ELSEVIER

Animal Feed Science and Technology
83 (2000) 205–221

ANIMAL FEED SCIENCE AND TECHNOLOGY

www.elsevier.com/locate/anifeedsci

An automated system for measuring gas production from forages inoculated with rumen fluid and its use in determining the effect of enzymes on grass silage

Z.S. Davies[a,b,*], D. Mason[a], A.E. Brooks[a], G.W. Griffith[b], R.J. Merry[a], M.K. Theodorou[a]

[a]*Institute of Grassland and Environmental Research, Plas Gogerddan, Aberystwyth, Wales SY23 3EB, UK*
[b]*Institute of Biological Sciences, Edward Llwyd Building, University of Wales, Aberystwyth,Wales SY23 3DA, UK*

Received 13 April 1999; received in revised form 3 November 1999; accepted 23 November 1999

Abstract

This paper describes an automated system that has been developed to measure the production of fermentation gas from ruminant livestock feeds inoculated with rumen fluid. The design of the apparatus and its method of use enables gas production to be determined from fresh, unprocessed plant material, as well as the more commonly used ground, particulate substrates, thus representing a closer simulation of forages consumed in vivo. The system consists of 48×140 ml bottles containing 100 ml buffered rumen fluid and 1 g of test substrate. Gas is produced as a consequence of the fermentation of the substrate. Gas, accumulating in the head-space of bottles, is released automatically, by use of pressure sensitive switches and solenoid valves, when a pre-determined pressure is reached. This prevents any build up of pressure in the fermentation bottle, which can affect the behaviour of the gas and the fermentation process. Gas accumulation profiles are produced as the fermentation proceeds and give information on forage digestibility and fermentation kinetics. In this paper, we describe the principles of the gas production technique and provide examples of how the automated system has been used in the evaluation of forages for ruminants. The results obtained show that the automated system is a useful tool for the determination of fermentation kinetics of ruminant feeds. It is simple to use and is considerably less labour intensive than manual gas measurement techniques.

Keywords: Gas production; Rumen; Digestibility; Silage; Fermentation

* Corresponding author. Tel.: +44-1970-828255; fax: +44-1970-828357.
E-mail address: zoe.davies@bbsrc.ac.uk (Z.S. Davies).

0377-8401/00/$ – see front matter
PII: S0377-8401(99)00138-8

1. Introduction

Since the late 1970s, measurement of in vitro gas production has become increasingly popular for determining forage digestion characteristics and the kinetics of fermentation (Theodorou et al., 1994, 1998). Gas production is measured from the in vitro digestion of forage with bicarbonate-buffered rumen fluid. Rumen micro-organisms ferment substrate to end-products including gases, carbon dioxide and methane, and volatile fatty acids (VFA), including acetate, propionate and butyrate (Beuvink and Spoelstra, 1992). As the culture medium used in gas production studies contains a bicarbonate buffer, and bicarbonate is present also in rumen fluid, the production of VFAs cause the release of carbon dioxide (titration gas) from the culture medium. Thus, the gas which is measured in gas production research contains a mixture of gases where the predominant gas (CO_2) is derived from both 'primary' fermentation and from the titration of acidic [H^+] fermentation end-products with basic [HCO_3^-] bicarbonate ion. The amount of gas produced depends on the amount of substrate fermented and the amount and molar proportions of the VFA produced (Beuvink and Spoelstra, 1992). The principles of techniques using gas production measurements have been reviewed by Theodorou et al. (1998) and Getachew et al. (1998).

A number of different systems have been used to measure gas production. Menke et al. (1979) described a method in which fermentations were conducted in 100 ml gas-tight, ground-glass syringe barrels and gas evolution was measured after 48 h of incubation. The technique was primarily used for end-point digestion studies, but by measuring the rate of assent of the plunger in the syringe barrel, information on the kinetics of digestion of the feedstuff was also obtained. More recently, Theodorou et al. (1994) described a simple gas production method using an electronic measuring procedure employing a pressure transducer to measure gas from incubations in 160 ml gas-tight culture bottles. Gas accumulated in the head-space of the bottles as the fermentation proceeded and was measured at regular intervals by a pressure transducer connected to a digital readout voltmeter, gas-tight syringe and needle. This method, although technically straight forward, was labour intensive since frequent readings were needed, especially over the initial stages of fermentation.

Several automated systems have been developed to measure gas production. Beuvink et al. (1992) used a liquid displacement system. In this system, a 100 ml serum bottle was connected to a water displacement bottle and collection vessel placed on a balance. Readings were taken every 25 min when the weight of liquid displaced by the gas was recorded and stored in a data-logger. The bottles were held in a shaking water bath throughout the fermentation.

Pell and Schofield (1993) described a gas production system using a series of closed 50 ml serum bottles, each with its own stirrer. Each bottle had its own individual pressure sensor that remained in place throughout the entire incubation. These pressure sensors were linked to an IBM-compatible computer.

In the system of Cone et al. (1994), each bottle was fitted with its own pressure transducer and electric micro-valve. The pressure transducer measured the pressure build up in each bottle until a pre-set upper value was reached (ca. 0.65 kPa). The valve then opened, allowing the pressure to fall back to a set limit (ca. 0.4 kPa). Every valve opening represented a known

amount of gas, so the number of valve openings was proportional to gas production. Each valve opened for just a fraction of a second (≈50 ms) (Cone et al., 1994, 1996; Cone, 1998).

These various systems have been used to monitor the fermentation characteristics of a range of different feedstuffs including silages (Beuvink and Spoelstra, 1994; Doane et al., 1997), tropical forages (Longland et al., 1995; Sileshi et al., 1996) straw (Prasad et al., 1994; Williams et al., 1996) and cereal grains (Opatpatanakit et al., 1994). Gas production has also been used to determine growth rates of anaerobic fungi on soluble and cellulosic substrates (Theodorou et al., 1995) and to elucidate the relative role of fungi and bacteria in the digestion of fibrous substrates (Davies, 1991; France et al., 1993). Gas production also allows the measurement of fermentation kinetics from soluble substrates, which is not possible by in situ techniques.

Due to the high labour input required for manual gas production systems, a new automated system, the automated pressure evaluation system (APES), was developed at the Institute of Grassland and Environmental Research (IGER) in the early 1990s. This was previously reported in abstract form by Davies et al. (1995). The system has since been modified and has now been used routinely for feed analysis and research for a number of years. The APES consists of 48×140 ml culture bottles connected to pressure sensitive switches and solenoid valves.

A comprehensive account of the APES is presented here, with examples of how it has been used in the evaluation of feeds for ruminants. The repeatability and reproducibility of the gas production profiles produced were assessed using freeze-dried and ground samples of perennial ryegrass (*Lolium perenne*). Examples are also given of the use of the APES in measuring gas production (i) from grass silages treated with cell-wall degrading enzymes and (ii) the effects that physical processing of samples has on the resultant gas production profiles.

2. Materials and methods

2.1. Rumen fluid inoculum

Two mature, rumenally-fistulated Clun forest sheep (mean weight 65 kg) were used as a source of rumen digesta. The sheep were fed Italian ryegrass (*Lolium multiflorum*) hay ad libitum, eating ≈1.5 kg per day, together with 0.15 kg per day of sheep coarse mix (Superstock, British Oil and Cake Meals (BOCM) Pauls, Ipswich, UK). Animals had free access to a clean supply of drinking water and a mineral block (Battle, Hayward & Bower, Lincoln, UK).

Rumen digesta were collected before the morning feed and immediately transported to the laboratory in a pre-warmed (ca. 39°C) vacuum flask. It was then strained through three layers of muslin, and the rumen fluid collected in a CO_2-filled flask. The solid residue remaining in the muslin was placed in a blender and homogenised for 60 s and then strained again through the muslin. This ensured that the resulting inoculum contained attached (fibre-associated) as well as unattached (free-floating) rumen micro-organisms. Throughout the process, the fluid was continually flushed with CO_2 gas and stirred with a magnetic stirrer and bar. The inoculum was dispensed immediately after preparation.

2.2. Culture media

A modified Van Soest medium was used, based on that described by Theodorou et al. (1994). This was prepared by mixing 500 ml distilled H_2O, 0.1 ml micro-mineral solution, 200 ml buffer solution, 200 ml macro-mineral solution and 1 ml resazurin solution (0.1%). A stream of oxygen-free CO_2 was bubbled through the medium for at least 6 h before use. The buffer solution contained 4 g NH_4HCO_3 and 35 g $NaHCO_3$ in 1 l of distilled water. The macro-mineral solution contained 9.45 g $Na_2HPO_4{\cdot}12H_2O$, 6.2 g KH_2PO_4 and 0.6 g $MgSO_4{\cdot}7H_2O$ in 1 l of distilled water. These were prepared freshly before use. The micro-mineral solution contained 13.2 g $CaCl_2{\cdot}2H_2O$, 10.0 g $MnCl_2{\cdot}4H_2O$, 1 g $CoCl_2{\cdot}6H_2O$ and 8 g $FeCl_3{\cdot}6H_2O$ in 1 l of distilled water. The micro-mineral and resazurin solutions were prepared beforehand and stored in the dark at 4°C until required. All chemicals were obtained from BDH, Poole, Dorset, UK, or Sigma, Poole, Dorset, UK.

2.3. Description of the automated pressure evaluation system (APES)

The automated pressure evaluation system (APES) consists of up to 12 racks of 4×140 ml Duran bottles (Schott Glaswerke, Mainz, Germany; Fig. 1). As the lids have the same diameter, the system may easily be modified to use Duran bottles of different

Fig. 1. One rack of four bottles of the automated pressure evaluation system (APES).

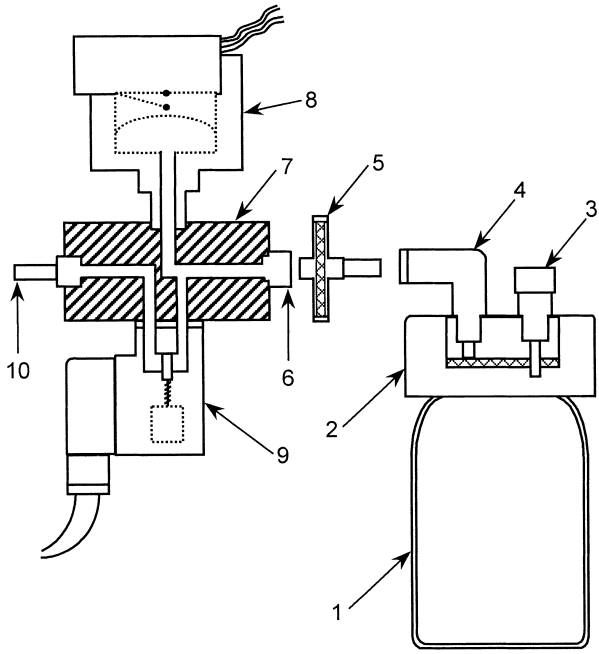

Fig. 2. Diagram showing the automated pressure evaluation system (APES). Key: (1) 140 ml culture bottle; (2) modified lid fitted with stainless-steel mesh and nylon gauze filter; (3) screw cap insert; (4) elbow union connection; (5) hydrophobic filter; (6) male luer lock fitting; (7) manifold; (8) pressure sensitive switch; (9) solenoid valve; (10) vent stem connector.

sizes if required. Racks were made from stainless steel sheets and were 210×290× 180 mm in dimension.

A cross-sectional diagram of the APES is shown in Fig. 2, with the individual components numbered. Culture bottles (1) were fitted with modified lids (2) which had an aperture through which microbial inoculum could be injected. A screw cap insert (3) (Drallion, East Sussex, UK) sealed this aperture during fermentation. A stainless-steel mesh and nylon gauze filter covered the inside of the lid. An elbow union connection (4) (R.S. Components, Corby, Northants, UK) connected the lid to a minisort hydrophobic filter (5) (Sartorius, Gottingen, Germany). The filter prevented any particles from the culture bottle reaching the pressure sensitive switch. A male luer lock fitting (6) (Omnifit, Cambridge, UK) connected the filter to the manifold (7) (IGER, Aberystwyth), and pressure sensitive switch (8) (Type 6731-65836-06, Herga, Bury St. Edmonds, Norfolk, UK). The pressure sensitive switch was connected to the solenoid valve (9) (R.S. Components) and vent stem connector (10) (R.S. Components) at the back of the manifold through which vented gas was released.

The racks of bottles were placed in a large incubator or temperature controlled room at 39°C. Each pressure sensitive switch was activated by a pressure of 4.5 kPa (0.65 psi), causing the solenoid valve to vent for three seconds, thus releasing the accumulated gas and returning the pressure in the head-space to ambient. The solenoid valves were linked

to a personal computer (Pentium 133, Pulsar, Penrhyncoch, UK) through parallel I/O cards (Racom, Cambridge, UK) for continuous monitoring of all bottles. The computer programme was designed and written at IGER in Quick Basic. It stored information from the inputs from each bottle then converted this into a form which gave vent profiles for each bottle. Results were displayed on the computer monitor throughout the fermentation, such that gas production could be monitored in real time. The progress of individual culture bottles could be monitored graphically and numerically, and the state of all bottles displayed simultaneously.

2.4. Operation of the APES

Culture fluid (85 ml) was dispensed using an automated dispenser (Accuramatic 5; Accuramatic, Watlington, King's Lynn, UK) into Duran bottles (capacity 140 ml) containing 1 g of substrate. Bottles containing no substrate were included as controls. Bottles were flushed with CO_2 gas before and during dispensing. Freshly prepared reducing agent (4 ml) was added to each bottle. This contained 0.625 g L-cysteine HCl, 4 ml 1 M NaOH and 0.625 g $Na_2S{\cdot}9H_2O$ dissolved in 100 ml of distilled water in a fume cupboard under nitrogen gas. Lids were tightly fitted onto the bottles and contained rubber ring inserts coated in silicone grease to provide a gas-tight seal. Pre-warmed bottles were inoculated with 10 ml of freshly prepared rumen fluid inoculum using a 10 ml syringe (Sterilin, Teddington, UK) fitted with a 0.8 mm×40 mm needle (Sabre International Products, UK). The bottles were incubated at 39°C until the end of the fermentation period, when gas production ceased (usually 2–5 days). Although gas profiles achieved an asymptote within 2–3 days, gas production, albeit minor, was sometimes still evident after this time, presumably due to lysis and fermentation of microbial cells under substrate-limiting conditions.

Each bottle was calibrated at the end of the fermentation period by measuring the exact volume of gas required to cause its switch to vent. This was done using a 5 cm calibration needle (blunt ended 18 gauge needle, Alfred Cox [Surgical], Coulsdon, Surrey) fitted to a three-way valve (Robinet three-way stopcock; Laboratories Pharmaceutiques, Vycon, BP 7-95440 Ecouen, France), with two 2.5 ml graduated plastic syringes (Sterilin, Teddington, UK) attached. Air was gently pushed into each bottle through the syringe until the switch was activated causing the valve to open. This was indicated both visually, by the green light located on the rack above each bottle which went out while the pressure switch was venting, and audibly by a click as the valve opened and closed. The volume of gas needed to cause the switch to vent was recorded (calibration volume). Measurements were taken in triplicate for each bottle. At all times during the development of this system we have paid attention to gas tight seals. Calibration at the end of the gas run ensures that if present, any leaks would be detected.

Measurements including VFA production (Merry et al., 1995), culture fluid pH and dry matter loss (Theodorou et al., 1994) were made at the end of the fermentation period.

2.5. Substrates

A sample of perennial ryegrass (*Lolium perenne*) cut after 4 weeks growth in July 1997 from field plots at IGER, Aberystwyth, was freeze dried and ground through a 1 mm dry

mesh screen. The grass sample was used to show the repeatability and the reproducibility of gas production profiles. Twenty-one replicate samples of ryegrass were used within the same experimental run, using the same rumen fluid to show repeatability of results within an APES fermentation experiment. The same ryegrass was then used to show the reproducibility of results between different fermentation experiments, using rumen fluid collected from the same sheep on different occasions. For this work, three replicate samples of ryegrass were used in each of seven different fermentation experiments.

Silage was prepared from perennial ryegrass (*Lolium perenne*; 320 g kg^{-1} DM) by Biotal, Cardiff. Herbage was chopped to 3–4 cm lengths then mixed and separated into 10 kg portions. Inoculants were applied as a fine spray, in 100 ml of liquid with thorough mixing of the herbage. Four treatments were used: (a) *Lactobacillus buchneri* plus a pectinase enzyme and a xylanase enzyme, with activities of 180 000 and 60 000 IU g^{-1}, respectively; (b) *Lactobacillus buchneri* plus a xylanase enzyme, activity 450 000 IU g^{-1}; (c) *Lactobacillus buchneri* plus a mixture of xylanase, β-glucanase, and amylase enzymes, with activities of 80 000, 45 000 and 10 000 IU g^{-1}, respectively; and (d) a distilled water only control. The *L. buchneri* inoculant was added at a rate of 10^5 cells per g of fresh herbage. Enzymes were added at a rate of 0.1 mg per kg of fresh herbage. All inoculants and enzymes were provided by Biotal. The treated herbage was packed into 15 l plastic barrels and sealed. After 90 days of ensilage the barrels were unpacked, the material well mixed and samples taken for fermentation using the APES. One 100 g sample of each of the four treatments was frozen but unprocessed (not ground) and another 100 g sample freeze dried and then ground to pass through a 1 mm dry mesh screen. Gas production from the inoculant and enzyme-treated and untreated silage samples was measured using both the unprocessed and dried, ground samples.

2.6. Curve fitting and statistical analysis

The APES produced a cumulative vent profile for each bottle, recording the number of vents and the time at which each vent took place. The cumulative vent total over time for each bottle was multiplied by the calibration volume for that bottle and divided by the dry weight of the sample to give the cumulative gas production over time per gram of dry matter. Cumulative gas production totals were then taken by linear interpolation at times throughout the fermentation, taking 30 data points (at time intervals of 0,1,2..6,8,10,12,15,18...72) and these data used for curve fitting. Gas production from blank bottles was not subtracted from the sample gas production profiles. The maximum likelihood programme (MLP; Ross, 1987) was used to fit curves to the gas accumulation profiles using the model of France et al. (1993):

$$y = A - BQ^t Z^{\sqrt{t}}$$

where $Q = e^{-b}$, $Z = e^{-c}$, and $B = Ae^{bT+c\sqrt{t}}$. In this equation, y denotes cumulative gas production (ml), t is incubation time (h), A the predicted asymptotic value for gas pool size (ml), T the lag-time (h), and b (h^{-1}) and c ($h^{-0.5}$) are rate constants. This model is fully explained in the paper by France et al. (1993).

The time taken to produce half the total gas pool (T_{50}) was calculated using the equation (France et al. (1999)):

$$t_p = \left[\frac{(-c/2 + \sqrt{[c^2/4 + b(bT + c\sqrt{T} - \log_e(1-n)]})}{b^2} \right]$$

The specific rate (K) at time (t) was calculated using the equation (France et al. (1993)):

$$K = b + \left(\frac{c}{2\sqrt{t^{0.5}}} \right)$$

Corrected R^2 was calculated, using the equation below, to show the fit of the model to the gas production profiles.

$$\text{Corrected } R^2 = \left(\frac{1 - \text{Residual mean squares}}{\text{Total mean squares}} \right) \times 100$$

The precision data, repeatability (r) and reproducibility (R) for the gas production profiles produced from triplicate samples of ryegrass in seven experiments were calculated using the statistical procedure of the International Standards Organisation (ISO, 1981). Repeatability errors measure the variabilities arising between replicates of a feed in a single run while reproducibility errors arise when analyses are performed on identical samples in different runs. According to the ISO (1981), repeatability (r) and reproducibility (R) are each the minimum value equal to or below which the absolute difference between single test results are expected to lie with a probability of 95%.

Differences between samples were analysed using the analysis of variance function in the Genstat statistical analysis package (Genstat 5 Committee, 1987). Comparisons between the treatments were made by calculating the least significant difference (LSD) using the standard error of the difference (s.e.d.) and the t-value at the appropriate degrees of freedom.

3. Results

3.1. Gas production from the fermentation of perennial ryegrass (Lolium perenne)

Twenty-one samples of dried, ground perennial ryegrass (*Lolium perenne*) were used in one fermentation experiment. To remove clutter from the graph showing the gas production profiles, the samples were split into seven groups of three replicates and the mean gas production profiles of these seven groups are shown in Fig. 3a. The values for parameters of the fitted curves are shown in Table 1. The perennial ryegrass showed a rapid fermentation. All the curves had a short lag phase of between 1.4 and 1.5 h and produced 50% of the total gas pool within 8 h of inoculation with rumen fluid. The derived asymptotes ranged from 251.9 to 267.9 ml of gas and the fractional rates at half asymptote were between 0.03 and 0.06. The VFA concentration of the culture fluid following gas production ranged from 72.2 to 80.1 mmol l^{-1}, with a mean of

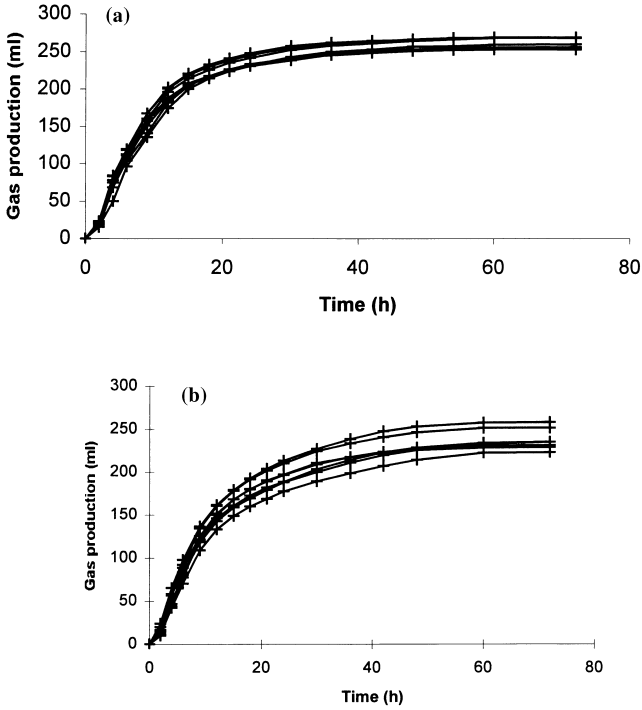

Fig. 3. Gas production profiles from (a) seven samples of perennial ryegrass within one fermentation series, and (b). from seven samples of perennial ryegrass using rumen fluid collected on seven different days (1–7). Lengend: mean values are shown (n=3). Standard errors were all <5% of mean. The standard deviation of the total gas production from all 21 samples in (a) were 236.4 and 13.6 ml, respectively, and in (b) were 259.6 and 9.8 ml, respectively.

75.5 mmol l^{-1}. The VFA molar proportions were 584 mmol mol^{-1} acetate, 277 mmol mol^{-1} propionate, 104 mmol mol^{-1} butyrate and 35 mmol mol^{-1} valerate. The DM loss ranged between 623 and 724 g kg^{-1} DM and the pH of the culture fluid from 6.5 to 6.6.

Fig. 3b shows the mean gas production profiles when three replicates of the dried, ground perennial ryegrass were tested on the APES on seven different occasions, using rumen fluid inoculum collected from the same animals but on different days. The values for parameters for the fitted curves are shown in Table 1. The perennial ryegrass samples again showed a rapid rate of fermentation, with lag times of between 1.3 and 1.7 h. Half the total gas pool was produced within 10 h for each sample. The derived asymptotes ranged from 224.3 to 260.7 ml of gas, showing a wider spread of values than when the samples were run within one experiment. Fractional rates at half asymptote ranged from 0.03 to 0.05. The repeatability (r) and reproducibility (R) values are also shown in Table 1. For all parameters, r is lower than R. The VFA concentration of the culture medium following gas production ranged from 67.8 to 89.2 mmol l^{-1}, with a mean value of 77.7 mmol l^{-1}. The mean molar proportions of VFA were 583 mmol mol^{-1} acetate, 0.277 mmol mol^{-1} propionate, 103 mmol mol^{-1} butyrate and 37 mmol mol^{-1} valerate.

Table 1
Fitted curve parameters from gas production curves of samples of perennial ryegrass[a]

Sample	Derived asymptote, A	Lag time, L	Time to reach 1/2 asymptote, T_{50} (h)	Fractional rate at T_{50}, K_{50} (h^{-1})
(a) Fitted curve parameters from seven sets of triplicate samples of 1 g of perennial ryegrass within one fermentation series				
1	267.4	1.5 a	6.5 a	0.06 a
2	267.9 a	1.5 a	6.6 a	0.05 a,b
3	266.7 a,b	1.5 a	7.0 a	0.05 a
4	255.2 a,b	1.5 a	6.6 a	0.05 a
5	249.9 b	1.5 a	6.9 a	0.05 a
6	251.9 a,b	1.4 a	6.8 a	0.04 b,c
7	258.2 a,b	1.5 a	7.6 b	0.03 c
s.e.d.	6.42	0.078	0.285	0.0063
(b) Fitted curve parameters from the incubation of 1 g of perennial ryegrass using rumen fluid collected on different days				
1	254.1 a	1.5 a	8.5 a	0.05 a
2	236.4 b	1.6 b	9.6 b,c	0.03 b
3	237.1 b	1.6 b,c	9.3 a,b,c	0.03b
4	232.6 b	1.5 b	8.2 a	0.04 c
5	228.5 b,c	1.7 c,d	8.6 a	0.04 c
6	260.7 a	1.3 e	8.8 a,b	0.03 b
7	224.3 c	1.7 b,d	10.0 c	0.03 b
s.e.d.	5.32	0.068	0.468	0.0024
r	43.76	0.424	1.750	0.0096
R	51.92	0.480	2.280	0.0215

[a] Mean values are shown (n=3). Values for each sample in the same column not bearing the same letters differ significantly (p<0.05). Corrected R^2 values for fitted curves were all over 99.5%.

The DM loss ranged between 687 and 794 g kg^{-1} DM and the pH of the culture fluid from 6.5 to 6.6.

3.2. Gas production from inoculated enzyme-treated grass silage

Gas production profiles were obtained from four dried, ground samples of grass silage inoculated with *L. buchneri* and given three different enzyme treatments, or untreated (Fig. 4a). The gas production profiles of the same silages, unprocessed are shown in Fig. 4b. The fitted parameters for these curves are shown in Table 2. When dried and ground substrates were used, all silages had similar gas production profiles. Lag times were low in all samples, between 0.5 and 1.6 h. The values for derived asymptotes ranged from 184.5 to 191.0 ml of gas and half the gas pool (T_{50}) was produced between 14.6 and 15.8 h. Fractional rates at half asymptote were all between 0.02 and 0.03. There were no significant differences ($p < 0.05$) in derived asymptotes or fractional rates between samples, though some differences in the parameters L and T_{50} were seen. Overall, the rates and extents of gas production from the dried, ground silage samples were not significantly different (p<0.05). However, when the same silage samples were evaluated

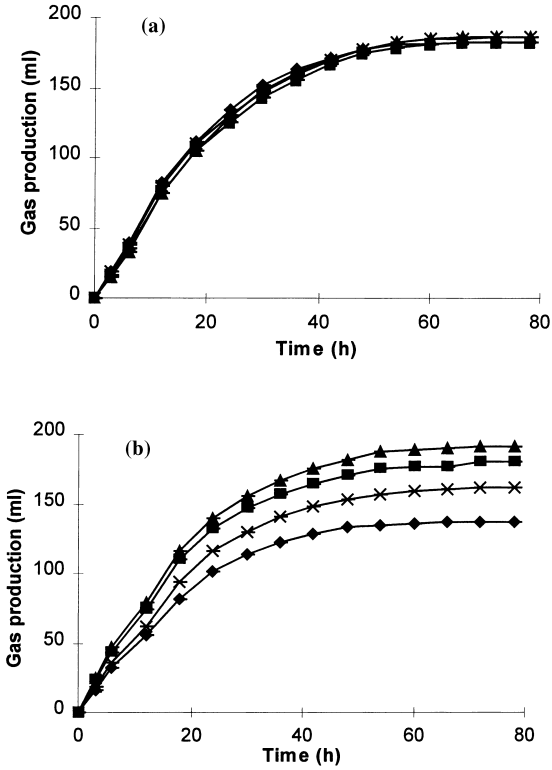

Fig. 4. Gas production profiles from (a) freeze-dried, ground grass silage and (b) from unprocessed grass silage. Legend: mean values are shown (n=4): -◆- untreated grass silage; -■- silage a treated with *L. buchneri*, pectinase and xylanase; -▲- silage b treated with *L. buchneri* and xylanase; -x- silage c treated with *L. buchneri*, xylanase, β-glucanase, galactomannanase and amylase. Standard errors were all <5% of mean.

using unprocessed material, significant differences in the gas production profiles of the different samples were apparent. Whereas the derived asymptotes for dried and ground substrates were not significantly different, all between 184 and 191 ml, those for the unprocessed samples ranged from 139 to 192 ml. The treated silage samples all produced significantly (p<0.05) more gas than the untreated silage. The derived asymptotes of silages treated with enzymes a, b and c were significantly higher (p<0.05) than the derived asymptote of the untreated silage. Lag times were between 1.2 and 1.8 h, with the untreated silage having the longest lag time. The times to reach half the asymptote were between 14.4 and 15.4 h and the fractional rates at half asymptote were between 0.02 and 0.03.

The mean VFA concentrations in the culture fluid following gas production, corrected for sample DM, for the unprocessed silages were 79.9, 86.2, 92.3 and 81.8 mmol l^{-1} for the control silage and silages treated with enzymes a, b, and c, respectively. These differences were significant (p<0.05) between the control and silages treated with enzymes a and b. The mean VFA concentrations in the culture fluid following gas

Table 2
Fitted curve parameters from freeze-dried, ground and unprocessed grass silage[a]

Sample	Derived asymptote, A	Lag time, L (h)	Time to reach 1/2 asymptote, T_{50} (h)	Fractional rate at T_{50}, K_{50} (h^{-1})
Dried, ground untreated	191.0 a	1.1 a,b	14.6 a	0.03 a
A	184.5 a	1.0 a,c	15.8b	0.02 a
B	187.7 a	1.6 b	15.8 b	0.02 a
C	188.2 a	0.5 c	15.2 a,b	0.02 a
s.e.d.	9.52	0.341	0.595	0.0010
Unprocessed untreated	138.7 a	1.8 a	14.8 a	0.03 a
A	179.8 b	1.2 b	14.4 a	0.03 a
B	191.9 b	1.2 b	14.5 a	0.03 a
C	162.1 c	1.7 a,b	15.4 a	0.02 a
s.e.d.	9.71	0.320	0.829	0.0020

[a] Mean values are shown, with standard deviations (n=4) from: untreated grass silage; silage A treated with *L. buchneri*, pectinase and xylanase; silage B treated with *L. buchneri* and xylanase; silage C treated with *L. buchneri*, xylanase, β-glucanase, galactomannanase and amylase. Values for each sample in the same column not bearing the same letters differ significantly (p<0.05). Corrected R^2 values for fitted curves were all over 99.5%.

production for the freeze dried and ground silages were 96.2, 96.0, 91.01 and 91.0 mmol l^{-1} for the control silage and silages treated with enzymes a, b, and c, respectively, and there were no significant differences between samples (p<0.05). The molar proportions of VFA in the culture fluid were 612 mmol mol^{-1} acetate, 274 mmol mol^{-1} propionate, 69 mmol mol^{-1} butyrate and 40 mmol mol^{-1} valerate. DM losses were between 750 and 810 g kg^{-1} DM and the differences between samples were not significant (p<0.05). The pH values of the culture medium following gas production was between 6.6 and 6.7 for all samples with no significant differences between samples.

4. Discussion

4.1. Comparison of APES with other gas measurement techniques

The APES was developed as a tool that could be used both for research and as a screen for feed evaluation. This method has the advantage of being less labour intensive than manual systems, such as the pressure transducer technique described by Theodorou et al. (1994), and thus it permits more cost-effective throughput. It is simple to set up and use and once running, needs no manual input until the fermentation is complete. The progress of fermentations can be monitored graphically in real time throughout the fermentation and this permits changes in the status of individual bottles to be seen instantly. This function could be particularly useful when investigating the effect of additives on rumen fermentation (Lowman, 1998).

In the APES, gas is automatically vented from the bottles by sensitive pressure switches when the pressure reaches a value of 4.5 kPa. The system of Cone et al. (1994) also vents automatically but uses pressure transducers and vent gas at 0.65 kPa. This is equivalent to ca. 2 ml of gas in the APES system and ≈0.7 ml in the system of Cone et al. (1994). From an economic standpoint, the pressure sensitive switches used in the APES are much less expensive than pressure transducers (£26 for pressure sensitive switches (Herga, Norfolk, UK) compared to £200 per bottle for digital pressure transducers (Digital Pressure Switch, SMC, Eastville, Bristol, UK). These systems contrast with that of Pell and Schofield (1993) where bottles are not vented during the fermentation. Many different types of switches were tested during the development of the APES, and the switches finally used (Herga, Norfolk, UK) were specially made by the company to our specification.

The APES system is calibrated after each run, as is the automated system of Pell and Schofield (1993). Calibration of bottles in the system devised by Cone et al. (1994) is fortnightly. Calibration after each run has the advantage that it takes into account small differences in the volume of liquid and substrate added and thus the head space volume between bottles.

In the rumen, the digesta are agitated by ruminal contractions and the automated systems of Pell and Schofield (1993) and Cone et al. (1994) simulate this by shaking or stirring the culture bottles. Pell and Schofield (1993) have suggested that agitation prevents supersaturation of solutions with CO_2. In the APES system however, the bottles are not shaken but as they are continually vented, supersaturation of solutions is unlikely to occur (Morris, 1983; Lowman, 1998). Bottle contents are mixed when the medium is added and adding the inoculum also mixes the contents of the bottle. The bottles are continuously vented and gas is driven out of solution so this encourages motion of the particles in the bottle throughout the fermentation as their buoyancy changes. Moreover, Lowman (1998) found that gas production was higher in bottles that were not shaken compared to bottles that were shaken either intermittently, after every gas reading as by Theodorou et al. (1994) or continually, on an orbital shaker at 115 rpm. This is possibly because the micro-organisms could not establish on the feed particles in shaken bottles as effectively as those in bottles which were not shaken. Reduced gas production in shaken bottles may also be the result of increased CO_2 solubility in the culture medium due to the increased gas–liquid interface produced by the shaking movement.

The APES may be conveniently housed in an incubator or temperature controlled room at 39°C, whereas the system of Cone et al. (1994) is placed in a water bath. Another difference between the APES and the system of Cone et al. (1994) is the quantity of substrate and culture medium used. The APES has 100 ml culture fluid in a 140 ml bottle. The system of Cone et al. (1994) uses 60 ml of culture fluid in a 250 ml bottle. These differences between the various gas production systems mean that precise comparisons of resultant gas profiles are difficult to make.

4.2. Repeatability and reproducibility of gas production profiles

The 21 samples of ryegrass tested within one fermentation experiment showed that the APES gave highly repeatable results. Differences in gas production profiles between

experiments were attributed to differences in rumen fluid taken on different days, therefore it is recommended that a standard sample is used to correct for differences between runs. The range of gas production values for comparable parameters between experiments was much greater than the range within an experiment. The range of VFA concentrations in the culture fluid was also greater between experiments than the range within an experiment. The results shown in Fig. 2 and Table 1 indicate that it will be necessary to include a standard feed sample as a control in each experiment in order to correct for differences due to variations in rumen fluid between experiments. All the values for the fitted curve parameters fell within the limits of the repeatability and reproducibility values, shown in Table 1.

4.3. Gas production from grass silage

No differences between gas production of inoculant and enzyme-treated silage and untreated silage samples were apparent when freeze-dried and ground substrate was tested. However, considerable differences between the gas production profiles of the treated and untreated substrates were evident when unprocessed (not ground, not freeze-dried) material was used. Silage samples which had been treated with cell-wall degrading enzymes had higher total gas pools than the untreated silage suggesting that the cell-wall degrading enzymes had increased the forage digestibility. There were also higher levels of VFA in the culture fluid from the enzyme-treated silages than the control silage. Silage b, treated with *L. buchneri* and xylanase enzyme showed the greatest increase in gas production compared to the control, and also had the highest VFA concentration. This treatment had the highest enzyme activity of the three treatments. These differences were not shown when freeze-dried and ground material was used, probably because in the ground substrates particle size reduction increased the surface area for attachment of rumen micro-organisms (Davies, 1991), thereby masking the significant differences between treated and untreated samples. Ruminants do not consume small dry feed particles so the use of intact material makes the evaluation closer to the situation in vivo. These results differ from those of Beuvink and Spoelstra (1994) who measured gas production from dried, ground grass silages treated with a range of cell-wall degrading enzymes. Their enzyme-treated silages had a shorter initial lag phase and lower maximum gas production rate than the untreated silages although the total amount of gas produced was not altered by enzyme treatment. The evidence in the literature on enzyme treatment effects is equivocal and effects are dependent on many factors including crop species, maturity, dry matter and ensiling conditions (van Vuuren et al., 1989; Jacobs and McAllan, 1991; Stokes, 1991; Adogla-Bessa and Owen, 1995). This study has shown that the effects of sample processing may have an influence, although further work is needed in this area.

Most previous in vitro gas production studies have used substrates which were dried and finely ground prior to fermentation (Pell and Schofield, 1993; Beuvink and Spoelstra, 1994; Theodorou et al., 1994). From the outset, the APES was designed to be used with intact forages and, thus wide-necked bottles were chosen for ease of charging with substrates. Nevertheless, one possible problem with the use of fresh or unprocessed material is that the degree of heterogeneity of the sample is increased. Care must be taken

to mix well before sampling. A larger sample may be needed to ensure a representative sample or a larger number of replicates could be used if variability of results became a problem.

5. Conclusions

The APES has been used routinely at IGER for several years, and is now being taken up by other laboratories within Europe. As readings are recorded automatically using pressure switches, which detect small increases in pressure, the method is less labour intensive and more sensitive than manual techniques. The gas build up in fermentation bottles on the APES is dependent on pressure rather than time, as in the manual pressure transducer technique (Theodorou et al., 1994). This means the pressure in the bottles never goes above the set level of 4.5 kPa. This avoids problems associated with the increased solubility of gas at increased pressure (Henry's law; Theodorou et al., 1998). The APES has considerable potential for use in the evaluation of the nutritive value of ruminant feed and feed constituents. Gas production data may be used along with rumen models to get the full benefit from the system.

Acknowledgements

M.S. Dhanoa is acknowledged for his help with curve fitting and statistical analysis and David Meade for his work on the APES electronics and computer. Part of this research was funded by a Teaching Company Scheme partnership between IGER, The University of Wales, Aberystwyth and Biotal Ltd.

References

Adogla-Bessa, T., Owen, E., 1995. Ensiling of whole-crop wheat with cellulase-hemicellulase based enzymes. 1. Effect of crop growth stage and enzyme on silage composition and stability. Anim. Feed Sci. Technol. 55, 335–347.

Beuvink, J.M.W., Spoelstra, S.F., 1992. Interactions between substrate, fermentation end-products, buffering systems and gas production upon fermentation of different carbohydrates by mixed rumen microorganisms in vitro. Appl. Microbiol. Biotechnol. 37, 505–509.

Beuvink, J.M.W., Spoelstra, S.F., 1994. In vitro gas production kinetics of grass silages treated with different cell-wall degrading enzymes. Grass Forage Sci. 49, 277–283.

Beuvink, J.M.V., Spoelstra, S.F., Hogendorp, R.J., 1992. An automated method for measuring time-course of gas production of feedstuffs incubated with buffered rumen fluid. Neth. J. Agric. Sci. 40, 401–407.

Cone, J.W., Beuvink, J.M.W., Rodrigues, M.A.M., 1994. Use and applications of an automated time related gas production test for the in vitro study of fermentation kinetics in the rumen. Rev. Port. Zootec. 1, 25–37.

Cone, J.W., 1998. The development, use and application of the gas production technique at the DLO Institute for Animal Science and Health (AD-DLO), Lelystad, The Netherlands. In: Deaville, E.R., Owen, E., Adesogan, A.T., Rymer, C., Huntington, J.A., Lawrence, T.L.J. (Eds.), In vitro Techniques For Measuring Nutrient Supply To Ruminants. Occasional publication No. 22 British Society of Animal Science, pp. 65–78.

Cone, J.W., van Gelder, A.H., Visscher, G.J.W., Oudshoorn, L., 1996. Influence of rumen fluid and substrate concentration on fermentation kinetics measured with a fully automated time related gas production apparatus. Anim. Feed Sci. Technol. 61, 113–128.

Davies, D.R., 1991. Growth and survival of anaerobic fungi in batch culture and in the digestive tract of ruminants. PhD Thesis, University of Manchester.

Davies, D.R., Theodorou, M.K., Baughan, J., Brooks, A.E., Newbold, J.R., 1995. An automated pressure evaluation system (APES) for determining the fermentation characteristics of ruminant feeds. Ann. Zootech. 44, 36.

Doane, P.H., Schofield, P., Pell, A.N., 1997. Neutral detergent fiber disappearance and gas and volatile fatty acid production during the in vitro fermentation of six forages. J. Anim. Sci. 75, 3342–3352.

France, J., Dhanoa, M.S., Theodorou, M.K., Lister, S.J., Davies, D.R., Isac, D., 1993. A model to interpret gas accumulation profiles associated with in vitro degradation of ruminant feeds. J. Theor. Biol. 163, 99–111.

France, J., Dijkstra, J., Dhanoa, M.S., Lopez, S., Bannink, A., 1999. Estimating the extent of degradation of ruminant feeds from a description of the gas production profiles observed in vitro 2: derivation of models and other mathematical considerations. Br. J. Nutr. (in press).

Genstat 5 Committee, 1987. Genstat reference manual. Statistics department, Rothamsted Experimental Station. Clarendon Press, Oxford, UK.

Getachew, G., Blummel, M., Makkar, H.P.S., Becker, K., 1998. In vitro gas measuring techniques for assessment of nutritional quality of feeds: a review. Anim. Feed Sci. Technol. 72, 261–281.

ISO, 1981. International Standard ISO 5725. Precision of test methods — Determination of repeatability and reproducibility by inter-laboratory tests, International Organisation for Standardisation, Switzerland.

Jacobs, J., McAllan, A.B., 1991. Enzymes as silage additives. 1. Silage quality, digestion, digestibility and performance in growing cattle. Grass Forage Sci. 46, 63–73.

Longland, A.C., Theodorou, M.K., Sanderson, R., Lister, S.J., Powell, C.J., Morris, P., 1995. Non-starch polysaccharide composition and in vitro fermentability of tropical forage legumes varying in phenolic content. Anim. Feed Sci. Technol. 55, 161–177.

Lowman, R.S., 1998. Investigations into the factors which influence measurements during in vitro gas production studies. PhD thesis, University of Edinburgh.

Menke, K., Raab, L., Salewski, A., Steingass, H., Fritz, D., Schneider, W., 1979. The estimation of digestibility and metabolizable energy content of ruminant feedingstuffs from the gas production when they are incubated with rumen liquor in vitro. J. Agric. Sci. Camb. 93, 217–222.

Merry, R.J., Dhanoa, M.S., Theodorou, M.K., 1995. Use of freshly cultured lactic acid bacteria as silage inoculants. Grass Forage Sci. 50, 112–123.

Morris, J.G., 1983. A Biologists Physical Chemistry, 2nd Edition. Edward Arnold, London

Opatpatanakit, Y., Kellaway, R.C., Lean, I.J., Annison, G., Kirby, A., 1994. Microbial fermentation of cereal grains in vitro. Aust. J. Agric. Res. 45, 1247–1263.

Pell, A.N., Schofield, P., 1993. Computerized monitoring of gas production to measure forage digestion in vitro. J. Dairy Sci. 76, 1063–1073.

Prasad, C.S., Wood, C.D., Sampath, K.T., 1994. Use of in vitro gas production to evaluate rumen fermentation of untreated and urea treated finger millet straw (*Eleusine coracana*) supplemented with different levels of concentrate. J. Sci. Food Agric. 65, 457–464.

Ross, G.J.S., 1987. MLP: Maximum Likelihood Program (A manual) Rothamsted Experimental Station, Harpenden, Herts.

Sileshi, Z., Owen, E., Dhanoa, M.S., Theodorou, M.K., 1996. Prediction of in situ rumen dry matter disappearance of Ethiopian forages from an in vitro gas production technique using a pressure transducer, chemical analyses or in vitro digestibility. Anim. Feed Sci. Technol. 61, 73–87.

Stokes, M.R., 1991. Effects of an enzyme mixture, an inoculant, and their interaction on silage fermentation and dairy production. J. Dairy Sci. 75, 764–773.

Theodorou, M.K., Davies, D.R., Nielsen, B.B., Lawrence, M.I.G., Trinci, A.P.J., 1995. Determination of growth of anaerobic fungi on soluble and cellulosic substrates using a pressure transducer. Microbiology 141, 671–678.

Theodorou, M.K., Lowman, R.S., Davies, Z.S., Cuddeford, D., Owen, E., 1998. Principles of techniques that rely on gas measurement in ruminant nutrition. In: Deaville, E.R., Owen, E., Adesogan, A.T., Rymer, C.,

Huntington, J.A., Lawrence, T.L.J. (Eds.), In vitro Techniques for Measuring Nutrient Supply to Ruminants. Occasional publication, No. 22 British Society of Animal Science, pp. 55–63.

Theodorou, M.K., Williams, B.A., Dhanoa, M.S., McAllan, A.B., France, J., 1994. A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. Anim. Feed Sci. Technol. 48, 185–197.

van Vuuren, A.M., Bergsma, K., Frol-Kramer, F., van Beers, J.A.C., 1989. Effects of addition of cell wall degrading enzymes on the chemical composition and the in sacco degradation of grass silage. Grass Forage Sci. 44, 223–230.

Williams, B.A., Chuzaemi, S., van Bruchem, J., Boer, H., Tamminga, S., 1996. A comparison of ten rice-straw varieties grown at two different altitudes during a wet and a dry season, using the in vitro cumulative gas production technique. Anim. Feed Sci. Technol. 57, 183–194.