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Examination of Methods for Enumerating Hemicellulose-Utilizing Bacteria in the Rumen

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Counts of colonies that developed after 4 days on agar medium containing 0.3% xylan and preincubated rumen fluid were similar to counts of xylanolytic bacteria obtained when total culturable counts were multiplied by the percentage of isolates capable of producing acid from xylan. Shortening the incubation period reduced the chance of including satellite colonies of non-xylanolytic organisms in the count. Nearly all of the xylanolytic isolates irrespective of the medium from which they were isolated degraded and utilized xylan extensively. The use of a culture medium containing a high concentration (3%) of xylan is also described. The number of colonies capable of producing clearings in this medium was less than 10% of the total culturable counts. Isolates from such colonies were shown to produce diffusible (extracellular) xylanases.

Despite the fact that hemicellulose is an important component of pastures and hence of ruminant feeds, few papers have appeared on methods for enumerating rumen bacteria which digest hemicellulose. J. G. de Wet of this laboratory (unpublished data) made counts of hemicellulolytic rumen bacteria by a method analogous to that used for counting cellulolytic bacteria (9, 12), i.e. by counting colonies producing clearings in an opaque medium containing 1 to 3% finely dispersed xylan. He assumed that only those colonies which produced clearings were xylanolytic and favored the medium containing 3% xylan because it gave the best defined clearings. P. N. Hobson and S. O. Mann at the Rowett Research Institute, Scotland, have for some time estimated the numbers of this group of bacteria routinely by counting all of the colonies developing on a 0.3% xylan medium (personal communication to N. O. van Gylswyk). More recently, while this study was in progress, Dehority and Grubb (5) published results on counts of hemicellulolytic rumen bacteria. They found that the specificity of the medium was enhanced by incubating the rumen fluid before including it in the medium.

The present study was designed to evaluate the merits of the above methods by using as a reference an indirect count of xylanolytic bacteria, which involved the testing of isolates from a nonspecific medium for ability to produce acid from xylan. Representative isolates from the various media were examined for differences in their action on xylan.

MATERIALS AND METHODS

Xylan. Xylan was obtained from Fluka A. G., Buchs, Switzerland (catalog no. 95590, batch no. 765331) and contained 72% xylose, 9% arabinose, and 19% glucose (neutral sugars).

Animals and diet. Two of the groups of sheep used and the diets fed were as described by Henning and van der Walt (6). A third group of sheep was fed a diet containing 10% molasses, 55% maize grain, 14% maize stalks, 15% fishmeal, and 6% minerals (half of this was $CaCO_3$).

Sampling and treatment of rumen ingesta. Rumen ingesta were sampled from one sheep on different days and treated as described previously (6).

Culture media. Rumen fluid used in media was collected from fistulated sheep 2 h after feeding, strained through a double layer of cheesecloth, and centrifuged at 1,000 × *g* for 30 min. A portion of the supernatant was incubated anaerobically at 39°C for 10 days before inclusion in a basal culture medium free of added carbohydrate (5, 6) and in a medium with 0.3% xylan as the sole added carbohydrate. The remaining supernatant was frozen and stored for subsequent use in standard culture media (13).

Total culturable counts were made on medium GCSX-2 (6). For xylan-containing media, an aqueous suspension of xylan was treated with a homogenizer (Ultra Turrax, Janke and Kunkel, Staufen i. Br., West Germany) for 90 s and included in media to give a final xylan concentration of 0.3 or 3% (wt/vol). For indirect counts of xylanolytic bacteria, all of the well-separated colonies appearing on GCSX-2 medium in roll bottles with about 20 colonies were isolated and tested for ability to produce acid from xylan in weakly buffered medium containing 0.5% xylan (only added carbohydrate), one-tenth the usual concentration of $NaHCO_3$, and the usual concentration of other constituents. The

gas phase was 88% N_2–10% CO_2–2% H_2. Bottles were incubated for 1 week, and a pH change of more than 0.3 units was taken as an indication of growth. Xylanolytic counts by the direct and indirect methods were made from the same dilution. The indirect counts were calculated by multiplying the percentage of isolates producing acid from xylan by the total culturable counts.

Degradation and utilization of xylan. Isolates were picked from GCSX-2 and 3 and 0.3% xylan media. Five of the cellulolytic strains studied by Dehority (3) (viz. A3c and B21a from B. A. Dehority and S85, C94, and 7 from M. P. Bryant) were included in this experiment. The isolates were inoculated into two bottles, each with 5 ml of well-buffered, 0.3% xylan-containing liquid medium. Total residual pentose was determined on one of the bottles. The other was acidified with acetic acid, and 4 volumes of ethanol was added to precipitate the hemicellulose (3, 11). The supernatant was analyzed for pentose soluble in 80% ethanol (3). The insoluble residue was brought into solution with 10% NaOH before analysis. Hemicellulose was estimated by the measurement of total pentose with the orcinol reaction (1). We calculated the extent of degradation of hemicellulose by the bacteria as the conversion of ethanol-insoluble pentose to an ethanol-soluble form and the utilization of hemicellulose as the loss in total pentose (3).

Xylanase activity in culture supernatant. Eight isolates from colonies that produced clearings in the 3% xylan medium and eight isolates from colonies that did not produce clearings were tested for production of diffusible xylanase. Cultures were grown for 18 h in 5 ml of well-buffered liquid medium containing 0.3% xylan and centrifuged at 27,000 × *g* for 20 min. The supernatant was removed with a 5-ml syringe and transferred to glass-stoppered tubes (purged with 98% CO_2–2% H_2) containing xylan to give a final concentration of 0.5%. Toluene was added to each tube to prevent bacterial growth (7). The tubes were incubated for 96 h. After the toluene layer was removed, the contents were centrifuged at 27,000 × *g* for 20 min. The supernatant was analyzed for soluble pentose. The pellet was dissolved in 10% NaOH and also analyzed for pentose. The amount of xylan solubilized was calculated from the loss of insoluble pentose compared with a control to which no culture supernatant was added. Those supernatant fractions which contained high concentrations of soluble pentose were acidified and separated into fractions which were soluble and insoluble in 80% ethanol. After centrifugation pentose was determined in both fractions.

RESULTS AND DISCUSSION

Table 1 shows that the number of clearings which were produced around some of the colonies after 7 days of incubation on 3% xylan medium inoculated with ingesta from sheep fed lucerne hay was much lower than the counts on 0.3% xylan medium or the indirect xylanolytic counts. This was also the case when the 3% xylan medium was inoculated with ingesta from sheep fed teff hay and concentrated diets. When roll bottles with the 3% xylan medium were incubated for 14 days or longer, the number of clearings increased somewhat, but they tended to merge and counts could no longer be made.

Despite the fact that colony counts on 0.3% xylan medium containing preincubated rumen fluid were lower than those on similar medium with untreated rumen fluid, they were appreciably higher than the indirect counts of xylanolytic bacteria. The background counts showed that the difference was not due to substrates other than the added xylan (Table 1). Roll bottles with 0.3% xylan medium incubated for 7 days also showed clearings around some of the developing colonies. This suggested that such colonies could produce diffusable xylanase and that non-xylanolytic satellite colonies could develop on the hydrolysis products of xylan. Such colonies would be included with the xylanolytic colonies, thus giving a high direct colony count. Colony counts obtained after 4 and 7 days of incubation (Table 2) showed that the 4-day period gave values which were in good agreement with the indirect counts. Colonies which were randomly isolated on these 2 days were tested for their ability to produce acid from xylan, and 23% of those from the day 7 were found to be non-xylanolytic. Hence the 4-day incubation period was adopted for all subsequent work.

From the above results there appear to be two populations of xylanolytic bacteria in the rumen. The first, which constitutes less than 10% of the total number, can produce clearings which are more easily discernible in a medium containing 3% xylan. The second, which cannot do this, includes the majority of the bacteria which can be more readily enumerated on a medium containing 0.3% xylan. To assess the significance of these two groups in the breakdown of hemicellulose in the rumen, the extent of degradation and utilization of xylan by representative isolates from both groups was examined (Table 3). Of the 49 isolates picked from the 3% xylan medium, 14 that produced clearings and 16 which did not produce clearings were selected. Also included in these tests were 16 of the 133 xylan-fermenting isolates from GCSX-2 medium and 15 of the 40 isolates from the 0.3% xylan medium. All of these isolates were selected on the basis of morphological differences. More information on presumptive identification of the isolates and their relative proportions in the rumens of sheep fed the different diets will be presented in a subsequent paper.

The isolates which formed clearings in the 3% xylan medium degraded 72% or more of the xylan (Table 3). Most of the isolates which did not produce clearings, as well as the majority

TABLE 1. *Total culturable, xylanolytic, and background counts of bacteria in rumen ingesta from sheep fed lucerne hay after a 7-day incubation period*

Medium type	Type of counts	No./g of rumen ingesta ($\times 10^8$)	% of total
GCSX-2	Total culturable colonies	41.9 ± 3.3[a]	100
0.3% xylan	Xylanolytic colonies	36.9 ± 3.1	88
0.3% xylan prepared with preincubated rumen fluid	Xylanolytic colonies	32.0 ± 2.5	76
Medium free of added carbohydrate and prepared with preincubated rumen fluid	Background colonies	0.4 ± 0.1	1
3% xylan	Clearings formed by xylanolytic bacteria	3.1 ± 0.3	7
Weakly buffered xylan medium	Xylanolytic bacteria by the indirect method	26.0	62[b]

[a] Mean and standard error of five trials, six bottles per trial.
[b] A total of 95 isolates from medium GCSX-2 were tested for their ability to produce acid from xylan.

TABLE 2. *Effect of incubation period on the number of colonies developing on 0.3% xylan medium inoculated with ingesta from sheep fed lucerne hay*

Method used	Expt no.[a]	Colonies after the following incubation periods: 4 days: No./g of rumen ingesta ($\times 10^8$)	4 days: % Of total[b]	7 days: No./g of rumen ingesta ($\times 10^8$)	7 days: % Of total[b]
Direct[c]	1	33.7	77	43.3	99
	2	23.3	70	31.0	94
	3	56.0	101	64.3	116
	4	55.3	67	76.0	92
	5	36.7	63	49.0	84
	6	29.3	82	34.7	95
	Mean	39.1	77	49.7	97
Indirect[d]			77		77

[a] Nine bottles per experiment.
[b] Total culturable counts were determined on medium GCSX-2 inoculated with the same rumen ingesta samples that were used in experiments 1 to 6.
[c] The medium contained 0.3% xylan and preincubated rumen fluid.
[d] Colonies that developed on medium GCSX-2 were counted and then isolated after 7 days of incubation; 80 isolates were examined.

(more than 80%) of the isolates from GCSX-2 and 0.3% xylan media, also degraded xylan extensively. Examination of those isolates that were poor degraders showed that they generally produced a small amount of acid from xylan. It was therefore not possible to distinguish between the two groups on the basis of xylan degradation.

The majority of the isolates were also capable of utilizing the solubilized pentose, and, except for isolates TF52, LU NCX 9, and AcHC8, the difference between pentose solubilized and utilized was less than 15%. Several of these isolates gave similar results when grown on hemicellulose isolated from teff hay (hemicellulose B; prepared by R. F. H. Dekker, University of Natal, Pietermaritzburg South Africa). The above results are in contrast to those of Dehority (3), who found that the amount of hemicellulose degraded and utilized was similar in only one of eight cellulolytic rumen bacteria. In Table 4 the results of tests on five of these bacteria, using xylan (Fluka), are compared with the results of Dehority (3). Utilization of the solubilized pentose by the ruminococci is apparently influenced by the nature of the hemicellulose, whereas the three strains of *Bacteroides succinogenes* were poor utilizers of all three substrates. *B. succinogenes* partially degrades hemicellulose, but when diluted cultures of strains A3c, B21a, and S85 were inoculated into roll bottles with 0.3% xylan medium, no visible colonies appeared. Hence, this species, and perhaps others which are also poor xylan utilizers, would be excluded from counts of xylanolytic bacteria by this method. Further evidence for the specificity of the 0.3% xylan medium is thus provided by this finding. The method of screening isolates from GCSX-2 medium would also have excluded bacteria of this type, and in a medium with 3% xylan they would not be able to form clearings.

Since differences in the ability of our isolates to degrade and utilize xylan were small, irrespective of whether they produced clearings in the 3% xylan medium, it was considered that such clearings could be produced by freely diffusable xylanases. Table 5 shows that the supernatant from cultures of bacteria producing clearings possessed considerable xylanase activity (71% of xylan solubilized). In contrast, the supernatant from isolates that had not produced clearings solubilized not more than 15% of the

TABLE 3. *Degradation and utilization of xylan by representative isolates of xylanolytic rumen bacteria*

Origin of isolate	Representative selected[a]	% Degradation	% Utilization	Origin of isolate	Representative selected[a]	% Degradation	% Utilization
Colonies producing clearings in 3% xylan medium	AcTF 2[b]	75	75	Colonies from 0.3% xylan medium prepared with preincubated rumen fluid	LU X17 1	73	75
	AcTF 4[b]	93	80		LU X17 2	2	9
	AcTF 6	93	88		LU X17 4	75	75
	AcTF 10[b]	93	88		LU X17 5	61	69
	AcLU 1	86	91		LU X17 6	61	59
	AcLU 2	77	79		LU X17 7	65	69
	AcLU 4	86	83		LU X17 8	86	82
	AcLU 5	86	82		LU X17 9	90	88
	AcLU 9	93	90		LU X17 10	49	53
	AcHC 1	72	67		LU X17 11	77	71
	AcHC 3	88	85		LU X17 12	71	71
	AcHC 7	93	82		LU X17 17	5	3
	AcHC 8	93	38		LU X17 19	65	69
	AcHC 9	97	85		LU X17 20	71	69
					LU X17 21	73	69
Colonies not producing clearings in 3% xylan medium	LU NCX 1	63	61	Colonies from nonspecific (GCSX-2) medium and screened for ability to produce acid from xylan	TF 7[b]	93	90
	LU NCX 2	73	72		TF 8[b]	88	78
	LU NCX 3	77	81		TF 22[b]	73	73
	LU NCX 4	76	76		TF 36[b]	66	72
	LU NCX 5	68	75		TF 41[b]	73	78
	LU NCX 6	78	79		TF 45[b]	87	80
	LU NCX 7	76	77		TF 47[b]	85	82
	LU NCX 8	47	57		TF 52[b]	97	80
	LU NCX 9	88	73		LU 1	74	75
	LU NCX 10	88	86		LU 7	74	74
	LU NCX 11	76	77		LU 15	93	90
	LU NCX 12	18	16		HC 1	9	6
	LU NCX 13	0	0		HC 12	52	52
	LU NCX 14	78	79		HC 34	13	0
	LU NCX 15	0	0		HC 43	89	89
	LU NCX 16	65	68		HC 47	7	0

[a] Abbreviations: TF, ingesta from sheep fed teff hay; LU, ingesta from sheep fed lucerne hay; HC, ingesta from sheep fed a concentrate ration; Ac, colonies which produced clearings in 3% xylan medium; NCX, colonies which did not produce clearings in 3% xylan medium; X17, colonies from 0.3% xylan medium which was prepared with preincubated rumen fluid.

[b] These isolates gave similar results when grown on hemicellulose isolated from teff hay.

TABLE 4. *Degradation and utilization of hemicellulose by pure cultures of cellulolytic rumen bacteria*

Organism	Source of hemicellulose					
	Flax[a]		Corn hull[a]		Xylan[b]	
	% Degradation	% Utilization	% Degradation	% Utilization	% Degradation	% Utilization
B. succinogenes A3c	66	4	41	4	66	23
B. succinogenes B21a	45	4	37	2	56	21
B. succinogenes S85	78	3	44	3	66	14
Ruminococcus flavefaciens C94	99	61	98	55	95	90
Ruminococcus albus 7	99	90	39	9	90	90

[a] These figures are from Dehority (3).

[b] The xylan was from Fluka.

xylan, even in the case of isolates which degraded more than 63% of this substrate in the growth experiment.

In supernatants containing high concentrations of the solubilized xylan, an average of 81% was present as ethanol-soluble pentose (Table 5). This agrees with the findings of other workers (2, 4, 8) in that mono- and oligosaccharides are produced on incubation of hemicellulose with extracellular enzymes.

The finding that less than 10% of the total culturable bacteria have diffusable xylanases and that the majority have bound xylanases may explain the controversy that exists in the litera-

TABLE 5. *Solubilization of xylan by growing cultures and enzymes present in culture supernatants*

Isolates from colonies which did not produce clearings			Isolates from colonies which produced clearings			
Isolate no.	% of xylan degraded by growing cultures[a]	% of xylan solubilized by culture supernatant	Isolate no.	% of xylan degraded by growing cultures[a]	% of xylan solubilized by culture supernatant	Amt of ethanol-soluble pentose (% of water-soluble pentose in culture supernatant)
LU NCX 1	63	15	AcTF 2	75	42	71
LU NCX 3	77	12	AcTF 4	93	75	87
LU NCX 5	68	10	AcTF 10	93	78	91
LU NCX 6	78	14	AcHC 1	72	73	68
LU NCX 10	88	3	AcHC 8	93	84	86
LU NCX 12	18	0	AcHC 9	97	73	85
LU NCX 15	0	0	AcLU 2	77	56	79
LU NCX 16	65	12	AcLU 9	93	82	83
Mean	57	8	Mean	87	71	81

[a] These values are from Table 3.

ture as to the presence of free hemicellulases in the rumen (10). Beveridge and Richards (2) found that extracellular hemicellulase activity in the rumen was at least an order of magnitude less than the activity released on disruption of the rumen microorganisms.

The specificity of the method for counting xylanolytic rumen bacteria in a medium prepared with preincubated rumen fluid and containing 0.3% xylan has been improved by shortening the incubation period. This reduces the chance of including satellite colonies of nonxylanolytic organisms in the count. This method gives values in good agreement with the tedious indirect method. Organisms that degrade xylan but cannot utilize the hydrolysis products are not included in such counts. Since the majority of bacteria so enumerated can degrade and utilize isolated xylan extensively, it provides the best method to date for counting rumen bacteria capable of digesting isolated hemicellulose.

Bacteria which produce clearings in 3% xylan medium, and hence freely diffusable xylanases, degrade and utilize isolated xylan to the same extent as organisms not able to produce clearings. Perhaps such bacteria are more effective in attacking hemicellulose in situ than those only possessing bound hemicellulases. Evaluation of the significance of such bacteria awaits the results of a study of their action on isolated forage cell walls, now in progress in this laboratory.

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LITERATURE CITED

1. **Ashwell, G.** 1957. Determination of pentoses. Methods Enzymol. **3**:87-89.
2. **Beveridge, R. J., and G. N. Richards.** 1973. Digestion of polysaccharide constituents of tropical pasture herbage in the bovine rumen. IV. The hydrolysis of hemicelluloses from spear grass by cell-free enzyme systems from rumen fluid. Carbohydr. Res. **29**:78-87.
3. **Dehority, B. A.** 1965. Degradation and utilization of isolated hemicellulose by pure cultures of cellulolytic rumen bacteria. J. Bacteriol. **89**:1515-1520.
4. **Dehority, B. A.** 1968. Mechanism of isolated hemicellulose and xylan degradation by cellulolytic rumen bacteria. Appl. Microbiol. **16**:781-786.
5. **Dehority, B. A., and J. A. Grubb.** 1976. Basal medium for the selective enumeration of rumen bacteria utilizing specific energy sources. Appl. Environ. Microbiol. **32**: 703-710.
6. **Henning, P. A., and A. E. van der Walt.** 1978. Inclusion of xylan in a medium for the enumeration of total culturable rumen bacteria. Appl. Environ. Microbiol. **35**:1008-1011.
7. **Howard, B. H.** 1955. Ruminal fermentation of pentosan. Biochem. J. **60**:i.
8. **Howard, B. H., G. Jones, and M. R. Purdom.** 1960. The pentosanases of some rumen bacteria. Biochem. J. **74**:173-180.
9. **Kistner, A.** 1960. An improved method for viable counts of bacteria of the ovine rumen which ferment carbohydrates. J. Gen. Microbiol. **23**:565-576.
10. **Morrison, I. M.** 1976. Xylanase (hemicellulase) activity in cell-free rumen fluid. Carbohydr. Res. **47**:129-139.
11. **Myhre, D. V., and F. Smith.** 1960. Constitution of the hemicellulose of alfalfa (*Medicago sativa*). Hydrolysis of hemicellulose and identification of neutral and acidic components. J. Agric. Food Chem. **8**:359-364.
12. **van Gylswyk, N. O.** 1970. A comparison of two techniques for counting cellulolytic rumen bacteria. J. Gen. Microbiol. **60**:191-197.
13. **van Gylswyk, N. O., and J. P. L. Hoffman.** 1970. Characteristics of cellulolytic cillobacteria from the rumens of sheep fed teff (*Eragrostis tef*) hay diets. J. Gen. Microbiol. **60**:381-386.