2.4. Anaerobic fungi

MICHAEL K. THEODOROU[1], JAYNE BROOKMAN[1] and ANTHONY P.J. TRINCI[2]
[1]*Institute of Grassland and Environmental Research, Plas Gogerddan, Aberystwyth, SY23, 3EB, UK*
[2]*School of Biological Sciences, 1800 Stopford Building, University of Manchester, Manchester, M13 9PL, UK*

Introduction

Although the rumen represents one of the most thoroughly investigated of all microbial ecosystems, more information is required about the size, diversity and function of the various cultivatable and non-cultivatable subgroups that constitute the rumen microflora. While microbial, molecular methodologies are developing at a considerable pace, and this will ultimately assist in the description of non-cultivatable forms, there is still a need to study the cultivatable forms, and to do this we need to grow and maintain their viability in axenic laboratory culture.

Anaerobic techniques and culture media

Many of the culture techniques used in rumen microbiology were first described by Hungate [15, 16]. Subsequently, Bryant [6], Miller and Wolin [24] and Balch and Wolfe [3] modified and added to them. With relatively few exceptions, these techniques, together with modifications of the anaerobic plating procedures of Leedle and Hespell [18], and the continuous-flow culture methodologies of Zhu et al. [37] are now used to culture the anaerobic fungi.

Anaerobic fungi are generally grown in small batch cultures in thick-walled glass tubes, serum tubes or serum bottles, sealed with butyl rubber stoppers. Cultures are grown with or without agitation, on soluble as well as insoluble carbon sources, under a head-space gas of 100% CO_2 or 70% CO_2 : 30% N_2. Mixtures of head-space gas are generally used to study the growth of anaerobic fungi in co-culture with methanogens [26]. Culture volumes generally range from 7 to 100 ml amounts, and colonized particulate material, as opposed to free zoospores, is preferred as the starting inoculum, as this leads to more vigorous culture growth and a substantial reduction in culture lag phase. In addition to growth in liquid and sloppy agar media [4, 20, 21, 28], agar roll tubes and Petri dish cultures have also been used to culture the anaerobic fungi [17, 19, 25].

The culture media used to grow anaerobic fungi are prepared using anaerobic procedures, and based on those used for the cultivation of rumen bacteria; for the

H.P.S. Makkar and C.S. McSweeney (eds.), Methods in Gut Microbial Ecology for Ruminants, 55–66.

most part and with the notable exception of the rumen fluid free medium of Lowe et al. [19], they are complex, non-defined media and contain rumen fluid. The media usually contain phosphate and/or bicarbonate buffers, the chemical reducing agents, sodium sulphide and/or L-cysteine hydrochloride, and resazurin, which is included as a redox indicator. Air is displaced from the medium by boiling and/or bubbling with O_2-free CO_2 gas. When using agar media and Petri dishes, air can be excluded during manipulations by use of an anaerobic chamber. Syringe and needle methods (see in what follows) are routinely used to transfer liquid and cultured materials from one container to another during inoculation and other manipulation procedures.

Medium C (modified Orpin's medium)

Medium C is used as a routine medium for maintenance of anaerobic fungi, as it supports good growth and is relatively simple to make. Use of a complex particulate carbon source (such as milled wheat straw), rather than just simple sugars (such as glucose or cellobiose), is advocated for routine cultures, as this provides a more vigorous and consistent growth and promotes greater stability of the fungal strains.

Solutions and reagents required
Clarified rumen fluid is prepared by squeezing rumen fluid obtained from fistulated cattle or sheep through muslin cloth and centrifugation at 25 000 *g* for 30 min at 4°C; the supernatant is retained. This rumen fluid is then stored at −20°C until required.
Salts solution I 3 g K_2HPO_4 in 1 l distilled H_2O. Store at 4°C in dark.
Salts solution II 3 g KH_2PO_4, 6 g $(NH_4)_2SO_4$, 6 g NaCl, 0.6 g $MgSO_4 \cdot 7H_2O$, 0.6 g $CaCl_2 \cdot 2H_2O$ in 1 l distilled H_2O; each component should be allowed to dissolve before addition of the next salt. Store at 4°C in dark.
Cysteine hydrochloride, Yeast extract, bacto-casitone and ammonium carbonate (see in what follows for amounts).
Resazurin solution (1000× stock solution). Dissolve 1 g in 1 l distilled H_2O. Store at 4°C in dark. The solution is blue under aerobic conditions and becomes pink then colourless with decreasing redox potential. Media are not used if they assume a pinkish tinge during preparation or storage. Inoculation sometimes gives a momentary pink colouration to the culture medium, but this should disappear within a few minutes.

Bottles and tubes. Serum bottles of 60 or 125 ml capacity for 45 and 90 ml media volumes and crimp-sealed serum tubes or screw-capped 'Hungate' tubes for smaller volumes (7–20 ml) are used. Serum bottles and serum tubes are sealed with butyl rubber stoppers plus crimped aluminium seals, whereas plastic caps are used for 'Hungate' tubes.

Carbon sources, such as glucose or cellobiose, should be added to the liquid medium at 4.5 and 5 g/l. Particulate substrates such as milled wheat straw should be placed in the tubes (1 g) or bottles (5/10 g) before dispensing of the liquid medium to give ∼10% (w/v) final concentration.

Preparation of medium C

1. Add 150 ml salt solution I; 150 ml salt solution II; 150 ml clarified rumen fluid; 2.5 g yeast extract; 10 g bacto-casitone; 6 g $NaHCO_3$ and 1 ml resazurin solution to a 2 l conical flask and make up to 1 l with distilled H_2O.
2. Place onto a heated stirrer and heat to 60°C with stirring (up to 1 h). Displace dissolved O_2 in the solution with O_2-free CO_2 bubbled vigorously through a submerged wide-bore (2 mm) stainless steel needle.
3. If using cellobiose or glucose as a carbon source, add to the medium.
4. Add 1 g cysteine hydrochloride to the medium to give a final reduction.
5. Prior to dispensing, flush tubes or bottles, with O_2-free CO_2 for ~1 min, then dispense medium, under a stream of CO_2 gas. After dispensing, gas tubes for a further minute (tubes) or two (bottles) to ensure that the head space is filled with CO_2 and then stopper firmly.
6. Sterilize media immediately by autoclaving at 115°C for 20 min.

Defined or semi-defined media are prepared in the same manner, and Lowe et al. [19] describe the composition of such media. Defined media are useful for experimentation where conditions need to be closely controlled such as radiolabelling studies or in work concerned with the addition of specific reagents and/or inhibitors. Medium C is perfectly compatible with most downstream procedures required from cultures, such as genomic DNA, RNA and protein extraction. Biomass for nucleic acid extractions is routinely produced by subculture of a wheat straw culture into a serum tube with cellobiose as the carbon source. After 3 d of incubation, the cellobiose culture is used to inoculate a 125 ml serum bottle with cellobiose as the carbon source, which is incubated for growth of biomass for 60 h for DNA preparation. Three time points are routinely used throughout the growth cycle of 20–24 h, 40–44 h and 60 h for RNA and/or protein samples.

Maintenance of cultures

Although Yarlett et al. [36] have developed cryopreservation methods for the long-term storage of anaerobic fungi, laboratory cultures usually require frequent subculturing in order to retain their viability. According to Milne et al. [25], most batch cultures of anaerobic fungi remain viable for 5 or 15 d in media containing glucose or wheat straw, respectively. Twice-weekly subculture is recommended, as described in what follows, although with experience, visual inspection of the cultures will determine the optimum frequency of subculture [20, 28, 29]. Fermentation gases will be produced during incubation of maintenance cultures grown on wheat straw, and this will raise the plug of wheat straw from the bottom of the tube to the top of the liquid phase. With increasing incubation, the fungal culture will enter stationary phase and the floating wheat straw plug will begin to sink to the bottom of the tube. The optimum time for use of the fungal culture as an inoculum for subculture is after the plug has reached the top of the liquid phase and before it begins to sink back again. These cultures are actively growing and approaching stationary phase, and they contain a

relatively high concentration of zoospores (ca. 10^3 zoospores/ml, [20]) or nucleated thalli. Slower growing cultures may take longer than the 3 d recommended above to be suitable for subculture.

Growth of cultures

Items required
A static incubator set at 39°C and CO_2 gassing apparatus are required for all subsequent procedures in this chapter but will not be specifically referred to below.
Tubes or bottles containing growth media, such as medium C, pre-warmed to 39°C for at least 1 h.
Hypodermic needles 4 cm long, wide-bore (0.2 mm) plus sterile plastic syringes (2 or 5 ml).

Inoculation
Inoculations are carried out on the bench using the Miller and Wolin [24] modifications to the method of Hungate [16] as below:
1. Flame the butyl rubber stoppers of both the inoculum culture and the fresh tube to be inoculated with 100% ethanol prior to insertion of the needle.
2. Shake the inoculum culture vigorously to disperse the fungal material within the culture. Tip the tube upside down and insert the needle into the tube. Withdraw 1 ml for inoculation of tubes or 2–10 ml for inoculation of serum bottles. Ensure that the inoculum is withdrawn at a rate which avoids the needle becoming blocked with particles of colonized wheat straw.
3. Inject the inoculum into the recipient tube or bottle and shake gently.
4. Incubate cultures at 39°C without agitation.

Preparation of long-term liquid nitrogen stocks

For long-term maintenance of anaerobic fungi, cultures are stored in liquid nitrogen using anaerobic glycerol as a cryoprotectant.

Items required
Cryovials, usually 1.8 ml (A/S Nunc, Roskilde, Denmark).
Sterile glass Pasteur pipettes and sterile disposable plastic loops.
10% glycerol: The 10% glycerol in double-distilled H_2O solution should be saturated with O_2-free CO_2 by bubbling for 10 min and then sterilized by autoclaving at 115°C for 20 min.

Preparation of stocks
1. Cultures for stock production are grown in tubes for 48 h with milled wheat straw as the major carbon source.
2. Under a stream of CO_2 and using aseptic technique, remove the plant/fungal plug from the culture tube using a sterile plastic disposable loop.

3. Transfer into a cryovial and cover in 1 ml 10% glycerol solution. Seal the vial and freeze by immersion in liquid nitrogen.
4. Cultures stored under liquid nitrogen remain viable for a considerable period of time; strains have been successfully resurrected after 10 years storage.

Reviving of cultures from liquid nitrogen stores

Items required
Tubes or bottles containing medium C growth media plus wheat straw, pre-warmed to 39°C for at least 1 h. To prevent bacterial contamination; the medium should be supplemented with chloramphenicol (5 mg/ml stock solution; 5 ml/l to give 0.025 mg/ml final concentration).
Sterile glass Pasteur pipettes and sterile disposable plastic loops.
1. Thaw vials at room temperature. As soon as the glycerol is melted, open the vial and remove the plug with a sterile glass Pasteur pipette.
2. Rapidly transfer the plant/fungal biomass into a pre-warmed serum tube (under a stream of O_2-free CO_2).
3. Gas the tube, replace the stopper and then incubate at 39°C without shaking.
4. Check the revival of the culture daily. Fermentation gases should be visible within 24–48 h of inoculation. Check the medium for bacterial contamination, which is manifested by clouding.
5. Subculture as above after 3–5 d.

Isolation procedures

The distribution of anaerobic fungi is restricted to the digestive tract of ruminant and non-ruminant herbivores where they are prevalent in fore-gut (rumen) and hind-gut digesta; they can also be isolated from saliva and faeces and from various other organs of the digestive tract, although their role in these situations remains unclear but is likely to be related to host transfer [8, 9, 23, 25].

Techniques to obtain fungal from these environments generally involve the use of antibacterial antibiotics to reduce bacterial numbers and some form of physical separation, such as the growth of isolated colonies on agar medium, to separate fungal colonies from bacterial contamination. Some procedures benefit by including a stage of enrichment culture, to increase the size of the fungal population relative to that of the bacterial and protozoal populations, prior to isolation. The single colony isolation methods of Bauchop and Mountfort [5] and Lowe et al. [19, 20] also ensure that axenic cultures are derived from just one fungal thallus or zoospore.

Sloppy agar media have been used for isolation procedures. For example, Orpin [28] overlaid particles of rumen digesta onto sloppy agar media and after incubation discarded the upper portion of the culture that contained fungal rhizoids while the lower portion, which contained migrating zoospores, was transferred to fresh sloppy agar medium. These new cultures were incubated and the earlier-mentioned process was repeated until successive subcultures yielded anaerobic fungi that were free from

contaminating bacteria. Orpin and his co-workers used the earlier-mentioned procedure to obtain *Neocallimastix*, *Piromyces* and *Caecomyses* isolates, and these fungi quickly became the subject of extensive research, from which many of the defining features and characteristics of the gut fungi were determined.

Joblin [17] developed a relatively straightforward procedure for the isolation of anaerobic fungi from rumen fluid. The method involved straining rumen fluid through muslin, preparing a dilution series of the filtrate and then mixing the appropriate, higher dilutions with a molten agar medium containing antibiotics. The inoculated agar medium was then used to prepare roll tubes [16], from which isolated fungal colonies could be obtained after a period of incubation. This technique was also used by Milne et al. [25], following enrichment culture, to obtain anaerobic fungi from faeces. The method outlined in what follows is a modified version of the procedure described by Joblin [17].

Isolation of axenic cultures of anaerobic gut fungi

Solutions and reagents required

Medium C agar: medium C containing 4.5 g/l glucose as a carbon source plus 18 g/l bacteriological agar.

Medium C with wheat straw and antibiotics: medium C as above plus 0.025 mg/ml chloroamphenicol from stock as above.

Medium C with cellobiose.

Sterile loops.

Rumen digesta or faeces: a source of fungi, such as rumen digesta contents from fistulated animals or a small amount of faecal material.

To isolate anaerobic fungi

1. Dispense 2 ml medium C agar into tubes under CO_2, seal and autoclave at 115°C for 20 min. Store at 4°C until required.
2. Inoculate source material into pre-warmed tubes containing medium C plus wheat straw and antibiotics. Incubate at 39°C for 3 d.
3. Melt medium C agar tubes at 50°C.
4. Add 0.5 ml mixed wheat straw culture to each agar tube. Immediately, roll the tube under cold water tap to give a thin layer of inoculated agar around the inside surface of the tube.
5. Incubate at 39°C for 2 d. Inspect for individual fungal colonies.
6. Open tube under CO_2 and pick individual colonies into medium C with cellobiose tubes.
7. Incubate at 39°C for 3 d.
8. Add 0.5 ml cellobiose culture to each agar tube. Immediately, roll the tube under cold water tap to give a thin layer of inoculated agar around the inside surface of the tube.
9. Repeat steps 5–8 again (on two occasions more) to obtain axenic, isolated cultures.

Enumeration procedures

Although it is difficult to estimate fungal biomass *in vivo*, counting procedures for the enumeration of fungal zoospores are relatively straightforward. By serially diluting samples of strained rumen fluid through antibiotic-containing molten agar in roll tubes and counting the thalli present after 20 h of incubation, Joblin [17] estimated that rumen fluid contained 2×10^4 viable zoospores/ml. This value was in good agreement with the zoospore population density of 3.5×10^4/ml as determined by counting zoospores with the aid of a microscope [30].

Theodorou et al. [33] developed most probable numbers (MPN) procedure for enumeration of anaerobic fungi as thallus forming units (TFUs). This technique, which relies on MPN statistical tables to provide a viable cell count, involves squeezing rumen contents through muslin and preparing a serial dilution of the filtrate in an anaerobic, antibiotic-containing, basal medium. Appropriate dilutions are then transferred to culture tubes containing a carbon source, incubated for up to 9 d and scored periodically for the presence or absence of anaerobic fungi. This procedure can be used with digesta and faecal samples, and the values obtained are generally equal to or higher than those recorded using either of the two zoospore-counting procedures described earlier. The centre of the raft region of the bovine rumen, for example, was found to contain 10^4–10^5 TFU/ml of rumen fluid while rumen fluid contained significantly lower populations.

Determining growth curves in batch culture

Optical density procedures have been used extensively as a method of choice for rapid and non-destructive determination of growth for microorganisms cultured on soluble substrates. However, where it is necessary to determine the growth of filamentous microorganisms on particulate substrates, optical density methods are often inappropriate. This is because of the spatial heterogeneity of the filamentous biomass and/or interference caused by the light scattering properties of the particulate substrates upon which the microorganisms are growing. This is a problem of considerable significance in the study of microbial ecology where it is often the norm for microorganisms to grow on, or at the expense of, particulate substrates.

Theodorou et al. [34] introduced a new technique to follow the growth of anaerobic fungi grown in batch culture on soluble and particulate substrates. This procedure uses a pressure transducer to measure and release the fermentation gases accumulating in the head space of culture bottles as a consequence of fungal growth in the liquid of the incubating cultures. The technique is straightforward and the equipment relatively inexpensive (Fig. 1), and as the method is non-destructive, it is possible to obtain an entire growth curve from individual culture bottles. Results from this technique are precise and reproducible [34], and the method has been used to demonstrate unique differences in the substrate colonization strategies adopted by rumen bacteria (surface erosion strategy) and rumen fungi (particle invasion strategy) [10]. The method

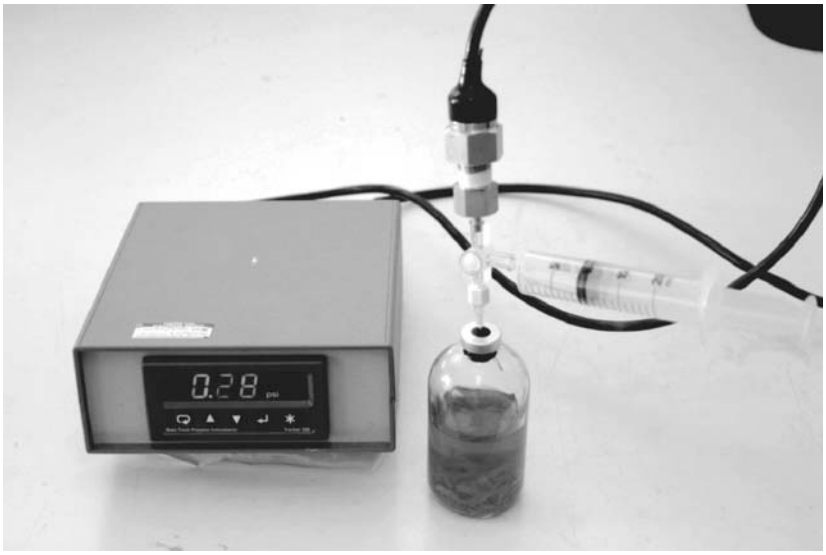

Figure 1. The pressure transducer assembly and a digital display unit for measurement of head-space gas pressure and volume (see Ref. [34] for further details).

can also be used as a screening technique to rank anaerobic fungi relative to their ability to degrade particular substrates [27] or to identify a particular time in the fungal growth cycle when cultures should be harvested, for example, for optimal nucleic acid extraction.

Continuous-flow culture

The rumen is an open ecosystem providing a relatively stable (steady-state) environment in which microorganisms grow at relatively high concentrations of digesta dry matter, within the region of 120 g/l [32]. For microorganisms to survive at such high substrate concentrations, it is essential to prevent the build-up of toxic end products in the rumen and reduce the development of adverse physiological conditions, including dramatic pH decline. This is achieved by salivary flow, the absorption of fermentation end products across the rumen epithelium and the flow of digesta to the lower tract. Under these conditions, and in the absence of any dramatic dietary perturbation, anaerobic fungal populations remain relatively constant, with an equilibrium between fungal biomass production in the rumen and the loss of fungi by death and/or passage from the rumen [11, 33].

In the laboratory, however, because of the difficulty of establishing rumen-like culture systems, anaerobic fungi are usually grown in closed batch cultures on particulate substrate concentrations of only 5–10 g/l dry matter, markedly below those found in the rumen. At these concentrations, growth of the fungus is initially rapid and not inhibited by the accumulation of fermentation end products [22]. As growth

proceeds, anaerobic fungi are subjected to a changing environment and ultimately fail to survive for more than a few days unless they are subcultured into fresh medium [21, 22, 25, 33].

A number of continuous culture systems have been used to grow rumen microorganisms. In general, they involve quite complicated pieces of apparatus in which rumen-like conditions are developed by using dialysis, sequestration or filtration techniques to remove culture liquid and preferentially retain plant and microbial biomass [1, 2, 12, 14, 35]. Although these systems have been used to grow rumen bacteria and protozoa, they have not been used to grow anaerobic fungi. The rumen simulation apparatus, Rusitec [7], has been used to grow anaerobic fungi on fragments of wheat straw in the presence of rumen bacteria [13]. A semi-continuous culture system has also been used for anaerobic fungi [31]; in this system, culture fluids were removed continuously while plant and fungal biomass were selectively retained.

Little attention has been given to the growth of anaerobic fungi on substrate concentrations approximating those found in the rumen. In a study by Zhu et al. [37], a simple continuous-flow culture system was used to investigate and compare the growth of *Neocallimastix hurleyensis*, on increasing concentrations of wheat straw. A unique feature of the continuous-flow system was its ability to operate up to 20 cultures at the same time, enabling comparative investigations of fungal growth against increasing levels of substrate concentrations (Fig. 2). Although not representative

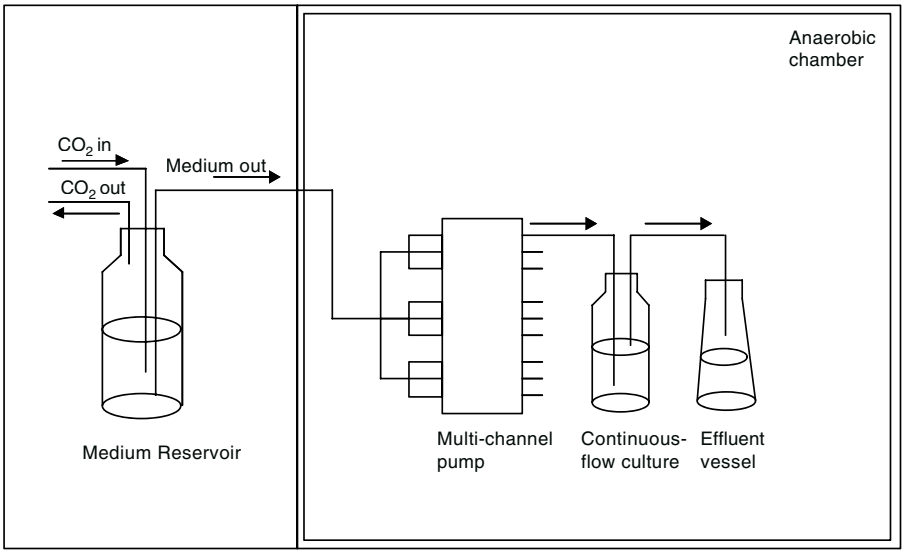

Figure 2. A schematic diagram of the continuous-flow culture system. A 20 l capacity medium reservoir is positioned external to an anaerobic chamber and constantly flushed with a stream of CO_2. Reduced medium from the reservoir is pumped by a multi-channel peristaltic pump to the continuous-flow cultures which contain various amounts of substrate. The culture vessels are operated at constant volume (100 ml), and effluent from the cultures collected individually in effluent collection vessels is kept inside the anaerobic chamber (see Ref. [37] for further details).

of a conventional continuous culture, continuous-flow culture provides a simple and effective means of growing anaerobic fungi on high concentrations of plant biomass approximating those found in the rumen.

Conclusions

The anaerobic fungi have stimulated considerable interest since the original communications of Orpin [28] and Bauchop [4]. Prompted by the initial-colonizer hypothesis of Bauchop [4] and by the uniqueness of the fungal anaerobes themselves, several research groups have developed the methodology for working with anaerobic fungi *in vitro* and *in vivo.* In this chapter, we have described some of the conventional techniques used for the isolation and maintenance of anaerobic fungi in laboratory culture alongside methods for estimating their growth and enumeration. This chapter is intended to provide a general overview of the methods employed to isolate, grow and maintain anaerobic fungi in axenic culture. It was also our intention to add some historical background to the subject area. While we recognize that the newer molecular methodologies add considerable new insight to the study of microbial ecology, it is currently impossible to elaborate phenotypic characteristics from genetic information alone. Consequently, the need to cultivate and study anaerobic fungi in axenic culture can never be completely replaced. Hence, this chapter is intended as a companion article to be read alongside other chapters in this manual which describe the subject area using more modern, molecular approaches and methodologies.

References

1. Abe, M., and F. Kumeno. 1973. In-vitro simulation of rumen fermentation: apparatus and effects of dilution rate and continuous dialysis on fermentation and protozoal population. *J. Anim. Sci.* **36:** 941–969.
2. Abe, M., and Y. Kurihara. 1984. Long-term cultivation of certain rumen protozoa in a continuous fermentation system supplemented with sponge materials. *J. Appl. Bacteriol.* **56:**201–213.
3. Balch, W.E., and R.S. Wolfe. 1976. New approach to the cultivation of methanogenic bacteria: 2-mercaptoethanesulfonic acid (HS-CoM)-dependent growth of *Methanobacterium ruminantium* in a pressurized atmosphere. *Appl. Environ. Microbiol.* **32:**781–791.
4. Bauchop, T. 1979. Rumen anaerobic fungi of cattle and sheep. *Appl. Environ. Microbiol.* **38:**148–158.
5. Bauchop, T., and D.O. Mountfort. 1981. Cellulose fermentation by a rumen anaerobic fungus in both the absence and presence of rumen methanogens. *Appl. Environ. Microbiol.* **42:**1103–1110.
6. Bryant, M.P. 1972. Commentary on the Hungate technique for culture of anaerobic bacteria. *Ann. J. Clin. Nutr.* **25:**1324–1328.
7. Czerkawski J.W., and G. Breckenridge. 1977. Design and development of a long-term rumen simulation technique (Rusitec). *Br. J. Nutr.* **38:**371–385.
8. Davies, D.R., M.K. Theodorou, A.E. Brooks, and A.P.J. Trinci. 1993. Influence of drying on the survival of anaerobic fungi in rumen digesta and faeces of cattle. *FEMS Microbiol. Lett.* **106:**59–64.
9. Davies, R.D., M.K. Theodorou, M.I.G. Lawrence, and A.P.J. Trinci. 1993. Distribution of anaerobic fungi in the digestive tract of cattle and their survival in faeces. *J. Gen. Microbiol.* **139:**1395–1400.
10. France, J., M.S. Dhanoa, M.K. Theodorou, S.J. Lister, D.R. Davies, and D. Isac. 1993. A model to inteprit gas accumulation profiles associated with *in vitro* degradation of ruminant feeds. *J. Theor. Biol.* **163:**99–111.

11. France, J., M.K. Theodorou, and D.R. Davies. 1990. Use of zoospore concentrations and life cycle parameters in determining the population of anaerobic fungi in the rumen ecosystem. *J. Theor. Biol.* **147:**413–422.
12. Gijzen, H.J., K.B. Zwart, P.T. van Gelder, and G.D. Vogels. 1986. Continuous cultivation of rumen microorganisms, a system with possible application to the anaerobic degradation of lignocellulosic waste materials. *Appl. Microbiol. Biotechnol.* **25**:155–162.
13. Hillaire, M.C., and J.P. Jouany. 1989. Effects of rumen anaerobic fungi on the digestion of wheat straw and the end products of microbial metabolism studies in a semi-continuous *in vitro* system, pp. 269–272. *In* J.V. Nolan, R.A. Leng, and D.I. Demeyer (eds.), *The Role of Protozoa and Fungi in Ruminant Digestion,* OECD/UNE International Seminar, Plenambul Books, Armidale, Australia.
14. Hoover, W.H., B.A. Crooker, and C.J. Sniffen. 1976. Effects of differential solid–liquid removal rates on protozoa numbers in continuous cultures of rumen content. *J. Anim. Sci.* **58:**692–700.
15. Hungate, R.E. 1966. *The Rumen and Its Microbes*. Academic Press, London.
16. Hungate, R.E. 1969. A roll tube method for the cultivation of strict anaerobes, pp. 117–132. *In* J.R. Norris and D.W. Ribbons (eds.), *Methods in Microbiology*, Vol. 3B. Academic Press, London.
17. Joblin, K.N. 1981. Isolation, enumeration and maintenance of rumen anaerobic fungi in roll tubes. *Appl. Environ. Microbiol*. **42:**1119–1122.
18. Leedle, J.A.Z., and R.B. Hespell. 1980. Differential carbohydrate media and anaerobic replica plating techniques in delineating carbohydrate-utilizing subgroups in rumen bacterial populations. *Appl. Environ. Microbiol.* **39:**709–719.
19. Lowe, S.E., M.K. Theodorou, A.P.J. Trinci, and R.B. Hespell. 1985. Growth of anaerobic rumen fungi on defined and semi-defined media lacking rumen fluid. *J. Gen. Microbiol.* **131:**2225–2229.
20. Lowe, S.E., G.W. Griffiths, A. Milne, M.K. Theodorou, and A.P.J. Trinci. 1987. Life cycle and growth kinetics of an anaerobic rumen fungus. *J. Gen. Microbiol.* **133:**1815–1827.
21. Lowe, S.E., M.K. Theodorou, and A.P.J. Trinci. 1987. Cellulases and xylanases of an anaerobic rumen fungus grown on wheat straw, wheat straw holocellulose, cellulose and xylan. *Appl. Environ. Microbiol.* **53:**1216–1223.
22. Lowe, S.E., M.K. Theodorou, and A.P.J. Trinci. 1987. Growth and fermentation of an anaerobic rumen fungus on various carbon sources and effect of temperature on development. *Appl. Environ. Microbiol.* **53:**1210–1215.
23. McGranaghan, P., J.C. Davies, G.W. Griffith, D.R. Davies, and M.K. Theodorou. 1999. The survival of anaerobic fungi in cattle faeces. *FEMS Microbiol. Ecol*. **29:**293–300.
24. Miller, T.L., and M.J. Wolin. 1974. A serum bottle modification of the Hungate technique for cultivating obligate anaerobes. *Appl. Microbiol.* **27:**985–987.
25. Milne, A., M.K. Theodorou, M.G.C. Jordan, C. King-Spooner, and A.P.J. Trinci. 1989. Survival of anaerobic fungi in faeces, in saliva and in pure culture. *Exp. Mycol.* **13:**27–37.
26. Mountfort, D.O., R.A. Asher, and T. Bauchop. 1982. Fermentation of cellulose to methane and carbon dioxide by a rumen anaerobic fungus in tri-culture with *Methanobrevibacterium* sp. Strain RA1 and *Methanosarcina barkari. Appl. Environ. Microbiol*. **44:**128–134.
27. Nielsen, B.B., W.-Y. Zhu, M.S. Dhanoa, A.P.J. Trinci, and M.K. Theodorou. 2002. Comparison of growth kinetics of anaerobic gut fungi on wheat straw in batch culture. *Anaerobe* **8:**216–222.
28. Orpin, C.G. 1975. Studies on the rumen flagellate *Neocallimastix frontalis*. *J. Gen. Microbiol.* **91:**249–262.
29. Orpin, C.G. 1976. Studies on the rumen flagellate *Sphaeromonas communis*. *J. Gen. Microbiol.* **94:**270–280.
30. Orpin, C.G. 1977. The occurance of chitin in the cell walls of the rumen organisms *Neocallimastix frontalis, Piromonas communis and Sphaeromonas communis*. *J. Gen. Microbiol.* **99:**215–218.
31. Teunissen, M.J., R.J.S. Baerends, R.A.G. Knelissen, H.J.M. Op den Camp, and G.D. Vogels. 1992. A semi-continuous culture system for production of cellulolytic and xylanolytic enzymes by the anaerobic fungus *Piromyces* sp. strain E2. *Appl. Microbiol. Biotechnol.* **38:**28–33.
32. Theodorou, M.K., S.E. Lowe, and A.P.J. Trinci. 1992. Anaerobic fungi and the rumen ecosystem, pp. 43–72. *In* G.C. Carroll and D.T. Wicklow (eds.), *The Fungal Community, Its Organization and Role in the Ecosystem*, 2nd ed. Marcel Dekker, Inc., NY.

33. Theodorou, M.K., M. Gill, C. King-Spooner, and D.E. Beever. 1990. Enumeration of anaerobic chytridiomycetes as thallus forming units: novel method for quantification of fibrolytic fungal populations from the digestive tract ecosystem. *Appl. Environ. Microbiol.* **56:**1073–1078.
34. Theodorou, M.K., D.R. Davies, B.B. Nielsen, M.I.G. Lawrence, and A.P.J. Trinci. 1995. Determination of the growth of anaerobic fungi on soluble and cellulosic substrates using a pressure transducer. *Microbiology* **141:**671–678.
35. Weller R.A., and A.F. Pelgrim. 1974. Passage of protozoa and volatile fatty acids from the rumen of the sheep and from a continuous *in vitro* fermentation system. *Br. J. Nutr.* **32:**341–352.
36. Yarlett, N.C., N. Yarlett, C.G. Orpin, and D. Lloyd. 1986. Cryopreservation of the anaerobic fungus *Neocallimastix patriciarum*. *Lett. Appl. Microbiol.* **3:**1–3.
37. Zhu, W.-Y., M.K. Theodorou, A.C. Longland, B.B. Nielsen, J. Dijkstra, and A.P.J. Trinci. 1996. Growth and survival of anaerobic fungi in batch and continuous-flow cultures. *Anaerobe* **2:**29–37.