2.1. Rumen bacteria

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Introduction

The rumen is the most extensively studied gut community and is characterized by its high population density, wide diversity and complexity of interactions. This complex, mixed microbial culture is comprised of prokaryote organisms including methane-producing archaebacteria, eukaryote organisms, such as ciliate and flagellate protozoa, anaerobic phycomycete fungi and bacteriophage. Bacteria are predominant (up to 10^{11} viable cells per g comprising 200 species) but a variety of ciliate protozoa occur widely (10^4–10^6/g distributed over 25 genera). The anaerobic fungi are also widely distributed (zoospore population densities of 10^2–10^4/g distributed over 5 genera). The occurrence of bacteriophage is well documented (10^7–10^9 particles/g). This section focuses primarily on the widely used methods for the cultivation and the enumeration of rumen microbes, especially bacteria, which grow under anaerobic conditions. Methods that can be used to measure hydrolytic enzymes (cellulases, xylanases, amylases and proteinases) are also described, along with cell harvesting and fractionation procedures. Brief reference is also made to fungi and protozoa, but detailed explanations for culturing and enumerating these microbes is presented in Chapters 2.4 and 2.5.

Anaerobic culturing techniques

The majority of bacteria, which colonize the gastrointestinal tract of ruminants are strict anaerobes, which require an environment with a low redox potential for growth. The anaerobic techniques of Hungate [12] as modified by Bryant [3] are used by all the major rumen microbiology laboratories and have been described by Stewart et al. [28]. A brief description will be given here, but it is recommended that experience be obtained in an anaerobic microbiology laboratory before attempting these procedures.

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Preparation of anaerobic media

Combine the mineral solutions, heat stable media ingredients and water (Tables 1, 2, 3 and 4) in a large vessel, which can be autoclaved. Heating the solution to boiling point in a microwave is recommended to help eliminate dissolved oxygen. After boiling, use a gassing probe/needle to purge the solution with food grade CO_2 while the medium cools. A reducing agent (e.g. cysteine HCl) can be added after gassing for at least 0.5 h (or when the redox dye such as resazurin has become colourless) and seal the vessel with a stopper that will prevent the entry or escape of gas from the bottle if the medium is to be autoclaved (15 min at 100 kPa, 121°C) in the preparation vessel. Other reducing agents are shown in Table 2. Reducing agents such as Na_2S should be added to culture tubes immediately before inoculation rather than during medium preparation. The most commonly used dyes to indicate oxidation–reduction state in media are resazurin and indigo carmine, which become colourless in the reduced state. The stoppered bottle can also be immediately transferred to a anaerobic hood (chamber) where it is aliquoted into smaller bottles or culture tubes equipped with gas-impermeable butyl rubber stoppers and then seal with a special crimper tool and aluminium seal (Bellco Glass Inc., Vineland, New Jersey, USA) before autoclaving. It is preferable to autoclave large volumes of medium in vessels, which are coated with a membrane (Schott Glas, Germany) that prevents shattering due to explosion or implosion during autoclaving. Filter-sterilized B-vitamins [18] can be added to melted agar medium and broth just prior to use for inoculation.

A more recent development in anaerobic microbiology has been the use of the flexible plastic anaerobic glove box chamber (Coy Laboratory products, Inc., Ann Arbor, Michigan), which is more convenient than the roll tube method of isolating anaerobic bacteria. The advantage of using an anaerobic chamber is that standard microbiological techniques can be used including agar spread plates, replica plating and dispensing of media in an anaerobic environment. However, media should be prepared using anaerobic techniques prior to transferring to an anaerobic hood for dispensing into plates or culture tubes. Media should not be made under aerobic conditions and placed in an anaerobic glove box to equilibrate.

Conventional techniques for enumeration of rumen bacteria, protozoa and fungi

The design of animal trials and rumen sampling methodology for quantitative analysis of microbial populations is discussed in Chapter 1.1 by A.R. Egan. For the purposes of this chapter, a representative rumen digesta sample (50 g) for analysis of microbial populations is collected from the rumen.

Bacteria

The rumen sample taken for bacterial counts is immediately transferred to an anaerobic hood containing a gas phase of CO_2/H_2. A subsample (5 g) is diluted 1 : 10 with cold anaerobic diluent (Table 1) and processed for 1 min with a homogenizer (Bamix,

Table 1. Media recipes widely used for the non-selective isolation and selective isolation and enumeration of culturable ruminal bacteria as well as recipes for the selective isolation of rumen fungi and bacteria based on hydrolytic properties (adapted from Stewart et al. [28] and McSweeney et al. [23])

	Non-selective medium					Selective medium						
	RGCA[a] [11]	Med10[b] [4]	Med2[c] [10]	HS[d] [13]	Anaerobic diluent	Total cultur-able	Xylanolytic	Cellulolytic	Proteolytic	Pectinolytic	Amylolytic	Fungi
Clarified rumen fluid (ml)	30	–	20	–	–	30	–	–	–	–	–	20
Depleted clarified rumen fluid (ml)						–	30	30	30	30	30	–
Peptone	–	–	–	–	–	–	–	–	–	–	–	–
Casitone	–	–	1.0	–	–	–	–				–	
Trypticase	–	0.2	–	–	–	0.05	0.05	0.05	–	0.05	–	0.05
Yeast extract	–	0.05	0.25	–	–	0.05	0.05	0.05	0.05	0.05	–	0.05
Soluble starch	0.05	0.05	–	–	–	0.05	–	–	0.05	–	0.25	0.07
Sodium lactate (70% w/v)	–	–	1.0	–		0.2	–	–	–	–	–	–
Glucose	0.025	0.05	0.20	–	–	0.05	–	–	0.05	–	–	0.03
Maltose	–	–	0.20	–	–	0.05	–	–	0.05	–	–	–
Cellobiose	0.025	0.05	0.20	–	–	0.05	–	–	–	–	–	0.03
Cellulose (ball-milled)	–	–	–	0.3	–	–	–	0.2	–	–	–	–
Xylan (birchwood/oat spelt)	–	–	–	–	–	0.05	0.2	–	–	–	–	0.07
Casein (soluble)	–	–	–	–	–	1.0	—-	–	1.0	–	–	–
Pectin	–	–	–	–	–	0.05	–	–	–	0.2	–	–
Xylose	–	–	–	–	–	0.05	– –	–	0.05	–	–	–
$(NH_4)_2SO_4$	0.1	–	–	–	–	–	–	–	–	–	–	–

Table 1. Contd...

	Non-selective medium					Selective medium						
	RGCA[a] [11]	Med10[b] [4]	Med2[c] [10]	HS[d] [13]	Anaerobic diluent	Total cultur-able	Xylanolytic	Cellulolytic	Proteolytic	Pectinolytic	Amylolytic	Fungi
$NaHCO_3$	–	–	0.4	0.5	–	0.8	0.6	0.8	0.6	0.6	0.6	0.8
Na_2CO_3 solution (8%) (ml)	–	5.0	–	–	5.0	–	–	–	–	–	–	–
Solution no. (Table 3) and volume (ml)	(1) 50	(2) 3.8	(4) 15	(4) 16	(2) 3.8	(4) 15	(4) 15	(4) 15	(4) 15	(4) 15	(4) 15	(4) 15
Solution no. (Table 3) and volume (ml)		(3) 3.8	(5) 15	(5) 16	(3) 3.8	(5) 15	(5) 15	(5) 15	(5) 15	(5) 15	(5) 15	(5) 15
Trace elements (ml, Table 4)				0.1								
Pfenning trace elements (ml, Table 4)						0.1	0.1	0.1	0.1	0.1	0.1	0.1
VFA mix 1 (ml)[e]	–	0.31	–	–		0.31	–	0.31	–	–	–	0.31
VFA mix 2 (ml)[f]	–	–	–	2.0	–	–	–	–	–	–	–	
Haemin (mg)[g]	0.5	1.0	–	100 μl	–	1.0	–	1.0	–	–	–	1.0
Vitamin solution[h]	–	–	–	100 μl		–	–	–	–	–	–	–
Vitamin K_1 (mg)	0.1	–	–	–		–	–	–	–	–	–	–
L-Cysteine HCl-H_2O[i]	0.05	0.025	0.05	0.02		0.05	0.05	0.05	0.05		0.05	0.05

$Na_2S{\cdot}9H_2O$[i]	–	0.025	–	0.1	–	–	–	–	–		–	0.025
Resazurin (mg)	0.1	0.1	0.1	1.2	–	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Indigo carmine solution (0.05%) (ml)					1.0	–	–	–	–	–	–	–
Agar	2.0	2.0	2.0	–		2.0	2.0	2.0	2.0	2.0	2.0	2.0
Antibiotics (ml)[j]												0.5

Ingredients in g unless otherwise indicated. Distilled H_2O added to final volume of 100 ml.

[a]Holdeman et al. [11].

[b]Caldwell and Bryant [4], cited in Holdeman et al. [11]

[c]Hobson [10]

[d]Hungate and Stack [13]

[e]VFA mix 1 : 17 ml acetic acid; 6 ml propionic; 4 ml *n*-butyric; and 1 ml each of *n*-valeric, *iso*valeric, *iso*butyric and 2-methylbutyric acid.

[f]VFA mix 2 : 9.845 g sodium acetate; 139 μl *iso*butyric acid; 163 μl 2-methylbutyrate; and 160 μl each of *n*-valerate and *iso*valerate made up to 100 ml with distilled H_2O.

[g]Haemin: Dissolve 50 mg haemin in 1 N NaOH; make up to 100 ml with distilled H_2O. Autoclave at 121°C for 15 min.

[h]Vitamin solution: 1 mg each biotin and cobalamin; 3 mg PABA; 5 mg folic acid; 15 mg pyridoxamine made up to 100 ml with distilled H_2O. After autoclaving the medium, add 100 μl (per 100 ml medium) of a solution containing 5 mg each thiamine and riboflavin per 100 ml distilled H_2O.

[i]When added together, prepared as a single concentrated solution in dilute alkali, pH around 10 [9].

[j]Benzyl penicillin (12 mg/ml) and streptomycin sulphate (2 mg/ml) dissolved in distilled water (previously boiled and bubbled with nitrogen until cooled) and filter-sterilize whilst being gassed with nitrogen.

Table 2. Reducing agents for anaerobic media

Compounds	E_0' (mV)	Concentration in media
Cysteine HCl[a]	−210	0.025%
Dithiothreitol[a]	−330	0.05%
H_2 + palladium chloride [1]	−420	
Titanium III citrate[b] [34]	−480	0.5–2 mM
Titanium III nitrilotriacetate[c] [25]		>30 μM
$Na_2S{\cdot}9H_2O$[a] (or H_2S)	−571	0.025%

[a] Stock solutions may be autoclaved and stored under anaerobic gas.
[b] Add 5 ml of 15% titanium trichloride solution to 50 ml of 0.2 M sodium citrate and neutralize with a saturated sodium bicarbonate solution. Filter, sterilize and add 30 ml/l of sterile oxygen-free medium in the appropriate concentration. The titanium III citrate complex is blue-violet when reduced and colourless when oxidized.
[c] Nitrilotriacetic acid (NTA, free acid 9.6 g) is added to 300 ml of anaerobic water (gassed with N_2). The pH is adjusted to 9.0 with concentrated NaOH. A total of 9.6 ml of 20% $TiCl_3$ is added slowly to the chelator solution (NTA). To prevent precipitation, the pH is kept above 2.0 by the addition of an anaerobic, saturated solution of $NaCO_3$. (Use of NaOH at this stage will precipitate the titanium.) The final pH is adjusted to 7.0 with $NaCO_3$, and the final volume was adjusted to 500 ml with anaerobic water. The Ti(III) NTA reductant is filter-sterilized into a sealed serum vial containing N_2 gas. The final solution is a slight bluish-green in colour, in contrast to the blue-violet colour of a Ti(III) citrate solution. The final component concentrations are 25 mM Ti(III) and 100 mM NTA.

Mettlen, Switzerland; Ultra-turrax Homogenizer, Janke and Kunkel, Germany), serially diluted in anaerobic diluent [19] and aliquots (20 μl) of 10^{-5}–10^{-8} dilutions are inoculated in triplicate onto agar selective media [20]. Media used in agar plates to enumerate total culturable, cellulolytic, xylanolytic, pectinolytic and proteolytic bacteria are described in Table 1. Rumen fluid used in agar media is pre-incubated to deplete fermentable substrates [16]. Control plates, which contain the same media constituents minus the selective nutrient, are also inoculated for each functional group of bacteria. All colonies growing on the selective media plates are counted after 5–10 d when they were larger than or at higher concentration than those on

Table 3. Mineral solution ingredients

	Solution number				
Mineral (g/1 distilled H_2O)	1	2	3	4	5
$CaCl_2$	0.2	–	–	–	0.6
$CaCl_2{\cdot}2H_2O$	–	–	1.6	–	–
$MgSO_4$	0.2	–	–	–	–
KH_2PO_4	1.0	6.0	–	3.0	–
KH_2PO_4	–	–	6.0	–	3.0
$NaHCO_3$	10.0	–	–	–	–
NaCl	2.0	–	12.0	–	6.0
$(NH_4)_2SO_4$	–	–	6.0	–	6.0
$MgSO_4{\cdot}7H_2O$	–	–	2.5	–	0.6

Table 4. Trace mineral solution ingredients

Mineral (mg/l distilled H_2O)	Trace mineral solution	Pfenning trace mineral solution
H_3BO_3	61.8	300
$ZnCl_2$	68.1	–
$ZnSO_4 \cdot 7H_2O$	–	100
$MnCl_2$	98.9	–
$MnCl_2 \cdot 4H_2O$	–	30
$CoCl_2$	95.2	–
$CoCl_2 \cdot 6H_2O$	–	20
Na_2MoO_4	36.3	–
$Na_2MoO_4 \cdot 2H_2O$	–	30
$Na_2S_iO_3$	21.2	–
$Na_2S_eO_3$	17.3	10
$NiCl_2$	12.9	20
$CuCl_2 \cdot 2H_2O$	–	10
Na_2WO_4	3.3	–
$FeCl_2 \cdot 4H_2O$	–	150
$Al_2(SO_4)_3$	–*	–

Note: Dissolve ingredients in 100 ml (0.25 M) HCl and then make up to 1 l distilled H_2O.
*One microlitre solution of 6.6 mg/1 distilled H_2O.

the control plates. Cellulolytic colonies are identified by zones of cellulose-clearing in the opaque agar media. The agar can be stained with Congo red to aid in the visualization of clearing zones (see below). Proteolytic colonies are counted as those with clearing zones after the casein agar plate is flooded with 1 M HCl. Cellulolytic bacteria can also be enumerated in broth medium using the most probable number (MPN) procedure described by Dehority et al. [7] and summarized below. The cellulose broth medium is the same as that used in the cellulose agar medium (Table 1) except trypticase and yeast extract are omitted and the ball-milled filter paper cellulose (Whatman No. 1, Kent, England; Sigmacell-20; Sigma Chemical Co., St. Louis, MO, USA) is replaced with either four pieces of cotton thread (1 mm diameter and 50 mm long; GIMP soft cotton; Coats, Brisbane, Australia) or four discs (5 mm diameter) of acid swollen filter paper (Whatman No. 1) [26] or both (see method below). After incubation for 5–10 d, tubes are scored positive for cellulolytic activity based on increased turbidity of the culture fluid from growth of cells and visual loss (>30%) of cellulose.

The techniques used to quantify cellulolytic populations often yield variable results. Cellulolytic bacteria have been enumerated on selective media plates with ball-milled cellulose as the substrate [15, 24]. However, we have found that this method also grows bacteria, which were not cellulolytic and other experiments (McSweeney, unpublished data) have shown that many strains of cellulolytic bacteria, such as *Fibrobacter succinogenes*, growing in broth culture will not grow on or within this type of selective agar medium. In our laboratory, the MPN procedure [7] based on cellulose degradation in broth culture was a more reliable method for enumerating cellulolytic bacteria, and acid swollen cellulosic filter paper supported the growth of

a larger population of cellulolytic bacteria than cotton thread. Cotton thread tends to select for *Fibrobacter* bacteria, whereas cellulosic filter paper is readily digested by *Ruminococcus* populations as well [29]. Therefore, enrichments with cotton thread may underestimate the total cellulolytic population.

Protozoa and fungi

Rumen samples collected for enumeration of protozoa and fungi are not homogenized prior to dilution. Direct counts of protozoa can be made from rumen digesta samples that are initially diluted 1 : 1 with 10% formal saline [22]. The preserved sample is diluted 1 : 10 with anaerobic diluent solution containing 30% glycerol (vol/vol) and a dye (e.g. methylene blue) counted using a glass counting chamber (Fuchs-Rosenthal, Weber, England; Sedgwick-Rafter). Counts of each sample are made from at least 32 fields of view in two chambers that were filled separately. A detailed procedure for counting total protozoal numbers and species identification of rumen ciliate protozoa is available in the Laboratory Manual written and illustrated by Dehority [6].

The total number of rumen anaerobic fungi are determined by counting colonies (thallus forming units, TFU) that formed from zoospores and thalli in roll tubes (3 ml agar medium in 25 ml Balch tube) using the technique of Joblin [14] as follows. A rumen digesta sample is taken and serial, 10-fold dilutions made in anaerobic diluent. For each rumen sample, a 0.2 ml aliquot of 10^{-1}–10^{-3} dilutions are inoculated into triplicate roll tubes of fungal medium. The fungal medium (Table 1) contains penicillin G (1.2 mg/ml), streptomycin sulphate (0.2 mg/ml) and chloramphenicol (25 μg/ml) to inhibit bacterial growth. Colonies are counted after incubation at 39°C for 5 d. A modification of this procedure is used to enumerate those fungi which are fibre degrading. The fibrolytic fungal population is quantified as TFU using a procedure developed by Theodorou et al. [32], which is based on an end point dilution procedure and the MPN technique. Rumen samples are diluted into culture medium containing antibiotics as described previously and inoculated into triplicate tubes of cellulose broth medium which contains ground plant material with a high fibre content (e.g. crop residue or grass hay; 50 mg/10 ml) rather than pure cellulose as substrate. Tubes are scored positive when there is visual evidence of colonization and degradation of the plant material by the fungi.

Most probable number enumeration procedure

The MPN method is laborious and statistically inefficient, but it is an advantage if the bacteria cannot be cultivated or grow poorly on solidified media. The number of viable microbial cells can be estimated by the MPN procedure, which involves the mathematical calculation of the viable cells in a sample based on the number of cultures that fail to show growth in a dilution series of tubes containing a specific broth medium for the growth of a particular organism. The method involves taking several replicate (at least 3–5 replicates per dilution and termed 3–5 tube MPN) dilutions and recording the number of tubes at each dilution which support growth until a dilution range is reached where there is no growth in any of the replicates. The number of viable

cells is calculated from MPN tables [31] that use statistical methods to combine data from different dilution levels when a specified number of tubes is run at each level. MPN table calculators can be downloaded from the Internet as free software packages.

Storage of cultures

The majority of rumen bacteria can be stored in anaerobic diluent at −70°C for at least several years. One reliable method is to prepare an anaerobic storage medium, which is comprised of (by volume) 20% each of solution 2 and 3 (Table 1), 30% glycerol and 30% water. This medium (3 ml) is dispensed into 10 ml glass serum bottles under anaerobic conditions, stoppered and autoclaved. Cultures to be stored are grown to mid log phase growth and then 4 ml of culture is added to the serum bottles containing the storage medium and immediately placed in a −70°C freezer. Deep-frozen cultures are recovered by rapid thawing in water at 32–35°C [9].

Assays for major functional microbial groups in rumen

The major nutritional components of conventional ruminant diets outside of North America are fibre (pectin, cellulose and xylan) and protein, which are degraded by enzymes from the predominant micro-organisms in the rumen. Enzyme assays are routinely used to quantify the cellulase, xylanase and proteolytic activity of pure and mixed cultures of organisms as well as total activity in crude digesta samples taken from the rumen.

Preparation of microbial cells for assays

Cell pellets are collected from cultures or a rumen fluid sample by centrifugation at 10 000 *g* for 5 min. The supernatant fluid is harvested and 1 M sodium phosphate buffer (pH 6.5) is added to give a final concentration of 50 mM (extracellular fraction). Residual culture supernatant fluid is removed from the cell pellet with a pipette. The pelleted cells are resuspended in 0.5 ml 50 mM sodium phosphate buffer (pH 6.5) by vortexing and can be lysed by either sonication or passage through a French pressure cell. Cell and extracellular fractions can be stored at −20°C until use, but should be assayed as soon as possible after preparation to avoid loss of activity.

Cellulase and xylanase assays

Cellulase assays can also be performed using the following substrates: 0.4% (w/v) carboxymethyl cellulose (CMC) (low viscosity), 0.05% (w/v) *p*-nitrophenyl cellobioside and 1% (w/v) amorphous cellulose which are supplied by Sigma (Sigma). Acid swollen cellulose and ^{14}C labelled cellulose can also be used as substrates (see procedure for preparation of these substrates below). The procedure used with Avicel as the substrate is as follows: an Avicel solution is initially washed by the addition of a

1/2 vol of H_2O to a 1% Avicel suspension and then centrifuged to remove any soluble cellulose. A typical reaction which is performed against the 1% Avicel contains 100 μl of ice-cold 200 mM Na-citrate pH 6.0, 250 μl of H_2O, X μl of enzyme and made up to a total volume of 400 μl with BSA (1 mg/ml). Samples are taken at appropriate time points for at least 2 h and are always removed by first centrifuging the Avicel solution and then removing 30 μl of reaction mixture. The digestion reaction is then resuspended and returned to the 39°C shaking incubator at 225 rpm for further hydrolysis.

Cellulase assays are also performed over an extensive pH and temperature range. Assays for evaluating the pH profile of enzymes can be performed using 50 mM Na-citrate buffer for a pH range of 4–7 and 50 mM Tris NaCl for a pH range of 7.5–10. Assays for a temperature profile are performed in 50 mM Na-citrate (pH 6.0) at a temperature range of 25–70°C. Rate of hyrolysis of cellulose is measured using a reducing sugar assay (see below).

Xylanase activity against 0.5% oat spelt xylan or birchwood xylan (Sigma) is carried out essentially as mentioned above for the cellulase assay. Xylanase activity is determined by measuring reducing sugar release from xylan substrate.

Reducing sugar assay

Reducing sugar assays are often performed according to the method described by Lever [17]. A typical assay requires a set of sugar standards based on the reducing sugar which is to be detected, i.e. glucose for Avicel, CMC and MUC; and xylose when xylan is used as a substrate. Thirty microlitre of digest is mixed with 20 μl of distilled H_2O and 150 μl of p-hydroxybenzoic acid hydrazide (PAHBAH) working solution, which is prepared from a 5× stock solution (2 g PAHBAH, 0.39 g $CaCl_2{\cdot}2H_2O$ in 40 ml 0.5 M HCl) by addition of 4 volumes of 0.5 M NaOH. The mixture is heated in a boiling water bath for 8 min. After cooling, the tubes are centrifuged and optical density measured in a spectrophotometer at 410 nm.

All enzyme assays are performed at least in triplicate. One unit of cellulase or xylanase activity is defined as the amount that released 1 μmol reducing sugar equivalents/min) from the cellulose or xylan substrate. Enzyme activity can also be expressed per unit of cell protein in the assay. Protein concentrations are determined by using a commercial kit (Pierce, Rockford, USA) such as the microbicin-chrononic acid assay kit [27].

Plate screening for cellulases and xylanases

Screening for enzyme activity from pure cultures or rumen fluid can be performed by plating cells (or rumen fluid) onto 0.8% (w/v) agarose plates containing 0.2% (w/v) CMC or 0.2% (w/v) oat spelt xylan/birchwood xylan. While both forms of xylan are often used as substrates in xylanase assays, it should be noted that oat spelt xylan is less soluble and more heterogenous than birchwood xylan in terms of composition and linkages. After a 12 h incubation at 39°C, the plates are stained for 10 min with 0.3% (w/v) Congo red for CMC-containing and 0.5% (w/v) Congo red

for xylan-containing plates [30]. The zones of hydrolysis are then revealed by destaining the plates with 1 M NaCl for 10 min at 25°C. Total crude, periplasmic and pure enzymes are screened for their activity by coring a well into the substrate agarose plates [(1% (w/v) agarose, 1% (w/v) substrate (CMC or xylan) in 50 mM Na-citrate (pH 6.0)] and applying 5–10 μl of enzyme into the well. The plates are then incubated at 39°C for 4–12 h and stained with Congo red and washed as mentioned above.

Swollen cellulose preparation

Preparation of H_3PO_4-swollen cellulose (Avicel) and filter paper discs (Whatman No. 1) is performed according to the methods described by Wood [33] and Rasmussen et al. [26], respectively. Avicel or filter paper discs (20 g) prepared with a paper punch are incubated on ice in 100 ml H_3PO_4 for 1 h with occasional stirring. The mixture is then transferred to 8 l of ice-cold distilled H_2O and left on ice for 30 min. The swollen cellulose product is then washed several times with cold distilled H_2O by decantation and then with 1% (w/v) $NaHCO_3$ solution. Finally, a 1% (w/v) suspension of swollen cellulose in distilled H_2O is adjusted to a pH of 6.5–7.0 with 1 N NaOH. The swollen cellulose is then collected by centrifugation at 1000 *g* for 30 min. To estimate the dry weight of H_3PO_4-swollen cellulose, a 0.5 g wet sample is dried in an oven (at 170°C) and re-weighed.

Preparation of [U-^{14}C] cellulose

This technique is based on the method developed by du Preez and Kistner [8] and employs *Acetobacter xylinum*, which synthesizes cellulose during growth and forms a pellicle on the surface of a broth culture. To maintain the organism, transfer the culture at monthly intervals by removing the whole pellicle with a sterile loop and placing it in a fresh broth of GluPPY medium. Cap the tube tightly and shake briefly before removing the pellicle. Incubate the loosely capped tube at about 25°C.

The method for synthesis of [U-^{14}C] cellulose is as follows:

1. Dispense 150 ml sterile Glucose-Phosphate-Peptone-Yeast (GluPPY) medium, pH 6.3 [(g/l): yeast extract (Difco), 5; Peptone (Difco), 5; KH_2PO_4, 6.8; Glucose, 10] into disposable plastic tissue culture flasks with surface area of about 175 cm^2 (e.g. cat. No. 1–56502; A/S Nunc, Kamstrup, DK 4000 Roskilde, Denmark).
2. Dilute required amount of D-[U-^{14}C]-glucose into sufficient glass distilled water to yield 1 ml of glucose solution for each flask. Filter this solution into a sterile container using a sterile membrane with 0.2 μm pores. Aseptically dispense 1 ml of filtered glucose solution into each flask.
3. Immediately after dispensing medium and radiolabel into flasks, inoculate each with a single pellicle from a 5 ml culture that has been incubated for 7–10 d. Incubate flasks in the flat position with loosened caps at 30°C for 17–18 d.
4. Remove pellicles from the flasks and wash with distilled water until the washes are clear. Boil the pellicles in 2 M KOH for 20 min and wash them with distilled water until the washes are neutral. Mascerate in suitable small quantities in a kitchen

blender at high speed for 30 s. Recover the ^{14}C-cellulose after freeze-drying the slurry or use as fresh material in assays.

Assay for total cellulase activity in growing cultures of rumen inoculum using ^{14}C-cellulose

Medium preparation

- In the anaerobic cabinet pipet 3.0 ml of well-blended ^{14}C-cellulose pellicle material into Balch tubes. Ensure the suspension is well mixed during this operation and use cut pipette tip to prevent straining.
- Add 6.0 ml basal medium containing 30% clarified rumen fluid prepared according to medium formulation, stopper, seal and autoclave.
  NB: Pink (oxidized) colour disappears prior to stoppering in the cabinet.
- Weigh out exactly 10–12 g of rumen sample into 300 ml beaker in cabinet and dilute exactly 1/10 with chilled diluent i.e. by weight. Blend for 1.0 min with bar mixer (clean between samples).
- Pipet 1.0 ml into diluent tube (9.0 ml amount) i.e. this is 10^{-2} dilution, using wide-mouth tip, stopper, crimp seal and remove from cabinet
- Prepare remainder of dilution series on bench using syringe and 18 gauge needles. Fresh syringe and needle for each dilution.
- Use 1.0 ml of 10^{-3} dilution to inoculate marked tubes of ^{14}C-cellulose medium. Do this in triplicate and record t_0.

Sampling protocol for ^{14}C-activity

- Adjust sampling times based on cellulase activity in samples. For example, sample at 8, 13, 17, 20, 23, 26, 29, 32 and 36 h after t_0. Optimize sampling schedule to get as many points on the linear increase portion of curve. Use 8.0 h sample as background (normally 20–250 cpm).
- Remove 0.5 ml sample from each tube with 1.0 ml syringe and 18 gauge needle. Ensure sample is homogenous with that in tube. Use new syringe and needle for each sample.
- Centrifuge at 14 000 g for 15 min to pellet residual cellulose.
- Remove 400 μl supernatant taking care not to disturb pellet.
- Add 2.5 μl INSTAGEL (Perkin Elmer, USA) to screw-cap counting vial. Close and store in cold/dark until sampling complete i.e. until no residual cellulose.
- Count in scintillation counter: 2 cycles (5 min counting time).
- Mean triplicates for each time and plot.

Specific activity of ^{14}C-cellulose

1. Do DM determinations on tubes of prepared medium or ^{14}C-cellulose suspension (n = 10).
2. Do radioactivity measurements on acid-hydrolyzed cellulose suspension. Calculate cpm/mg cellulose.
3. Use max release of activity from live assay as an approximate analysis for this.

Amylase activity assay

Amylase activity is measured as an indication of starch degrading capacity. A modification of the dextrinogenic assay is used to estimate amylase activity [21]. A 1% solution of soluble starch is prepared in 0.1 M acetate buffer (pH 4.6). To each of two test tubes is added buffered starch substrate (5 ml), 0.1 M acetate buffer (pH 4.6, 3 ml) and 0.5 M $CaCl_2$ (1 ml). A third tube is used as a reagent blank. All tubes are equilibrated at 39°C, enzyme is added to one of the substrate tubes and incubated at 39°C for a further 10 min and then 1 N HCl (2 ml) is added to all three tubes. Enzyme is then added to the reagent blank and the undigested starch control tube. After thorough mixing, an aliquot (0.2 ml) from each tube is placed in volumetric flasks (50 ml) containing 1 N HCl (0.5 ml) and distilled H_2O (40 ml). Colour is developed in each flask by the addition of an iodine solution (0.1 ml; 3% KI or 0.3% I_2). Absorbance of the resultant blue solutions is measured at 620 nm.

One unit of alpha amylase activity is that amount of protein which will hydrolyse 10 mg starch per min. Specific activity is expressed as units per mg protein.

Protease activity assays

Cells for protease assays are separated from culture fluid by centrifugation (7000 *g* for 20 min at 4°C), washed and suspended in 0.1 M Bis-tris (*bis* [2-hydroxy-ethyl]imino-tris[hydroxymethyl]methane, pH 7) and re-centrifuged as described above. The cell pellet is resuspended in 0.01 its original volume and disrupted twice by ultrasonication with an ultrasonic disintegrator (Ultrasonic Industries Pty Ltd, Sydney) at 60 W for 10 min at a time. Centrifuged culture fluid was also assayed for protease activity.

Proteolytic activity in cell associated and extracellular fractions of cultures can be determined spectrophotometrically with azocasein as the substrate [2]. Controls are performed by incubating enzyme samples and the azocasein substrate separately and by combining these solutions at the time of acid addition as described by Cotta and Hespell [5]. One proteolytic enzyme unit equalled 1 μg of azocasein digested per h at 39°C. Extracellular proteolytic activity can be expressed per ml of culture fluid per unit absorbance of culture prior to centrifugation. Cell associated proteolytic activity can be expressed per g protein. Enzyme activities in culture fluid can be expressed per mg of microbial protein.

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