Method for Measuring Gas Production Kinetics

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Introduction

Methodology can play a critical role in the measurement of digestion kinetics, especially when the objective is to define kinetic parameters for feeds when formulating rations or modeling animal responses. Measurement of gas production kinetics provides the opportunity to evaluate the rate of digestion of the soluble, more rapidly fermenting fractions of feeds. However, differences among procedures that have little impact on digestion of dry matter after 48 h of incubation may have dramatic effects on fermentation of soluble matter during the first 20 h. Our objective was to develop a method for measuring the kinetics of gas production that would minimize any detrimental effects associated with the in vitro system and provide estimates of digestion kinetics that can be used to both describe feeds for ration formulation systems and provide parameters for models of ruminal digestion.

Method Description and Justification

The capacity of the incubator, stirring system, and data acquisition system developed at Cornell University was increased from 16 to 40 vials to accommodate experimental designs that include multiple treatments and blanks within runs. During inoculation, vials are kept upright to prevent particles of the sample from sticking to sides of the vial and being out of contact with the fermenting media. After several uses, needles attached to the transducers became dull and often were bent when inserted into incubation vials. Attachment of needles to transducers was modified so needles could be replaced easily. Luer lock adapters were glued to the pressure transducers by using the end of a plastic tuberculin syringe as a connector. A thin coating of stopcock grease applied to the inside of the hub of each 18 gauge needle provided a gas-tight seal. Needles were replaced after 4 uses and the gas-tight seal of new needles was verified by injecting 10 mL of air into an empty vial attached to each needle and observing no drop in pressure overnight.

Transducers that measure pressure relative to ambient were used because they were more economical than absolute pressure transducers. However, relative pressure transducers are affected by building and ambient barometric pressure changes. Four sealed vials were used to monitor changes in background pressure and the data were used to correct for these changes. Pressure transducers were calibrated by injecting pre-heated (39ºC) CO_2 gas into vials containing 10 mL of in vitro media and recording voltage changes. Five injections of 10 mL of CO_2 gas were spaced at 3.5 h intervals to generate calibration curves. Voltages, adjusted for background pressure changes and differences in vial volume were regressed against the mL of CO_2 injected to obtain a calibration coefficient for each transducer. Media containing phosphate-bicarbonate buffers, trace minerals, macrominerals, ammonia, and trypticase was used that could maintain pH above 6.2 when 40-60 mg of substrate was fermented and gasses were not released during a 96 h fermentation.

On the day before inoculation, 40 to 100 mg of sample was weighed into a 50 mL serum bottle (previously weighed and calibrated for volume) containing a small (1015 mm) stirring bar of known volume. On the morning of inoculation, 400 mL of media containing rezasurin was mixed and a 0.7 mL of media was added to each vial to wet the sample and minimize floating. Vials were swirled and placed in a water bath (39ºC). Each vial was connected to a manifold and continuously purged with CO_2. An additional 5.0 mL of media was added to each vial while they were in the water bath. Reducing solution (sodium sulfide and cysteine) was prepared and 0.3 mL was added to each vial. Vials were kept in the water bath, but were disconnected from the manifold and lightly capped. Reduction of the media and sample prior to inoculation is critical to minimize the shock to strictly anaerobic micro-organisms and the resulting lag period.

While samples were reducing, 1200 mL of ruminal fluid and 800 mL of solids were collected from a fistulated cow into a warmed insulated container. In the lab, the top 20 cm of solids were discarded and the remaining fluid and solids were squeezed through 2 layers of cheesecloth. One hundred grams of squeezed solids were placed in a blender with 200 mL of previously chilled and reduced media and blended for 45 sec. Two hundred mL of strained ruminal fluid was filtered through 4 layers of cheesecloth, the contents of the blender were added and squeezed tightly. Blending of solids with chilled media instead of ruminal fluid eliminates potential damage to bacteria in ruminal fluid during blending and improves the likelihood of detaching particle-bound bacteria. The inoculum was warmed to 39ºC while being continuously stirred and purged with CO_2.

After samples were reduced as indicated by colorless rezasurin indicator, 4 mL of inoculum was added while purging each vial with CO_2. Vials were capped with flanged butyl rubber stoppers and sealed with aluminum crimps. Two people can inoculate and connect 40 samples within 20 min., and the time between collection of ruminal contents and inoculating the last sample should be less than 40 min. After 6 h, all vials were gently swirled to mix material that was floating and to loosen the ring of residue from the sides of the vial. Vials were swirled every 24 h until the end of fermentation being careful to insure that material did not creep up the side of the vial. Voltages were recorded using a computer data acquisition system every 0.01 h for the first 0.5 h, every 0.10 h for the next 5 to 6 h and every 0.5 h until 96 h of fermentation. When fermentation was complete, vials were equilibrated to room temperature for 1-2 h and centrifuged at 220Xg for 30 min. The stopper was removed, a sample of the fluid was collected for VFA analysis, the contents were mixed and pH was taken as rapidly as possible. Dry matter and neutral detergent fiber of the residue remaining were determined.

Discussion

Evaluating in vitro methodology is complicated because the reference value to be used for comparison is arbitrary and because there are many interactions between substrates and the *in vitro* system that make it difficult to insure that any system is acceptable in all situations. The criterion of maximal digestion kinetics suggests that any in vitro method that does not achieve maximal rates and extents of fermentation and minimal lag times clearly does not measure digestion kinetics that is limited by feed characteristics. In previous experiments, in vitro system using continuous gassing of flasks obtained the fastest fractional rates, shortest discrete lag times, and smallest indigestible residues for fiber digestion compared to other systems. The use of reducing agents to remove oxygen from the sample and media before inoculation, blending of ruminal solids to detach bacteria, and including trace minerals and trypticase in the media also have been observed to maximize fiber digestion.

In the in vitro gas production system, the use of chilled media to detach bacteria from ruminal solids with blending was observed to slightly decrease lag and increase rates of gas production. Complete reduction of the media and rapid inoculation of the samples resulted in no lag time for gas production. An increase in gas pressure was observed within 1 min. of inoculation. It appears that stirring at approximately 1 min. intervals with swirling 6 h after inoculation and every 24 h thereafter also maximized gas production. A small but inconsistent increase in gas production was observed when gas was manually released from the vials. The lack of a significant effect due to gas pressure build-up in this system may be related to the large head-space to sample ratio. Typically less than 60 mg of substrate is fermented in 10 mL of media with a head-space volume in the serum bottles of 48 mL which results in a maximum pressure of twice ambient after 96 h of fermentation.

Conclusions

If the objective of an in vitro method is to provide kinetic parameters that characterize the feed, it is evident that the method itself should not limit fermentation. The complete reduction and equilibration of samples and media before inoculation, the rapid and thorough preparation of an inoculum that maximizes detachment of particle-bound microbes, and the rapid inoculation of samples in the method described insures that lag time is minimized and fermentation rate and extend are maximized.