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Isolation and Cellulolytic Activities of Bacteria from a Cattle Waste Anaerobic Digester and the Properties of Some *Clostridium* Species

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ABSTRACT

Bacteria were isolated from a cattle-waste digester on eleven occasions by dilution culture in solid media containing powdered barley straw, 'delignified' straw or filter-paper. Clearing of the medium around colonies was taken as primary evidence for cellulolytic activity and 390 isolations were made from different samples and dilutions on this basis. Most (367) of the isolates were screened for cellulolytic activity by incubating with filter-paper strips and a range of activities was found. A number (128) of the isolates of higher activities and two isolates of low activity were tested for their ability to degrade straw and again, a range of activities was found. There were only slight correlations between filter-strip disintegration activities, or initial isolation medium, and straw degradation activities.

The results show that a cattle-waste digester contains a heterogeneous cellulolytic bacterial population. Of the eleven groups investigated in detail, five had properties which suggested that the bacteria were strains of the known Clostridium *species* butyricum, beijerinckii, acetobutylicum, bifermentans *and* sporogenes.

INTRODUCTION

The production of biogas in anaerobic digestion is a process carried out by a mixed culture of bacteria degrading some primary substrate to a mixture of methane and carbon dioxide, with the concomitant formation

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of bacterial cells using nitrogen sources present in the digester feedstock. The degradation takes place in a number of steps, promoted by different groups of bacteria. In recent years, much attention has been given to the bacteria and reactions involved in the final steps, the production of methane from fermentation and breakdown products of carbohydrates and lipids in the primary substrates. Less attention has been given to the bacteria responsible for the production of substrates for the methanogenic bacteria.

Except, perhaps, for some factory wastewaters, the primary 'substrate' in digester feedstocks, from which methanogenic substrates are derived, is heterogeneous in composition and it may be dissolved, or in suspension, or a mixture of both. Dissolved substrates are rapidly fermented and the rate-limiting factor in the digestion of such substrates is the inherently slow growth rates of the methanogenic bacteria. Substrates in suspension are broken down and fermented slowly and the breakdown of solids can then become the rate-limiting step in the digestion (Hobson *et al.* 1974).

Amongst the most recalcitrant of suspended digester-feedstock components are the lignified-carbohydrate residues of vegetable matter found in the excreta of farm animals, particularly ruminants, where all easily degradable carbohydrate has been removed by digestive secretions and microbial action in the animal gut. Degradation of this residual cellulose and hemicellulose is the overall rate-limiting reaction in the digestion of animal excreta (Hobson, 1983).

Few isolations of cellulose-digesting bacteria have been made from any anaerobic digesters (Hungate, 1950; Maki, 1954; Hobson & Shaw, 1974). The present paper describes the isolation of cellulolytic bacteria from a cattle-waste digester using media and techniques designed to encourage both the growth of bacteria able to digest prepared cellulose and of natural fibres and to determine the number of such bacteria in the digester over a period of time. The predominant bacteria in the digester were rod with properties which suggested that they could be cellulolytic varieties of five known species of *Clostridium* and these bacteria are described here

METHODS

For all samplings, dilutions, cultures and preparation of media, oxygen was excluded by gassing techniques based on those of Hungate (1950) with gases deoxygenated by passage over heated copper (unless otherwise

stated). Culture and dilution tubes were closed by an open-centre screw cap covering a butyl-rubber septum ('Hungate Tubes', Bellco Glass Inc., Vineland, NJ, USA).

Digester and sampling

The digester consisted of a 150-litre stainless steel, continuously mechanically mixed vessel fed intermittently by a Lina-Flow pump from a stirred feedtank and with a gravity overflow to a stainless steel tank. The interval between 'on' periods of the feed pump was determined by the retention time required and, for 20 day retention, was about 20 min. The whole system, except for the feed vessel, was gas-tight, the biogas being led off via a condensate trap to a gas meter. The digester had been previously used with other feedstocks (Bousfield *et al.* 1979; Hobson *et al.* 1980). Before the present sampling the digester had been running at 35 °C for 247 weeks, and at the time of sampling had been for some weeks at a retention time of 21 days, on the diluted whole waste from dairy cattle fed on silage and concentrates. The feed slurry contained 5·8 % TS (Total Solids) with VS (Volatile Solids) 76 % of the TS. The TS contained 32·7 % cellulose, 15·8 % hemicellulose, 11·8 % lignin, 1·6 % nitrogen and 8·2 % ash. The digester was functioning correctly with only acetic, at about 300 mg litre^{-1}, as the residual acid and producing a gas containing about 62 % CH_4, 33 % CO_2, 4 % N_2, plus traces of H_2S (See Summers & Bousfield (1980) for analytical methods).

Samples of digester contents were obtained in mid-mornings by suction into a sterile McCartney bottle through an 8 mm tube ending about halfway down the digester vessel. The stirring rate of the digester was increased from 50 to 60 rpm before drawing the sample and CO_2 was introduced into the digester to increase the positive pressure of the gas in the headspace. Six preliminary samples were taken to flush out the tube before taking the working sample of 30 ml into a fresh bottle which had been flushed out with CO_2.

Isolation of bacteria

The 30-ml sample from the digester was transferred under CO_2 to 270 ml of dilution fluid in a 500-ml flask, mixed by swirling and poured into a sterile, gassed Waring Blender under an aluminium-foil covering. The gassing jet was withdrawn and the covered contents were blended for 40 s

at room temperature. The gassing jet was reintroduced and 1 ml of this, × 10 dilution transferred to 9 ml of diluent in a tube. The cap was secured and the contents of the tube agitated for some seconds on a 'Whirlimixer' (Fison's Scientific Apparatus, Loughborough, Leicestershire, Great Britain) before higher dilutions were made by transferring 1 ml to further tubes. Portions (0·5 ml), from 10^6 downwards, of serially diluted sample were transferred to 4·5 ml of molten agar medium in tubes and the agar dispersed by mechanically rolling the tubes in cold water. When the agar had solidified the tubes were incubated upright for 3 weeks and examined at intervals. Dilution series on the different substrates were duplicated. Cultures were incubated at 39 °C as a matter of convenience. This temperature is within the region of optimum activity of mesophilic digesters.

Examination of tubes for clearing of the cellulosic substrates was begun at 5–7 days and continued through the 3 weeks. The highest dilution showing a colony with a clear zone was recorded as the number of bacteria present attacking that substrate, as it was not possible to count effectively colonies which often formed big, overlapping, clear zones. Colonies were picked from various dilutions and transferred to liquid cellobiose medium 1. They were then purified by serial dilution in 10^6 in roll tubes of medium 1 with 2·5 % agar to prevent spreading growth of some isolates.

Substrates for media

Untreated straw was barley straw dried at 80 °C overnight, ground to coarse particles and then ball-milled for 48 h. Treated ('delignified') straw was made by adding NaOH at 200 mg g^{-1} to dried straw, adding sufficient water to soak the material and autoclaving at 121 °C for 15 min. The straw was then washed to a neutral pH, squeezed to remove excess water, dried at 80 °C overnight, coarsely ground and ball-milled for 48 h. Filter paper (Whatman No. 1) was used as strips or as powder made by soaking the paper in concentrated HCl at room temperature for 24 h, washing to remove the acid and wet ball-milling for 48 h (Hungate, 1950).

Media

Medium 1 was based on medium 2 of Hobson (1969) and the medium of Scott & Dehority (1965). It contained Bacto Casitone, yeast extract, sodium bicarbonate, minerals I and II cysteine HCl, as in medium 2, plus VFA and vitamins as in Scott and Dehority's medium except that acetic

acid was replaced by DL-2 methylbutyric acid at 8 mg per 100 ml of medium. Each 100 ml of medium also contained resazurin (0·1 mg), agar (2 g) and 20 ml of liquid from the digester which had been centrifuged in a Sharples centrifuge at 25 000 rpm for 20 min, plus 1 g of treated or untreated straw or 600 mg of paper powder. For purification of the isolates the carbohydrate was 0·2 g of cellobiose and the agar was increased to 2·5 g (per 100 ml).

Dilution fluid was as medium 1 above except that the carbohydrate, vitamins, VFA and agar were omitted and 0·01 % Tween 80 was added.

Medium 2 was modified from that of Patel *et al.* (1980). Constituents were as Patel *et al.* except that KH_2PO_4, $CaCl_2$, borate and pyridoxin were omitted, cysteine HCl was increased to 1 g litre^{-1} and the VFA of medium 1 were added.

Medium 3 was modified from that of Mann (1968) by including dithiothreitol (0·01 %) and the VFA and vitamins of medium 1 and 10 % centrifuged digester fluid in place of rumen fluid. A filter-paper strip (0·8 × 9·5 cm) was added to each 10 ml tube of medium but was not weighed and suspended from the bung as described by Mann, and the strip was sterilised with the medium.

All media were adjusted to pH 7–7·2. Sterilisation was at 121 °C for 15 min or by filtration as appropriate.

Maintenance medium was medium 1 with cellulosic substrates replaced by 0·5 % cellobiose and with agar reduced to 0·7 % to give a semi-solid medium. Glycerol (0·5 %) was added as a cryoprotectant and cultures were stored at −20 °C or −50 °C.

Screening for cellulolytic activities

Filter-strip method

Tubes of medium 3 were inoculated with 1 ml of overnight growth in liquid medium 1 with cellobiose and incubated for 21 days. The degree of disintegration of the paper strip was estimated visually on a scale from 1 for slight attack to 5 for complete disintegration of the strip to a fine powder. A standard scale was originally made by stopping a culture at different stages of disintegration of the paper and photographing the tubes.

Loss in weight of cotton or paper powder

A culture grown in medium 2 for 10 days with a known amount of dewaxed cotton or filter paper powder (about 25 mg 10 ml^{-1}) was

transferred to a weighed centrifuge tube and spun at 2000 rpm for 20 mi: The supernatant was removed and 3 ml of a solution of 15 ml 80 % glaci. acetic acid plus 1·5 ml concentrated nitric acid was added to the residue The tube was covered and heated in boiling water for 30 min. Afte cooling and centrifuging, absolute alcohol (6 ml) was added to th sediment which was resuspended and then centrifuged. The alcoh treatment was repeated and the residue dried at 80 °C overnight an weighed (Uppdegraff (1969) modified).

Loss in weight of straw

A culture in medium 2 containing a known weight (about 50 mg 10 ml^{-1} of ground, untreated straw was incubated on a shaker (150 rpm) for 15 days. The culture was transferred to a weighed centrifuge tube and centrifuged at 2000 rpm for 45 min. The supernatant was removed and the sediment washed twice, with centrifuging for 30 min, with a similar volume of distilled water and then dried to constant weight at 80 °C. Medium 2 with cellobiose was used for the inoculum culture for all these determinations and the mean results of triplicate cultures for each isolate recorded.

Identification of clostridia

Media

Cultures were grown anaerobically in sealed tubes under a CO_2.N_2 (20.80) gas mixture unless stated otherwise. Where growth on plated media was possible the plates were prepared in air and incubated in anaerobic jars under 10 % CO_2/90 % H_2 with a cold catalyst. Cultures were incubated at 39 °C The medium constituents and methods of carrying out the tests as described in *The Anaerobe Manual* (1977, except as noted below where some modifications were made.

For fermentation tests and analysis of fermentation products. PY medium. adjusted to pH 7·2, that of the digester, was used. Carbohydrates were usually added in 1 % concentration and fermentation was determined by observation of growth and fall in pH.

For optimum temperature of growth PY medium was used, and for optimum pH. sodium bicarbonate was omitted from PYG medium and oxygen-free nitrogen was used as the gas.

Hydrogen sulphide production was tested in modified SIM medium containing (g or ml $litre^{-1}$): tryptone, 2·0; peptone, 6·1; ferrous ammonium sulphate, 0·1; sodium thiosulphate, 0·2; yeast extract, 4·0;

haemin solution, 10; vitamin K_1, 0·2; sodium ascorbate, 5·0; agar, 3·5; salts solution (as PY) 500; resazurin solution, 4·0: pH adjusted to 7·2. The haemin solution contained 50 mg per 100 ml, dissolved in alkali and autoclave sterilised. Resazurin solution was 25 mg per 100 ml. Fermentable sugar was not added to the medium as acid production may suppress the enzymes involved in H_2S production (Bulmash & Fulton, 1964). Ascorbate was used in place of cysteine to reduce possible H_2S production from sulphur amino acids.

PY glucose medium was used for the heat test. Timing was started when a control tube put into the water bath reached the bath temperature and, after the requisite time, the culture was rapidly cooled and incubated.

For testing aerobic growth the modified medium of Patel *et al.* (1980) was used with 1% glucose and without cysteine. The medium was prepared in air, dispensed into plates, streaked and incubated aerobically.

Analyses

Gas analyses were done by GLC with a Pye Unicam GCV (Pye-Unicam, Cambridge, Great Britain) chromatograph with a column packed with silica gel.

Fermentation products were determined with a Pye 104 gas chromatograph using a column packed with Chromosorb 101 (Carlsson, 1973). The preliminary anion- and cation-exchange resins treatment of Carlsson (1973) was found not to be necessary and samples were acidified to pH 2 with 50% metaphosphoric acid. Methylation of samples for determination of lactic and succinic acids was done by the method in *The Anaerobe Manual* (1977). Acid and alcohol solutions of known concentrations were used to prepare standard curves and as external standards with each set of culture analyses.

Sugars from cellulose hydrolysis were determined by a TLC method using 3 ml of the supernatant from a culture containing ball-milled filter paper which had been incubated for 3 months. Glucose, cellobiose, cellotriose, cello-tetraose, xylose and rhamnose were used as standards. Only spots identified with these standards are mentioned in the 'Results' Section: in some cases other spots were also present.

$G + C$ values were determined essentially by the procedure described by Gibson & Ogden (1979).

Spores were stained by Schaeffer and Fulton's method with 5% aqueous malachite green, or were observed under phase contrast illumination or as unstained objects in Gram-stained films.

RESULTS

Isolation and cellulolytic activities of the bacteria

Dilution cultures were incubated for 21 days as longer incubation did not make any difference to trial counts. The treated straw and cellulose powder media tended to give the highest counts as on 7 of the 11 sampling days cultures from 10^6 dilutions of digester contents showed cellulolytic bacteria while, on the other days, 10^5 dilutions were the highest with cellulolytic bacteria. With untreated straw, two samples gave cellulolytic activity in the 10^6 dilution cultures, six gave activity at 10^5 dilution and in three, 10^4 was the highest dilution showing activity. Since the digester was loaded every 20 min, no large variation in numbers of bacteria with time such as might occur with less frequent loading, would be expected. In preliminary tests dilution fluids with 0·01 % methyl cellulose (Minato & Suto, 1981), Triton X-100 and Tween 80 were used to try to dislodge bacteria from fibres in digester samples. The same results were obtained with each and so Tween 80 was used for the main counts. It was assumed that, even if the total cellulolytic bacteria were not being counted, a constant proportion of them was. Approximately 390 colonies were subcultured from the primary dilutions and 367 were tested for cellulolytic activity. The morphological characteristics of each isolate were recorded.

The isolates were initially divided into eleven groups on the basis of morphology. Later, they were examined in more detail and representative strains taken for full testing for identification. Of these latter, eleven groups were identified with known species or were assigned new species names, and some others were regarded as unidentified. The properties of all these isolates were recorded by Sharma (1983) and some are given in this paper.

The eleven morphological groups and subgroups were, briefly, as follows; most were Gram-negative, a few Gram-variable: (1) Pleomorphic rods up to about 4·5 μm long. (2) Large straight or usually slightly curved rods, mainly with rounded ends, up to about 7 μm long. Large rods with rounded ends producing an extracellular gum. Similar rods not producing gum. (3) Small to medium rods, up to 3·5 μm long. (4) Rods of various lengths occurring mainly in pairs. (5) Medium, thick rods, up to about 4 × 1 μm. Medium, thin rods up to about 0·5 μm wide. (6) Rods in a palisade arrangement. (7) Thin rods, thin rods in chains, filamentous

(5–100 μm) thin rods. (8) Short, pleomorphic rods. (9) Cocco-bacilli. (10) Rods with beaded appearance. (11) Cocci.

Cellulolytic activities

A method of initial testing of isolates for cellulolytic activity was required which did not take a lot of manipulative time but which would give at least a semi-quantitative measure of activities. A number of methods were tested but the cellulose-strip method proved the most reproducible.

Preliminary tests showed that the high organic nitrogen content of isolation medium 1 was not needed for good cellulolysis, so Mann's medium of lower N content was used. The numbers of isolates with different cellulolytic activities are shown in Table 1: there was no discernible pattern of activities of isolates from the different media, so isolation media are not distinguished. There also seemed to be no relationship between morphologies and cellulolytic activities determined by this method. The specific gas production (gas per kilogram of solids fed in the digester) was lower on days 2, 3, 4, 5 and intervening days than the average for days 1 and 6 to 11, but it is difficult to say whether the percentages of isolates with activities 4 and 5 on these days bear any relationship to the gas production in the digester. The numbers of cellulolytic bacteria seemed to have no relationship.

Isolates of activities 3, 4 and 5 (128) and two isolates of activity 2 were

TABLE 1

Numbers of Isolates with Different Cellulolytic Activities

Filter-strip activity[b]	*Day of isolation from digester*[a]											*Per cent of total isolates*[c]
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	
	Number of isolates with particular activity[d]											
5	2	1	3	0	0	0	4	4	1	4	00	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

[a] With two intervening weekends.
[b] Scale for visual disintegration of filter strip. See 'Methods': 5 = complete disintegration.
[c] All isolates with activity as first column as per cent of 367.
[d] Total isolates tested 367.

selected for testing for hydrolysis of straw where both cellulolytic and hemicellulolytic activities might be involved. As above, preliminary tests showed that extra organic nitrogen (Casitone or yeast extract) added to medium 2 inhibited, or did not increase, straw digestion. Loss in weight of straw varied from 0% to 50% for the different isolates, but the triplicate cultures for each isolate gave very close results. Thirty-eight isolates gave less than 10% loss in weight with 8 showing 0% (or 1% on average) loss; sixteen isolates gave between 10% and 20%; twenty-one between 20% and 30%; nine gave over 40% and forty-six between 30% and 40%. For each value of filter-strip disintegration there was a wide variation in straw digestion. However, there was a slight correlation in that the cultures giving a filter-strip disintegration of 5 gave losses of weight of straw quite evenly distributed between 22% and 46%, with one isolate at 50% and one at 13%, while isolates of filter-strip activity 4 were distributed between 0% and 44% straw degradation, and isolates of activity 3 were distributed between 0% and 40% straw degradation. The two isolates of filter strip activity 2 gave straw degradations of 3% and 21%. While all media gave some isolates with straw disintegration of above 40%, there seemed to be a slight bias towards the isolates of higher activity coming from the untreated straw medium as only 6% of these had straw disintegration below 10% (all were 0%) whereas 34% of the isolates from the treated-straw medium and 44% of the isolates from the cellulose-powder medium had activities less than 10%. The final pH values in the straw medium were generally 6·3–6·6, although, in a few cases, they were between 5·6 and 6·0.

Properties of presumptive clostridia

The present Group numbers are those given to the final, identified groups of bacteria and do not necessarily correspond with the preliminary, morphological groupings described previously.

Group 1

Members of this Group were found on all days of isolation and in the highest or near-highest dilution cultures. The powdered filter-paper medium gave most isolates, followed by treated and untreated straw. However, although some 65% of the isolates had filter-strip cellulolytic activities of 3–4, the remainder, most of which were isolated from one day's digester sample, had activities of only 1 or 2 units. The maximum

TABLE 2
Properties of Representative Strains of Groups 1–5

Test	*Strain and growth or reaction*					
	Group 1 *T278*	*Group 2* *C440*	*Group 3* *T421*	*Group 4* *C387*	*Group 5* *U61*	*C249*
Glucose	+	+	+	+	+	+
Fructose	+	+	+	+		(+)
Mannose	+				−	−
Cellobiose	+	+	+	(+)	(+)	−
Lactose	+	+	+	−	−	−
Maltose	+	+	+	(+)	+	+
Sucrose	+	+	+	−	−	−
Melibiose	+	+			−	−
Raffinose	+			−		−
Trehalose				−	−	−
Ribose	+		+		−	−
Erythritol	(+)			−		−
Dextrin	(+)	−	+		(+)	
Starch	+	+	+	−	−	−
Xylan	(+)	(+)	−	(+)	(+)	(+)
Mannitol		+				(+)
Melezitose		(+)			−	−
Aesculin		+		+	+	+
Gelatin	−	−	−	+	+	+
Milk	AC	AC	AC	C	D	D
Meat	−				D	D
BS		(+)	−	+	−	+
Indole	−	−	−	(+)	−	−
Catalase	−	−		−		−
Lipase	−				+	+
Haemolysis	−					β
Urease	−			−		−
H_2S	−	−	−	+	+	+
Motility	+	+	−	+	+	+

Blank, not tested. (+) slight positive. A, C, D: acid, clot, digestion. BS, bile salts medium. Reactions of U153 were the same as those of T278 (Group 1). C387 was nitrate reduction −, acetylmethylcarbinol (amc) −.

C249 was xylose −, adonitol −, dulcitol −, inositol −, salicin, −, sorbitol −, nitrate −, amc −.

U61 was arabinose −, salicin (+), xylose +.

degradation of straw by members of this Group was about 35%, but many showed only about 5% degradation.

Thirty-eight isolates were placed in this Group after preliminary testing and two, T278 and U153, were selected for extensive testing while some of the more important tests were also done on isolates C302, C304 and C305. T278 was isolated on treated straw medium and U153 on untreated straw medium, on different days, while C302, 304 and 305 were isolated on paper-powder medium on the same day. T278 showed a filter-strip disintegration of 4, but virtually no degradation of straw. U153 had a filter-strip disintegration of 3 and straw degradation of 31%. C302, 304 and 305 had filter-strip disintegrations of 3, 4 and 1 with 5% and 8% (305 not tested) degradations of straw.

The properties of T278, the same as U153, are shown in Table 2 and fermentation products from glucose shown in Table 3.

TABLE 3

Fermentation Products from Glucose of Representative Strains of Groups 1–5

Product	*Strain and production (mM)*							
	Group 1		*Group 2*	*Group 3*	*Group 4*	*Group 5*		
	T278	*U153*	*C440*	*T421*	*C387*	*C249*	*C346*	*C347*
Ethanol	1·0	0·4	0	0	10·9	20·0	21·2	5·2
Formate	P	P	P	0	0	P	P	P
Acetate	3·2	8·2	12·0	13·2	9·5	15·3	10·5	30·2
Propionate	0·6	3·0	1·0	0	4·1	2·1	8·4	5·0
Butyrate	13·0	9·0	15·0	14·9	0	1·1	4·2	0
i-butyrate	0	0	0	0	1·7	1·8	1·9	4·0
Valerate	0	0	0	0	0	0·4	1·0	5·7
i-valerate	0	0	0	0	1·2	1·3	2·6	3·8
Caproate	0	0	0	0	0	0	0	2·2
i-caproate	0	0	0	0	0	0·8	2·6	11·9
Lactate	16·5	8·3	P	P	32·3	25·0	P	P
Succinate	6·1	T	P	P	22·6	12·4	P	P
H_2	+	N	+	N	+	+	N	N
CO_2	+	N	+	N	+	+	N	N

C440, T421 and C249 formed butanol (10·0, 5·0, 5·0 mM).
T, P, present in trace or small amounts but not quantified.
Averages of duplicate or triplicate cultures incubated for 5 days.
N, gases not analysed.

T278 was an anaerobic, Gram-negative. pleomorphic rod, 0·75–1·25 × 2–4·5 μm, others in the Group were similar. In 10 h cultures Gram-positive cells were seen. Uni- or bi-polar staining was seen in 24–48 h cultures. In electron micrographs of sectioned bacteria a Gram-positive cell wall was present. In general, cells had rounded ends, but when central swelling occurred the ends appeared more pointed. Spores were sub-terminal. Cells were generally single, but a few pairs and chains were sometimes found. The bacteria were motile, and although flagella were difficult to distinguish, they appeared to be peritrichous.

Colonies in glucose and cellulose roll-tubes were circular, slightly irregular, white, convex, 1–3 mm diameter and, in young cultures, glossy but becoming drier later, possibly because of sporulation. In starch roll-tubes colonies were 1–15 mm diameter, white, but matt with irregular margins. In liquid medium turbidity was initially uniform, but growth later sedimented. In spent-cellulose cultures cellobiose, cellodextrins and some xylose, but not glucose, were detected. The effects of temperature on growth are shown in Fig. 1. Other groups gave similar results. Cultures survived 80 °C for 10 min. As with other isolates tested, the optimum pH for growth was about 7·0 to 7·5. There was growth from pH 6 to 8 but not at 5·5.

T278 gave good growth in glucose–mineral salts–biotin medium, showing that it had no requirements for growth factors other than biotin, a property that seems to separate it from *C. beijerinckii*. Considering all the properties, it was concluded that T278 and others of this Group, which were similar in properties tested, were strains of *Clostridium butyricum*.

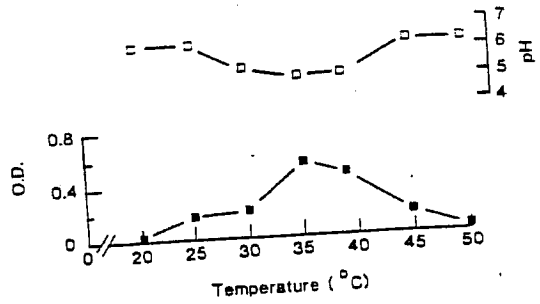

Fig. 1. The effects of temperature, at initial medium pH of 7·2, on growth of isolate T278. Culture 20 h in PY glucose medium. OD at 640 nm of diluted cultures. The upper graph shows the final pH of cultures.

Group 2

This Group appeared to form one of the minor components of the flora in that they did not seem to be present in every sample and, where found, were not above the 10^4 dilution. Most isolates were from the powdered cellulose medium and four from this and one from treated-straw medium were selected, with one, C440, studied in most detail.

The isolates all had filter-strip cellulolytic activity of 3, but degradation of straw was only from 4·7 to 9·4 %. C440 gave 7·3 % straw degradation.

Properties of C440 are shown in Table 2 and products of glucose fermentation in Table 3.

The Group were anaerobic, Gram-negative, pleomorphic rods, 0·5–0·75 × 1·5–3 μm, with rounded ends. Some cells in young culture were Gram-positive. Spores were subterminal.

Colonies in cellulose roll-tubes were pinhead, circular or oval, with entire to slightly undulate margins, and light tan in colour. In starch roll-tubes, colonies were 2–3 mm, white, matt and convex with slightly undulate margins. Growth in liquid medium was initially uniform, but later sedimented, and cellobiose, with a trace of glucose and cellodextrins, was found in spent cellulose medium.

There was no growth in glucose, mineral-salts, biotin medium and the isolates seem very similar to *Clostridium beijerinckii*. *Bergey's Manual* (8th edn) says *C. beijerinckii* does not hydrolyse starch, but this is contradicted by *The Anaerobe Manual* (1977).

Group 3

Members of this Group were isolated on all substrates and in dilutions up to 10^5. Seven isolates, from all substrates, were selected for further investigation. The isolates had filter-strip activities of 3-4 and gave from 4·7 % to 35·3 % degradation of straw. Isolate T421, with filter-strip activity 4 and 35·3 % straw degradation, was characterised extensively.

Properties of T421 are shown in Table 2, and fermentation products from glucose in Table 3.

The bacteria were anaerobic, Gram-negative, straight to curved rods with rounded ends, 0·6–0·85 × 2–4 μm. Occcasional cells were 10 μm long and a few cells showed Gram-positive staining. Pairs and some chains were present, but the cells were often arranged side by side.

Colonies in cellulose roll-tubes were mainly circular with an occasional oval, 0·5–7·5 mm, cream, but lightly tan-coloured in transmitted light. A slight mucoid zone, presumably polysaccharide, was seen around the

irregular margins of colonies. Growth in liquid medium was uniformly turbid, with a slight ropiness in shaken up sedimented growth which dispersed on further shaking. In spent cellulose medium only the cellodextrins were found. The isolate survived 80 °C for 10 min, but spores were difficult to detect with certainty.

The tests suggested that the isolates were strains of *Clostridium acetobutylicum*.

Group 4

These were isolated on straw and cellulose powder media in dilutions up to 10^5, and seven isolates were taken for testing. Like other isolates, although filter-strip activity was 3–4, straw degradation varied; from 1·9 % to 33·8 %. Isolate C387, with filter-strip activity of 3 and straw degradation of 1·9 %, was selected for detailed examination.

Properties and fermentation products of C387 are shown in Tables 2 and 3.

The Group were anaerobic, Gram-negative, rods with rounded ends, 0·5 × 1·5–3·0 μm, occurring singly, in pairs or short chains. Some cells were Gram-positive, and in older cultures cells were pleomorphic with a tendency to bi-polar staining. Spores were difficult to detect, but were sub-terminal.

Colonies in glucose and cellobiose roll-tubes were ellipsoidal, 0·25–0·5 mm, white, non-mucoid. In cellulose roll-tubes, colonies were circular, convex; 0·25–0·5 mm and tan-coloured in transmitted light. Growth in liquid medium was uniformly turbid, but as it sedimented it tended to clump, suggestive of the presence of extracellular poly-saccharide. Cells were sluggishly motile. C387 survived 70° and 75°, but not 80 °C, for 10 min.

The properties suggested that C387 and the Group be classified as strains of *Clostridium bifermentans*, although, like many of the digester bacteria, C387 produced H_2S in sulphate medium.

Group 5

These were isolated on straw and cellulose powder media from up to the 10^5 dilution. Four isolates were selected for further testing. U61 was isolated in 10^5 dilution on straw medium and had a filter-strip activity of 4 and straw degradation of 44·1 %. C249 was isolated from 10^3 dilution on cellulose medium and had filter-strip activity of 3 but gave only 8·8 % degradation of straw. C346 and C347 had filter-strip activities of 4, but were not tested on straw.

Properties and fermentation products of the isolates are shown in Tables 2 and 3.

C249 and U61 were both anaerobic, Gram-negative rods, 0·25–0·5 × 1·5–3·5 μm, with U61 being generally shorter and thinner than C249; others in the Group were generally similar. Some Gram-positive cells were seen in young cultures of C249. The rods occurred in pairs with some short chains and formed sub-terminal spores. Long filaments were occasionally found and the growth of C249 was somewhat slimy.

In cellulose roll-tubes colonies had a filamentous margin and were from 0·2 to 1 mm diameter. The isolates grew in an anaerobic jar and on plates of nutrient agar plus glucose and cysteine they formed matt, white to yellow colonies with a raised centre and 2·5 mm in diameter with rhizoidal margins. Similar colonies were also produced on egg-yolk and blood-agar.

Early results suggested that these isolates might be similar in some properties to *Desulfotomaculum nigrificans* and *Clostridium thermosaccharolyticum*, so type cultures of these were tested with the present isolates. The two latter organisms were not cellulolytic, although *C. thermosaccharolyticum* did grow at 39 °C, and they differed in some other aspects from the present isolates. Although the $G+C$ ratio of 29·7% determined for C249 is rather higher than the 26% given in *The Anaerobe Manual* (1977) for *C. sporogenes*, considering all the properties of the isolates it is suggested that they are strains of *Clostridium sporogenes*.

Fermentation gases

Some results of analyses of gases in the headspace of cultures on glucose medium, incubated under N_2/CO_2 (90/10), are shown in Table 3. All the isolates tested produced H_2 and CO_2 as shown by an increased proportion of the latter gas. A decreased amount of N_2 in the gases, particularly in the case of isolate C440, suggested that N_2 was being fixed by these bacteria.

DISCUSSION

Degradation of most of the available (not the total) cellulose and hemicellulose in cattle-waste digester feedstocks can take 20 or 25 days and such digesters are usually run at these retention times (Bousfield *et al.*

1979). In contrast, in the cattle rumen the cellulose and hemicellulose is degraded in a system with retention time for liquids and fine particles measured in hours and a retention of larger solids of about 2 days at the maximum. This leaves a residue of the most resistant cellulose and hemicellulose which forms the substrates for the digester bacteria. Although both are mesophilic systems with essentially the same reactions occurring in each, because of the different conditions pertaining in anaerobic digesters and the rumen it might be expected that different cellulolytic bacteria would develop in each habitat, although both are open to the same sources of inoculum bacteria. The work previously reported, and the present work, seem to support this hypothesis.

Hungate (1950) found 0·8 to $2{\cdot}0 \times 10^3$ cellulolytic bacteria per millilitre of sludge from a domestic sewage digester. He isolated three strains of Gram-negative, cellulolytic rods which were not named but differed from the Gram-negative rods he had isolated from the rumen using similar media and techniques. These rods were non-sporing. Maki (1954) reported $1{\cdot}6 \times 10^4$ to $9{\cdot}7 \times 10^5$ cellulolytic bacteria per millilitre of digesting sewage sludge in samples taken from the same digester at intervals of a few days, or, in one case, some months later. He isolated ten different rods divided into two groups of high and low cellulolytic activities. Hobson & Shaw (1971, 1974) found 4×10^3 cellulolytic bacteria per millilitre in another sewage digester. The variations in counts could reflect differences in the cellulose contents of the sewage-sludge feedstocks, as Hobson & Shaw (1974) found that when their digesting sewage sludge was adapted to a feedstock of piggery waste of higher cellulose content than the sewage sludge the number of cellulolytic bacteria increased to 4×10^5 ml^{-1}. From these piggery-waste digesters Hobson & Shaw (1974) isolated eleven types of cellulolytic bacteria of which ten were Gram-negative rods and coccobacilli and one was a Gram-positive rod. The morphologies and fermentation products were described, but the isolates were not further characterised, although none was apparently similar to rumen species. All the digesters mentioned above were mesophilic. These results seemed to suggest that digester populations contained a wider variety of cellulolytic bacteria than the rumen and that these bacteria did not include the three or four species, *Bacteroides succinogenes*, *Ruminococcus albus* and *R. flavefaciens*, and sometimes *Butyrivibrio fibrisolvens*, which seem to be universally of most importance in the rumen.

The above isolates were made on media containing powdered filter-paper, a substrate which has been generally used in the isolation of rumen cellulolytic bacteria. The present work was designed to see if a more extensive survey on a digester treating a feedstock of higher fibre content than those previously tested would confirm the apparent diversity of cellulolytic species, and lack of rumen species, in digesters, and whether the use of a culture substrate closer in physical and chemical structure to the digester feedstock than cellulose powder would give a better assessment of numbers and types of bacteria. A general analysis of barley straw is 42·5 % cellulose, 26·6 % hemicellulose, 10·3 % lignin (Morrison, 1979) with about 2–5 % crude protein and 10–15 % ash (*cf.* analysis of digester solids). Bacteria with specific properties, e.g. cellulolysis, have also been counted by testing large numbers of strains initially isolated on a non-specific medium designed to culture all bacteria in a habitat. However, Hobson & Shaw (1974) noted that no cellulolytic bacteria could be detected in the isolates growing on their non-selective medium for 'total' viable counts (this contained glucose, cellobiose, maltose and lactate) although it cultured about 8×10^6 bacteria per millilitre of digester fluid; about ten times the counts on the cellulose medium. Ianotti *et al.* (1982) used a medium with glucose, cellobiose, starch and pyruvic acid as energy sources for viable counts of bacteria in a pig-waste digester. Although this medium cultured a greater proportion of the total count of bacteria than the non-selective medium of Hobson & Shaw, and the bacteria in cultures differed from those of the latter workers, again no cellulolytic or hemicellulolytic activities could be found in the 130 isolates tested. So, because of the numbers of cellulolytic bacteria relative to the high numbers of non-cellulolytic bacteria, and possibly other reasons, it would seem that a medium containing a cellulosic substrate which will tend to select for the cellulolytic bacteria, and in which a visible change in substrate can be detected, is necessary for counting and isolating such bacteria. However, the present results suggest that within the limits of experimental errors the choice of cellulosic substrate makes little difference to the counts recorded. These were similar to, or rather higher than, the counts of Hobson & Shaw (1974). This latter might be expected as the present digester feedstock was of higher TS and fibrous solids content than that of Hobson & Shaw. Some of the isolates were able to degrade 40–50 % of the untreated straw and about 50 % is the calculated degradation of straw in a digester at infinite retention time, with about 35–40 % the more practical degradation (Hobson, 1979). Overall, the

bacteria showed widely differing abilities to hydrolyse the different substrates, even the ones contained in the medium on which they were isolated. This confirms and extends the observation of Maki (1954) of varying ability to hydrolyse cellulose in his isolates.

Anaerobic digesters, then, contain mixed populations of cellulolytic bacteria, even after months or years of running on the same feedstock. Inherent ability to degrade cellulose well does not seem to be enough to cause dominance of one or two species in the long term. This suggests that many more factors than provision of energy source govern the growth of bacteria in digesters, although continued inoculation of the digester from the feedstock, air or other sources of contamination may play a part in keeping the population heterogeneous. On the other hand, the rumen is subject to the same continuous inoculation, but here the cellulolytic population is limited to a few species. In spite of the overall similarities in reactions between the rumen and anaerobic digesters, the different conditions in the digester (e.g. higher pH, longer retention time, lower Volatile Fatty Acid concentrations) would seem to suppress the growth of rumen bacteria and encourage growth of other types. On the other hand, the short retention time of the rumen would not seem to be the factor excluding the digester bacteria from this habitat. In pure culture the rates of growth and cellulose degradation of the digester bacteria were comparable to those of rumen bacteria on the same substrates (Stewart *et al.*, 1979). The composition of the feedstock must be the cause of the slow fibre degradation in digesters.

Clostridium isolates

In the rumen, from which is derived much of the material of the feedstock of cattle-waste digesters, there are few, if any, clostridia. Two cellulolytic clostridia—*C. longisporum* and *C. lochheadii*—were isolated in low numbers from a cow (Hungate, 1957) and, earlier, a cellulolytic *C. cellobioparus* was isolated from some rumen contents (Hungate, 1944), but these have rarely, if ever, been found in other investigations of the rumen flora. A non-cellulolytic *C. butyricum* was once isolated from a sheep fed on flaked maize (Masson, 1951).

In systems of long retention time and slow overall growth rate of organisms, spore-forming bacteria could have an advantage over non-sporing bacteria such as those of the rumen, where cessation of growth

because of nutrient shortage or other factors is followed by lysis (Hobson 1965, 1971). Spore-forming bacteria made up a major proportion of the digester cellulolytic bacteria isolated and tested, although sporulation was often difficult to detect, and the present Groups seemed to be best identified with known species of *Clostridium*. Not all these species have been previously shown to have cellulolytic strains, but there is other evidence that clostridia are abundant in anaerobic digesters.

In early work on the bacteriology of anaerobic digesters Cookson & Burbank (1965) isolated *C. carnofoetidum*, McCarty *et al.*, (1962) and Torien (1967) found proteolytic activity to be associated with sporing rods and, of forty-three proteolytic isolates characterised by Siebert & Torien (1969), twenty-eight were *Clostridium* species, although some of these isolates were made from laboratory digesters fed on artificial feedstocks. Hobson & Shaw (1971, 1974) isolated *C. perfringens* and other proteolytic, amylolytic possible clostridia from a domestic sewage digester and also found proteolytic and amylolytic clostridia as major components of the flora of a piggery-waste digester. Some of the latter were identified as *C. butyricum*.

One of the cellulolytic bacteria isolated by Maki (1954) definitely formed oval spores on first isolation, but sporulation became more and more difficult to detect on continued subculture of the bacterium. The three isolates tested formed, mainly, ethanol and acetic acid from cellulose. A non-cellulolytic butyric acid-forming *Clostridium* sp. was present in high numbers in the digester and this increased growth and cellulolysis in co-culture with the other bacteria. Of the eleven types of cellulolytic bacteria isolated by Hobson & Shaw (1974) none was completely identified, but some could have been clostridia, although no sporing was observed. In their group of unidentified bacteria Iannotti *et al.* (1982) briefly mention a Gram-positive, anaerobic, spore-forming rod producing a large amount of butyric acid and two other Gram-positive, sporing rod isolates.

Khan *et al.* (1981) reported on a consortium of bacteria derived by treating, at 80 °C for 10 min, the contents of an anaerobic digester starting from an inoculum of digesting sewage sludge and fed on a chemically defined medium containing Whatman CF11 cellulose. The bacteria of the consortium appeared to be mainly cellulolytic spore-formers and produced acetic acid and CO_2 and some propionic acid, H_2 and ethanol from cellulose. Later, Khan & Miller (1983) said that a new strain of *C. butyricum*, presumably cellulolytic, had been isolated from the mixture.

These digester bacteria were all from mesophilic digesters, but *C. thermocellum* is probably important in thermophilic digesters.

Enzymes of the cellulase complex have been found in an industrial strain of *C. acetobutylicum* used in starch fermentation (Allcock & Woods, 1981) and other clostridia with cellulolytic activity have been reported elsewhere; for instance, in soils. Cellulolysis by clostridial strains could thus be widespread, and particularly so in anaerobic digesters where there is a large and heterogeneous population amongst which transfer of genetic information might easily occur.

The possible nitrogen fixation reported here is in line with the known properties of *Clostridium* spp. Although, while nitrogen from air often occurs in digester gases, the high ammonia concentrations in digesting sludges might suggest that little actual nitrogen fixation could occur. The ranges and optima of pH and temperature for growth of the Group 1 bacteria correspond very well with conditions for optimum activity of mesophilic digesters. These have a maximum operational temperature of about 45 °C before there is a fall in activity until thermophilic digestion can be developed above 50 °C, and the optimum pH of a digester is about 7·2–7·4 (Summers & Bousfield 1980; Hobson, 1983). Of the fermentation products formed in pure cultures, hydrogen, acetate, propionate and butyrate are converted to methane by single methanogenic bacteria or consortia in digesters. Under the influence of hydrogen removal by methanogenesis, lactate and succinate would probably not be formed by the bacteria in a digester, but lactate-fermenting bacteria have been isolated from a digester (Hobson *et al.*, 1974) and the presence of succinate-fermenting bacteria might be inferred.

Sugars from cellulolysis are unlikely to persist in digesters, but the residues were examined here as Hungate (1950), Prins *et al.* (1972) and Ng *et al.* (1977) used the sugars in spent cellulose medium as characters in descriptions of *C. cellobioparus*, *Eubacterium cellulosolvens* and *C. thermocellum*.

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