleared zones were subcultured and purified by dilution in similar media containing cellobiose (0.2%) as energy source. Details of the isolation medium, which contained centrifuged digester fluid, vitamins, and volatile fatty acids as growth factors, are given [17]. Cultures were incubated for 21 days at 39°C.

Cellulolytic activities were determined by the extent of disintegration of filter paper strips, based on the method of Mann [16], and by loss in weight of filter-paper powder or untreated-straw powder, in cultures under standard conditions based on the method of Updegraff [21], but with residual fibers determined gravimetrically, as described in [17]. A known amount, about 25 mg paper powder or 50 mg straw, was included in each 10 ml culture, and the mean results of triplicate cultures were recorded.

Isolates were identified by standard tests for anaerobic bacteria based primarily on those in The Anaerobe Laboratory Manual [20]. Full details of all media and tests are given in reference [17] and V. K. Sharma (1983) Isolation and characterisation of cellulolytic bacteria from a cattle-waste digester. PhD thesis, Aberdeen University.

Interaction Tests

Filter-strip disintegration was tested in 10 ml cultures inoculated with the same volume (0.1 ml) of one or more overnight cultures of the bacteria in a medium with cellobiose as substrate.

Culture filtrate effects were tested with bacteria grown in PYG or in GMB medium [20]. Both media contained glucose as energy source, but the GMB medium had only ammonia as N source. One culture was grown for 10 days, and the cells were removed by filtration through fritted glass, P 1.6. A portion (0.1 or 0.5 ml) of the cell-free filtrate was added, to give a total of 5 ml, to the same medium containing a 0.1 ml inoculum of an overnight culture of another bacterium. This inoculum provided a tenfold higher concentration of cells (10^6–10^7 cfu ml^{-1}) than that recommended for testing minimum inhibitory concentrations of antibiotics [23], and the number was similar to the counts of cellulolytic bacteria in the digester. The culture was incubated and growth was compared by optical density with that of a control culture with no added filtrate. The sterility of the cell-free filtrate was confirmed by checking that no growth occurred in media inoculated with filtrate alone.

Results and Discussion

Cellulolysis by Pure Cultures

In the isolation and counting of cellulolytic bacteria from the digester, zones of cellulolysis often spread to encompass a number of adjacent colonies and made exact counts difficult. Thus, the highest tenfold dilution cultures showing cellulolysis were recorded. On this basis, the counts showed a fairly steady population of cellulolytic bacteria over the 15 days of sampling. There was a greater tendency for lower counts of bacteria to degrade untreated straw than to degrade the more available forms of cellulose (Table 1).

However, when isolates were tested for cellulolysis by ability to grow on and degrade filter paper, it was evident that the digester population contained strains with a wide spectrum of cellulolytic activities (Table 2). There seemed to be no relation between morphology or isolation medium and cellulolytic activity determined by this method, which was used as a rapid method of screening many isolates.

All the isolates scoring 3, 4, or 5 on the filter-strip activity scale and 2 isolates

Table 1. Dilution counts of cellulolytic digester bacteria on different days and with different substrates

Medium	Day of isolation and highest dilution showing cellulolysis										
	1	2	3	4[b]	5	6	7	8	9[b]	10	11
US	6[a]	5	4	5	4	5	6	4	5	5	5
TS	6	6	6	6	6	5	6	6	5	5	5
C	5	6	6	6	6	6	6	6	5	5	5

[a] Dilution, n = 10^n
[b] Samples taken on successive days except for where a weekend intervened
US, untreated straw medium; TS, NaOH-treated straw; C, filter-paper powder. Cultures incubated for 21 days at 39°C

scoring 2 (Table 2) were tested for ability to degrade untreated straw. In this case, xylanolytic activity as well as cellulolytic activity might be involved, but almost all the isolates, when later tested in detail for classification, showed some ability to ferment a commercial xylan.

The maximum straw degradation by the isolates was 45–50%, which is the limit of degradation of this straw in an animal-waste digester at infinite retention time [7], but there was a wide variation in straw degradation. Thirty-eight isolates produced less than 10% loss in weight of straw (8 with 0 or 1%), 16 gave 10–20%, 21 gave 20–30%, 46 gave 30–40%, and 9 gave over 40% loss [17]. Variation in straw degradation was also found in isolates with the same filter-strip activity, but there seemed to be some relationship between degradation and filter-strip activity. Isolates that scored 5 on the activity scale degraded between 22 and 46% of the straw, with one isolate giving 14% and one 50% and a mean of 34.5% degradation. Straw degradation by isolates that scored 4 were between 0 and 44% with a mean of 23.8%. Straw degradations of isolates that scored 3 were between 0 and 40%, with a mean of 15.6%, and the two isolates that scored 2 gave straw degradations of 3 and 21%, with a mean of 12.0%.

General examination of all isolates, followed by detailed testing of representative strains, suggested that there were at least 12 groups that could be identified as known genera or species or for which new genera or species assignments could be suggested. Of these, *Clostridium* spp. seemed to be in the majority, but in each group cellulolytic activities varied widely [17].

These results suggested that the fiber-digesting population in anaerobic digesters is diverse in type and activity, and this accords with the results of Hobson and Shaw [13], who isolated 11 types of cellulolytic bacteria from pig-waste digesters, and those of Maki [15] who isolated 10 cellulolytic rods from a sewage digester, although none of these bacteria was studied in detail. This situation is unlike that in the rumen where three types (*Bacteroides succinogenes, Ruminococcus albus,* and *R. flavefaciens*), with a possible fourth (*Butyrivibrio fibrisolvens*), are the predominant cellulolytic bacteria. Other cellulolytic bacteria have been isolated from rumens, but only very occasionally. Clostridia, cellulolytic or noncellulolytic, have rarely been reported in the rumen. The present digester had been running for almost 5 years on cattle waste, and the digesters

Table 2. Distribution of filter-paper degrading cellulolytic activity among isolates of cellulolytic bacteria

Filter-strip activity[a]	Number of isolates with activity Day of isolation 1	2	3	4	5	6	7	8	9	10	11	% of total isolates[b]
5	2	1	3	0	0	0	4	4	1	4	0	5.2
4	3	6	2	1	1	2	13	3	5	3	1	10.9
3	2	7	4	4	7	7	13	6	3	7	9	18.8
2	5	10	3	5	6	3	13	15	18	14	13	28.6
1	6	7	6	6	9	3	21	15	11	16	16	31.6
0	0	2	1	1	0	0	11	0	0	0	3	4.9

Results from triplicate cultures incubated for 21 days at 39°C
[a] Scale for visual disintegration of filter strip: 5 = complete disintegration; 0 = no disintegration
Data from Sharma and Hobson [17]
[b] Number of isolates with degree of activity as given in first column, as % of the total (367)

of Hobson and Shaw [13] had been running for a long period on pig waste. So in each case one would have thought that any dominant species would have attained an equilibrium population. The suggestion is that the digester bacteria attain a "dynamic" steady state in which no species becomes dominant. Cellulolytic activity *per se* does not produce dominance. Although the isolates differed in their rate of attack on the various cellulosic substrates, as shown by the extent of degradation produced in a fixed culture period, their maximum rates of growth on easily available substrates were much higher than their overall growth rate in the digester and comparable with rumen bacteria. It could be suggested that the rate of fiber degradation in the digester is determined by the lignified structure of the fibers and that the amounts of cellulase produced by all the bacteria are sufficient to saturate the few substrate sites available, so variations in cellulase production are not involved in survival of the bacteria. Thus, some interactions among the cellulolytic flora were investigated.

Cellulolysis by Mixed Cultures

Some experiments on the interactions of rumen bacteria with cellulolytic and hemicellulolytic activities have demonstrated increased breakdown of forages by mixed cultures. Other tests have shown interactions between rumen bacteria able to hydrolyze hemicellulose or pectins, but not able to use the hydrolysis products and nonhydrolytic, fermentative bacteria [3–5]. These interactions involve fibers more complex in composition than those in digester feedstocks and mixtures of hydrolytic enzymes or removal of inhibitory hydrolysis products. Volatile fatty acid fermentation products of some bacteria have been shown to be growth factors for other rumen cellulolytic and noncellulolytic bacteria [2].

Although cellulolytic bacteria are found in the digester liquid, when actively metabolizing substrate, they are attached to the surface of fiber particles and

Table 3. Loss in weight of cellulose powder in pure and mixed cultures

Group	Isolate	Wt. loss (%)[a]	Group	Isolate	Wt. loss (%)
1	C49	29.5	6	C458	15.9
	C58	9.7		C453	15.9
	C59	7.6		M	19.9
	M	16.3			
2	C47	13.2	7	C449	19.4
	C48	16.2		C451	24.1
	M	15.8		M	2.5
3	C120	15.6	8	C407	12.5
	C121	13.8		C408	18.0
	C122	7.6		M	26.7
	C123	15.0			
	M	20.5			
4	C124	18.9	9	C459	5.8
	C125	24.0		C448	3.8
	C128	11.7		M	12.1
	M	18.1			
5	C400	23.8	10	C460	12.4
	C401	26.5		C461	4.5
	C402	5.6		C462	12.4
	M	27.3		C463	8.8
				C430	12.4
				C464	5.6
				M	13.2

Isolates in each group were obtained from the same dilution of digester contents taken on 1 day and cultured in a cellulose-powder medium. M, mixture of isolates above
See text for brief description of isolates
[a] Averages of two replicate cultures incubated for 10 days at 39°C

form micro-colonies [9]. In these micro-environments there are more possibilities of interactions through diffusion of excreted materials than there are with bacteria suspended in a liquid medium. In the present case, the digester population contained bacteria with various cellulolytic activities, and so the experiments tested the possibility that the combined actions of the enzymes of some of these bacteria might result in enhanced cellulolysis. It is now well established that the degradation of highly ordered cellulose by aerobic fungi is brought about by a "cellulase" complex of three enzymes (endo- and exo-glucanase and cellobiase). The components of the complex exist in forms of different molecular weight, and synergistic actions can be between components from one, or in some cases, from two or more fungi [24]. The position with bacteria is less clear-cut as comparatively little work has been done on bacterial cellulases. Leatherwood [14] reported a synergistic action between variants of a strain of *Ruminococcus* which apparently had different effects on cellulose agar in that either a clear zone, a hazy zone, or no zone was formed around colonies. Interactions of substances diffusing from colonies without zones or with hazy zones produced clear zones.

In the first tests, 53 mixtures from 111 isolates were tested for filter-strip disintegration. Each mixture contained strains isolated from the same dilution of digester sample taken on a particular day. Of these 53 mixed cultures, 33 showed less cellulolysis than the individual components, or in some cases complete inhibition of cellulolysis. In 18 cultures, cellulolysis was the same as the highest individual cellulolysis, and only in two cultures was cellulolysis enhanced.

Eleven mixtures were then tested for their ability to degrade cellulose powder, a more accurate method of determining cellulolysis. The results from 10 cultures are shown in Table 3. The eleventh mixture contained two strains of bacteria which individually produced 3.5 and 1.7% loss in weight of cellulose, but this gave widely varying results in repeat cultures. So, although the mixture seemed to have enhanced cellulolysis, the results are not included in the table. Group 6, with enhanced cellulolysis, was two gram-positive cocci. Group 8 contained two coccobacilli, one gram-positive the other mainly gram-negative in staining. Group 9 contained a gram-variable coccus and a gram-negative, pleomorphic rod. Group 3 had gram-negative rods with a gram-variable rod. Group 7, showing decreased cellulolysis, contained a strain of gram-positive coccus similar to those in Group 6, and a gram-negative rod. Other groups in these tests were mixtures of rods.

The results of the tests suggested that many of the isolates could grow in co-culture with no apparent interactions. However, cellulolysis by the cocci, which were a minority of the total isolates, was enhanced in cultures of two cocci. On the other hand, some of the rods suppressed cellulolysis by both cocci and other rods. More than one interaction could, of course, have been taking place in some of the mixed cultures. The provision of a suitable mixture of endo- and exo-glucanases in the cellulase complex could enhance cellulolysis. The presence or absence of cellobiose in the complex could enhance or decrease cellulolysis, as cellobiose is inhibitory to cellulolysis. However, all the isolates fermented cellobiose, so this could not have been a factor.

Growth or inhibition factors not connected with the cellulase system seemed most likely to be involved. Growth of the bacteria was difficult to determine quantitatively in the cultures containing cellulose, so the ability of some bacteria to produce extracellular substances affecting growth of other bacteria on glucose, a sugar used by all isolates and a product of cellulose degradation, was tested.

Culture Filtrate Effects

Testing a large number of the isolates for identification showed that the majority of the groups of rods were *Clostridium* spp. Five groups were identified as cellulolytic varieties of known *Clostridium* spp., and *C. butyricum* was a major group [17]. Hobson and Shaw [13] identified noncellulolytic *C. butyricum* as a major group in pig-waste digesters and noncellulolytic butyric acid-forming, sporing rods have been found in digesters by other workers. Maki [15] reported that a butyric acid-producing, sporing rod enhanced cellulolysis in co-culture with one of the cellulolytic rods he isolated from a sewage digester. So, a cellulolytic *C. butyricum* isolate and isolates of *C. sporogenes*, another common

Table 4. Actions of cell-free filtrates of digester clostridia on growth of digester and rumen bacteria

Source of isolation[a]	Genus or group[b]	Isolate tested[c]	Source of filtrate and its effect		
			Inhibition	Stimulation	No effect
a	1	T278			C249
	2	C249		T278	
	2	C346			
	2	C347	C249, C346		T278
	1	C305	C249, C346		
	3	C266		T278, C249	
	4	U311	C249, C346	T278	
	4	U191	C346		
	5	T421	T278, C249		
	6	C440		T278	C249
	6		T278		
	7	C387	T278, C249		
	8	U33		T278, C249	
	9	C245	T278		C249, C346
	10	C456			T278, C249, C346
	11	T365	T278		C249
	12	T111	C249	T278	
	13	U151	C249	T278	
	14	C343		T278, C249	
	15	C56			T278, C249
b	16	GC14			MG6
	16	GC17	MG6		
	17	MC3			MG6
	17	MC8			MG6
c	*Ruminococcus flavefaciens*	F278			C249
	Bacteroides succinogenes	C249			T278
	Butyrivibrio fibrisolvens	T278		C249	
	Butyrivibrio fibrisolvens				T278
	Eubacterium cellulosolvens			C249	

[a] a, digester; b, cotton enrichment culture of digester bacteria; c, rumen
[b] Isolates of 1, *Clostridium butyricum*; 2, *C. sporogenes*; 3, *Clostridium* sp.; 4, *Clostridium* sp.; 5, *C. acetobutylicum*; 6, *C. beijerinckii*; 7, *C. bifermentans*; 8, *Sporolactobacillus* sp.; 9, sporing streptococcus; 10, 11, *Sarcina* spp.; 12–15, unidentified gram-rods; 16, strains of similar gram-, acetate and lactate producing rods; 17, strains of similar gram-, ethanol and acetate producing rods. For details of these cultures see [17], V.K. Sharma, (1983), Isolation and characterisation of cellulolytic bacteria from a cattle-waste digester. PhD thesis, Aberdeen University
[c] Isolate number. MG6, Group 2

cellulolytic *Clostridium* in the digester population, were used as test organisms here.

The results are shown in Table 4. The bacteria used in the tests were representatives of the principal groups of digester isolates, which were identified

as known species or for which new names could be suggested. C266 was one of a group whose properties were determined but could not be classified; the most likely classification was a new species of *Clostridium*. C245, C456, and T365 were representatives of the three groups into which most of the coccal isolates could be placed. T111, U151, C343, and C56 were rods that were not included in groups extensively characterized and so were not named. The tests with T278 and C249 were done first, followed by some tests with C346, so all possible combinations were not tested in section (a) of Table 4. The bacteria in section (b) of Table 4 were from the mixed population of bacteria enriched from a digester liquid inoculum by over 1,000 hours of continuous culture in a medium containing cotton as sole carbon and energy source. GC14 and GC17 were similar acetate- and lactate-producing, gram-negative rods, and MC3 and MC8 were similar ethanol- and acetate-producing rods, but they were not named.

The bacteria of section (c), *Ruminococcus, Bacteroides* and *Butyrivibrio*, were strains of the predominant cellulolytic rumen bacteria. *Eubacterium cellulosolvens* (formerly *Cillobacterium cellulosolvens*) was a strain isolated from a rumen, but the species seldom seems to occur in ruminants.

The results are by no means clear-cut. Although there is a tendency for the two more common species of digester bacteria to inhibit growth of less numerous species, only two out of the three representatives of the minority coccal species were inhibited. Some species were stimulated by the test clostridia, but again, this was not clear-cut; a species could be inhibited by one *Clostridium* and stimulated by the other.

The cellulolytic rumen bacteria most likely to be found in cattle-waste digester feedstock seem to be generally inhibited by the test clostridia, and this may be a factor contributing to their absence from digester floras. However, the results with the *Butyrivibrio* show that, as with the digester bacteria, the effects of the clostridia are dependent on the strain of the other bacterium. The results with C347 suggest that strains of bacteria classified as the same species can have inhibitory interactions.

Maki [15] showed stimulation of cellulolysis only in co-culture of two organisms and did not give a reason for this stimulation. In the present tests the increased or decreased cellulolysis found in mixed cultures seems unlikely to be an effect deriving from a mixing of cellulolytic enzymes; there is a direct effect on growth of the bacteria. The effects on growth do not seem likely to be caused by gross metabolic products. There was no relationship between fermentation products and growth effects, and in a number of cases fermentation products of the two bacteria were similar or the same. Ammonia is unlikely to be implicated as stimulant or depressant of growth, as farm digester liquids usually have quite high ammonia concentrations, and digester bacteria can adapt to ammonia concentrations inhibitory to growth under some circumstances [22]. The effects of T278 were found in some tests in which T278 and the affected bacterium were grown in a medium with excess ammonia as sole nitrogen source. An amino acid analysis showed the culture filtrate of T278 to contain aspartic and glutamic acids, methionine, glycine, histidine, alanine, and lysine. However, the PYG medium used in the main tests contained large amounts of protein hydrolysate and other sources of amino acids, so it would not seem that provision of amino acids was a stimulant to growth.

Whatever the factors involved in these synergystic or antagonistic reactions, they are effective in small amounts, as 10 (or 2%) of the test bacterium culture liquid in the second culture medium produced the effects. Smaller volumes of culture were not tested. The antagonistic agents seem generally to be the same type as bacteriocins, as they are secreted by the cell and can affect other strains of the same species or different species. The nature of the stimulatory agent is not known.

It would seem, though, that inhibitory or stimulatory effects are probably only localized or short-lived in the digester contents. For instance, if proteins are involved, these could be degraded by proteolytic bacteria, many of which appear to be clostridia [13, 18]. A localized effect is suggested because the digester populations continue as mixed floras, and although antagonism was found in the isolates from the continuous culture, that mixed flora had continued to exist after 11 retention times (1,050 hours). Although the mixed flora in a digester exists in a gross "steady-state" this is most likely a dynamic state in which numbers of particular species and strains fluctuate. Serological tests showed changes with time in types in a numerically constant species of bacterium in the rumen [12]. Similar changes probably take place in digester populations due to antagonisms, stimulations, and mutations, together with inoculations from the feed sludge. On this basis, factors other than the antagonisms shown must cause suppression of rumen bacteria. These are nonsporing and tend to lyse during resting phases or very slow growth [6]. The ability to form spores shown by a majority of the cellulolytic digester bacteria could be an advantage in survival in a system of slow degradation of lignified fibers, and possibly in a culture where there are substances antagonistic to growth.

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