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In vitro gas production as a surrogate measure of the fermentability of cellulosic biomass to ethanol

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Abstract Current methods for measuring ethanol yields from lignocellulosic biomass are relatively slow and are not well geared for analyzing large numbers of samples generated by feedstock management and breeding research. The objective of this study was to determine if an in vitro ruminal fermentation assay used in forage quality research was predictive of results obtained using a conventional biomass-to-ethanol conversion assay. In the conventional assay, herbaceous biomass samples were converted to ethanol by *Saccharomyces cerevisiae* cultures in the presence of cellulase enzymes. Cultures were grown in sealed serum bottles and gas production monitored by measuring increasing head space pressure. Gas accumulation as calculated from the pressure measurements was highly correlated (r^2>0.9) with ethanol production measured by gas chromatography at 24 h or 7 days. The same feedstocks were also analyzed by in vitro ruminal digestion, as also measured by gas accumulation. Good correlations (r^2~0.63–0.82) were observed between ethanol production during simultaneous saccharification and fermentation and gas accumulation in parallel in vitro ruminal fermentations. Because the in vitro ruminal fermentation assay can be performed without sterilization of the medium and does not require aseptic conditions, this assay may be useful for biomass feedstock agronomic and breeding research.

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Introduction

Herbaceous biomass has been suggested as a promising feedstock for ethanol production. Biomass can be converted to ethanol by pretreating the material and fermenting the residual solids with *Saccharomyces cerevisiae* in the presence of cellulase enzymes and a β-glucosidase; this type fermentation is termed simultaneous saccharification and fermentation (SSF). The yeast ferments glucose as rapidly as it is released by the enzyme mixture, which avoids end product inhibition of the cellulase system (Lynd et al. 2002). Current standard methods for measuring ethanol yield by this process (United States Department of Energy 1995) are time-consuming and are not geared for analyzing the large numbers (thousands) of samples that can be produced in agronomic and plant breeding research to develop improved feedstocks. Laboratory tests for analyzing herbaceous biomass or hay for feed quality traits are available including in vitro ruminal (IVR) digestion tests, and most forage research programs are currently using these procedures but often lack the capability to conduct biomass-to-ethanol conversion using SSF. A common forage quality test involves IVR fermentations in sealed glass serum bottles and determination of fermentative gas production by measuring the increase in pressure in the vial head space (Theodorou et al. 1994; Schofield and Pell 1995). If head space volume is known, pressure measurements are easily converted to accumulated gas volume using empirical equations or the ideal gas law. For yeast fermentations, the

amount of gas produced is stoichiometrically related to ethanol production (1 Glc → ethanol + 2 CO_2).

The objective of this study was to examine IVR digestibility assays as an indirect predictor of ethanol yields. The IVR assay employs a mixed ruminal inoculum, added at high microbial cell density to an unsterilized sample, and implicitly assumes that this inoculum will greatly predominate over any microbial activity by the indigenous microflora. The hypothesis that the IVR assay will serve as a surrogate of SSF bioconversion for the purpose of rank-ordering samples based on fermentability, is based upon a recognition that both the SSF enzyme cocktail (of fungal origin), and the anaerobic ruminal microbes (primarily anaerobic bacteria), face similar challenges in degrading plant cell walls and have evolved under similar selective pressure. For this study a variety of samples of three forage species were fermented by a traditional yeast SSF with direct and indirect measurement of ethanol and by an IVR fermentation. Results from each method were compared to judge their predictor value relative to the traditional SSF methodology.

Materials and methods

Forage samples

Air-dried forage samples for evaluation by SSF were selected from a large sample set used in a biomass screening program. The set for which in vitro ruminal gas production had been measured included switchgrass [*Panicum virgatum*, cultivar Cave-in-Rock, 250 samples representing two locations and harvested from one to eight times during 1994–1996 (Vogel et al. 2002)]; eastern gamagrass (*Tripsacum dactyloides*, 408 samples representing six different cultivars grown at seven locations and harvested from one to four times during 2001); and big bluestem (*Andropogon gerardii* Vitman, 220 samples representing five locations and harvested one or two times during 2001). Eastern gamagrass and bluestem samples were ground through a 1 mm Wiley mill, while switchgrass samples were successively ground through a 2 mm Wiley mill then a 1 mm Udy mill.

Gas pressure sensor and calibration

Gas pressure measurements were made with a digital pressure gauge (model SEDPGB0015PG5 sensor unit, SenSym, Milpitas, Calif.; Fig. 1) having a 0.01 lb/in^2 (or psi; 1 psi =0.06805 atm) sensitivity. The male 1/4" (0.635 cm) normal pipe thread (NPT) stainless steel fitting at the bottom of the sensor body was connected to a threaded adapter fitting that terminated to a female Luer-Lock fitting. To prevent gas leaks, the threaded adapter was connected to the sensor using Teflon tape, and the top of the plastic hub of a disposable 22 gauge, 1" (2.54 cm) hypodermic needle was coated with a light coating of petrolatum prior to screwing the hub tightly into the fitting. The sensor was allowed to equilibrate in a 39°C room prior to all readings. The disposable needles were replaced after every six stopper penetrations.

In vitro ruminal assays

For in vitro ruminal assays, the sensor was calibrated using serum bottles (~60 ml) of known volume, measured to 0.01 ml by filling the tared vials to the brim with water and determining the net weight of water (assuming a density of 1.00 ml per g). These volumes were adjusted for the volume displaced by the flanged butyl rubber stopper (1.87 ml) and the volume of included forage, buffer, and inoculum. Bottles that contained 10 ml of Goering-Van Soest (1970) buffer under CO_2 gassing were sealed with new stoppers and were injected with varying amounts (0–50 ml) of a mixture of 36% CH_4/64% CO_2 at 22–24 °C, and pressures were read after equilibrating the vials overnight in a 39 °C room. After correction for thermal expansion of the gas mixture, a calibration line was constructed by linear regression (mean r^2=0.998) of gauge reading (in psi) versus volume fraction (F_v) of added gas, where F_v= milliliters added gas/milliliters bottle volume. The calibration equation permitted calculation of milliliters of gas in experimental vials as the product of bottle gas phase volume and the slope of the calibration line, which ranged from 11.571 to 11.682 ml gas/volume fraction of gas/psi gauge reading over the course of this study.

Ruminal inoculum and in vitro incubations

The ruminal inoculum for in vitro runs was obtained from two fistulated, lactating Holstein cows that were milked twice daily. Immediately after the morning milking, the cows were offered ~1.5 kg [dry matter (DM) basis] of a

Fig. 1 Digital gauge used for measurement of gas pressure in sealed vials

mixed grass hay for ~1 h prior to the once-daily feeding of a total mixed ration (TMR) that contained 30% alfalfa silage, 30% corn silage, 30% corn grain, and 10% soybean meal, to which were added supplemental vitamins and minerals. Ruminal samples were collected ~3 h after offering TMR. The mean pH values and standard deviations of the ruminal samples from the two cows were 5.79±0.26 and 5.83±0.28. Methods for preparing the composite ruminal inoculum have been described previously (Mourino et al. 2001). Use of two donor cows for preparing a composite inoculum has been shown to improve the performance and reproducibility of the ruminal inoculum (Mertens et al. 1998).

In vitro ruminal experiments were conducted using a single replicate of each sample (36–64 samples per run), and replication was achieved through a second in vitro run. Duplicates of a standard ground alfalfa stem material were included in each run. Incubations were conducted in nominal 60 ml serum bottles (volume-calibrated to 0.01 ml) and that contained ~100 mg (weighed to 0.1 mg) of forage, 6.7 ml of Goering and Van Soest buffer, 0.3 ml of cysteine-sulfide reducing agent (6.25 g/l each of cysteine HCl and $Na_2S{\cdot}9H_2O$) and a CO_2 gas phase. The flanged butyl stoppers were inserted halfway into the necks of the bottles while the reducing agent removed the last traces of O_2 as the vials were incubated at 39 °C. All inoculations and incubations were conducted in a 39 °C room. The diluted ruminal inoculum, under continuous stirring and continuous sparging with CO_2 in a water-jacketed vessel, was transferred by hypodermic syringe and added to the incubation bottle after loosening the stopper while the neck of the bottle was held under a CO_2 gas stream. The bottles were tared before adding the inoculum (3.0 ml) and weighed immediately thereafter to determine the exact amount of ruminal fluid added (to 0.01 g). Each bottle was then sealed and its gas pressure immediately measured with the sensor. Additional gas pressure readings were made at 24 h and at 96 h. To prevent the development of leaks, only fresh, degreased stoppers were used for each vial. Following inoculation and the 24 h gas pressure reading, vials were briefly and gently rolled to facilitate mixing and to maximize contact of the inoculum with the forage, which exhibited a slight tendency to adhere to the glass above or below the gas-liquid interface.

Gas accumulation was calculated for all vials, and net gas accumulation at 24 h and at 96 h was determined by subtracting the gas accumulation from the mean gas accumulation in six blank vials that contained reduced buffer and ruminal inoculum but no biomass sample. The resulting data were normalized for differences in net gas accumulation across runs, using mean values from paired samples of the alfalfa standard included in each run to calculate the ratio of mean gas accumulation from standards within a run divided by the mean gas accumulation of all standards across runs. The normalization factor varied from 0.812 to 1.156 at the 24 h time point, and from 0.863 to 1.156 at the 96 h time point. Gas accumulation in each sample is expressed on a DM basis, using DM values determined by near infrared reflectance spectroscopy from a calibration generated from a subset of 30 randomly chosen samples.

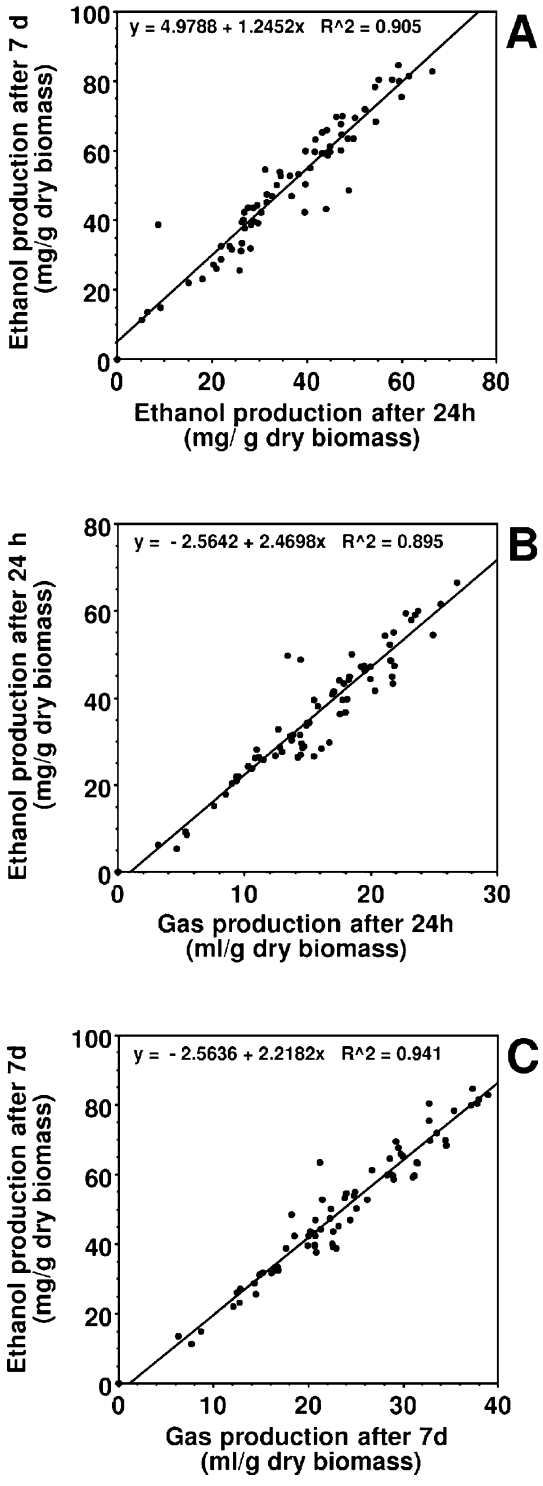

Fig. 2 Ethanol production and cumulative head space gas production in SSF of all samples (eastern gamagrass, bluestem, switchgrass). **a** Ethanol production at 24 h and at 7 days. **b** Ethanol production and gas production at 24 h. **c** Ethanol production and gas production at 7 days

SSF assays

To select samples for SSF analysis, the mean normalized gas accumulation from the IVR assay for each sample within a species was rank-ordered (based on mean gas accumulation at the 24 h and 96 h time points), and two subsets of 10–12 samples from each forage were selected that represented approximately equal spacing of these mean gas accumulation values across their entire range. SSF fermentations were conducted under aseptic conditions in volume-calibrated 60 ml serum vials. To each vial was added 1.00 g (dry matter basis) of forage sample and 17.0 ml H_2O. After gassing with N_2, the vials were sealed with butyl stoppers and aluminum closures, and then were autoclaved for 15 min at 121 °C. To each vial was then added sterile solutions of citric acid buffer (1 ml of 1 M, pH 4.8; sterilized by filtration), 2 ml of 10x YP (100 g yeast extract +200 g peptone/l, sterilized by autoclaving), and 0.12 ml of enzyme solution (5 FPU/g dry biomass). The enzyme solution was a filter-sterilized mixture (1:1 ratio) of Cellulclast and Novozyme 188 β-glucosidase (both produced by Novozyme, Denmark) that contained 60 International filter paper units (FPU) of activity per ml. The vials were then inoculated to an initial OD_{600} of 0.5 with a culture of *Saccharomyces cerevesiae* Y-2034 that had been grown overnight at 30°C in YPD medium (10 g yeast extract, 20 g peptone, and 50 g glucose/l) under aerobic conditions, then centrifuged and the cell pellet aseptically re-suspended in PBS [11.8 mM phosphate buffer (pH 7), 200 mM NaCl, and 27 mM KCl] to an OD_{600} of 21. The vials were incubated at 32 °C with shaking (100 rev/min). Periodically during the fermentation, head space pressure readings were taken and the head space pressure then vented. Ethanol and residual glucose were directly assayed by HPLC (Dien et al. 2002). In initial experiments, gas accumulation was measured at 18, 48, 92, and 168 h, and ethanol measured at 168 h. In later studies, head space pressure was measured at 24, 48, 96 and 168 h, and ethanol measured at 24 h and 168 h.

Statistics

For each forage, gas accumulation and ethanol production data were compared by linear regression using the General Linear Model of SAS (SAS, Cary, N.C.), Data from all samples within each forage were pooled for analysis. If the line intercept differed significantly from zero, a studentized *t*-test was applied to identify outliers (defined as data points that yielded a ratio of residual sums of squares/mean square error that was >2.5 or <–2.5) that were then removed from the data set; this corresponded to 0, 2, and 3 samples for eastern gamagrass, bluestem and switchgrass, respectively.

Results

Yeast SSF cultures

Cultures were sampled and ethanol directly measured after 24 h and 7 days. Ethanol production after 24 h (E1d) was highly correlated with final ethanol yield obtained after 7 days (E7d) (r^2=0.905, Fig. 2a). Ethanol production and gas accumulation were also highly correlated (Fig. 2) across all forage species at 24 h (r^2=0.895; Fig. 2b) and at 7 days (r^2=0.941, Fig. 2c). Gas accumulation from samples of eastern gamagrass and big bluestem at a variety of time points from 18 to 168 h also was found to predict E7d, with r^2 values in all cases of at least 0.92, but generally increasing with time (data not shown).

IVR fermentations

In vitro ruminal gas accumulation at 24 h (IVR24) was well correlated with in vitro ruminal gas accumulation at 96 h (IVR96), with r^2=0.806 across all samples (data not shown). For both eastern gamagrass and bluestem, IVR gas accumulation at both 24 h and 96 h was also well correlated with E7d in the yeast SSF system (Table 1). Fitting of these data to second-order polynomial equations usually yielded slightly higher r^2 values than did linear regression analysis, but visual inspection of the data

Table 1 Linear and quadratic regression of ethanol production after 1 day (*E1d*) or 7 days (*E7d*) in SSF versus in vitro ruminal gas accumulation after 24 h (*IVR24*) and 96 h (*IVR96*). *ND* Not determined (sugar accumulation not measured at 1 day)

	r^2 for E1d vs IVR24		$r^2$for E7d vs IVR24		r^2 for E7d vs IVR96	
Biomass material	Linear	Quadratic	Linear	Quadratic	Linear	Quadratic
Eastern gamagrass	0.862	0.869	0.824	0.853	0.816	0.909
Bluestem	0.631	0.697	0.718	0.791	0.806	0.823
Switchgrass						
Uncorrected	0.021	0.040	0.110	0.223	0.055	0.241
Corrected[a]	ND	ND	0.633	0.634	0.502	0.554

[a] Corrected for sugars released by saccharification but not fermented by the yeast. Additional ethanol production is predicted as the (measured mmol of glucose remaining in the incomplete fermentation) × (2 mmol ethanol/mmol glucose) × (46.1 mg ethanol/mmol ethanol)

revealed that the quadratic equations showed their poorest fit for samples having high levels of gas and ethanol production (i.e., the best-fermented materials, which are those of most interest in a biomass screening program). Consequently, linear regression was used for subsequent comparisons. The fit of the linear regression equations for E7d versus IVR24 was stronger for eastern gamagrass than for big bluestem (Table 1, Fig. 3). In the case of switchgrass, the fits of the regression equations for ethanol production by SSF and gas accumulation by IVR were poor (Table 1, Fig. 4a), apparently due to the presence of compounds that inhibited the yeast fermentation but not the ruminal fermentation. Inhibition of the yeast fermentation was indicated by additional experiments with these samples that yielded similar results, and by the observation that inhibited fermentations in which sugar had accumulated did not produce additional ethanol when reinoculated with additional yeast. However, reanalysis of the data to take into account the theoretical increase in gas yield that would accrue from complete fermentation of the accumulated sugars revealed a much better fit (r^2 improved from 0.110 to 0.633; Fig. 4b).

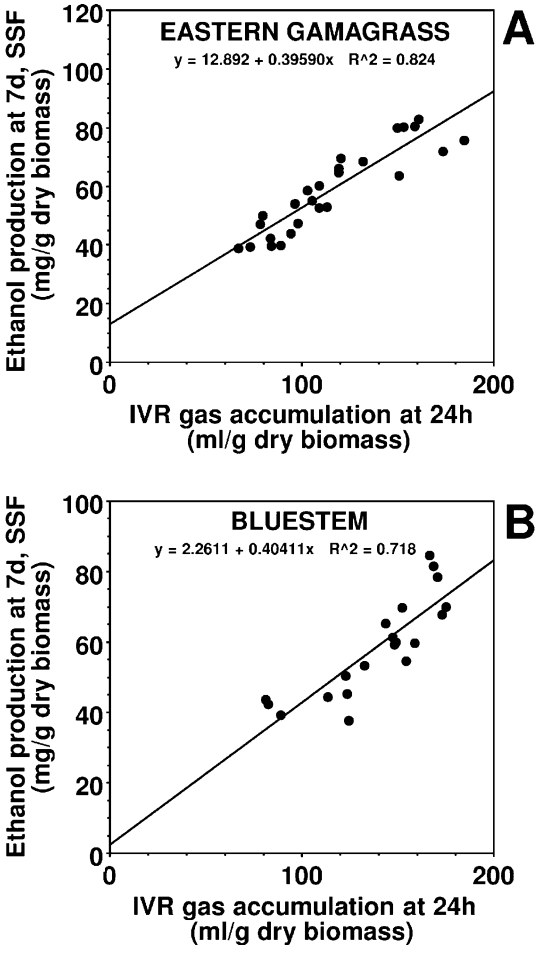

Fig. 3 Ethanol production from SSF after 7 days incubation, and gas accumulation in an in vitro ruminal fermentation assay after 24 h for eastern gamagrass **a** and bluestem **b**

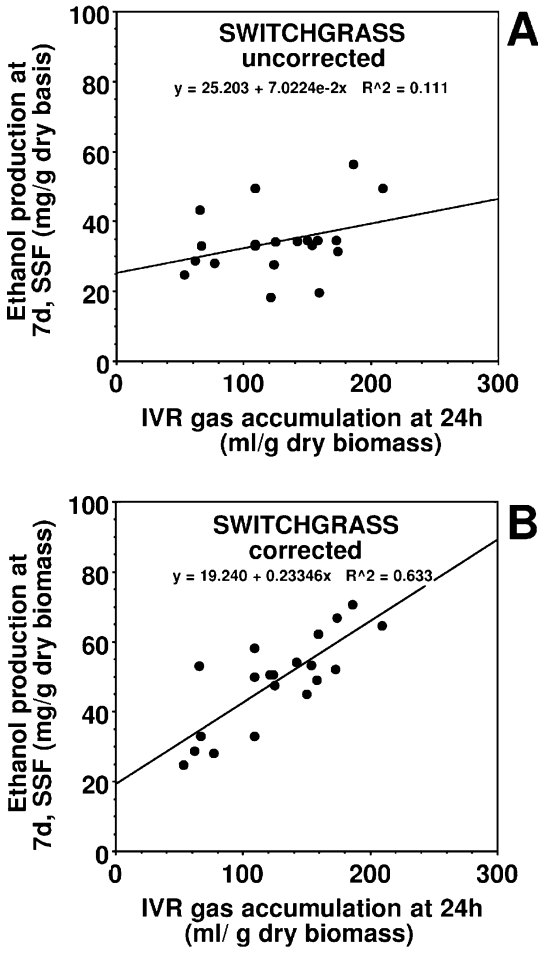

Fig. 4 **a** Ethanol production from SSF after 7 days incubation, and gas accumulation in an in vitro ruminal fermentation assay after 24 h for switchgrass. Accumulation of glucose in many SSF samples resulted in variable ethanol yields and poor correlation with gas production in the parallel ruminal fermentation. **b** Predicted ethanol production based on expected conversion of accumulated glucose at theoretical stoichiometries (see Table 1, footnote a)

Discussion

Experimental evaluation of the bioconversion of cellulosic materials to ethanol by SSF is not amenable to rapid processing of large numbers of samples, due to the requirements of setup and operation under aseptic conditions and of sample processing for ethanol analysis via GC or HPLC. The present study was conducted to evaluate the ability of a simple gas production measurement system to predict ethanol yield in a SSF system, thereby avoiding the need for expensive analytical instruments. To develop a more convenient method for predicting ethanol yields from biomass samples, these SSF results were correlated with fermentations, conducted in parallel, using mixed ruminal microbes as the fermentative agent. These latter cultures do not require asepsis and have shorter fermentation times. Use of accumulated gas pressure has been widely em-

ployed as a rapid method to assess anaerobic fermentation of organic matter (e.g., Menke et al. 1979; Shelton and Tiedje 1984), and has been extensively used to evaluate forage quality for ruminant feeding (Davies et al. 2000; Lee et al. 2002; Schofield and Pell 1995; Theodorou et al. 1994).

In the enzyme/yeast SSF system itself, there was a strong positive relationship between measured gas accumulation and ethanol production. Gas accumulation in the enzyme/yeast SSF system would be expected to show a strong correlation with ethanol production, as ethanol and CO_2 are produced in stoichiometrically equivalent amounts (1 Glucose $\rightarrow$ 2 ethanol + 2 CO_2). Based on the data from Fig. 2, we conclude that use of gas accumulation measurements and shorter incubation times thus represents a facile and inexpensive alternative to direct quantification of ethanol for SSF experiments (e.g., for evaluating effectiveness of pretreatments, enzyme formulations, and yeast strains) conducted in volume-calibrated vials. Previously, gas production in ethanol fermentations was measured based upon weight loss-from escaping CO_2 (Dien et al. 2002). Such a technique, while simple, requires a substantial sample size and a considerable amount of gas and ethanol production for accurate measurement. By contrast, the sensitivity of the gas pressure transducer allows the assays to be conducted at smaller scale, enhancing sample throughput. The protocol also allows for fermentation kinetics to be determined if pressure changes are measured more frequently.

The feasibility of using the IVR gas accumulation method as a substitute for measuring gas or ethanol production by the SSF method is less clear-cut, as it presents both advantages and disadvantages. A primary advantage is its non-aseptic operation, which permits higher sample throughput. A second advantage is high sensitivity, as approximately five times more gas is liberated from fermentation of biomass samples in the IVR system than in the SSF system (cf. Fig. 2 with Fig. 3 and 4). Several factors are responsible for the higher response in the IVR assay. In SSF, only the glucosyl and possibly the galactosyl fraction of the biomass is converted to ethanol and gas, as *Saccharomyces* strains cannot utilize pentoses and some hexoses. By contrast, in the IVR system a wide variety of polysaccharide hydrolases release a broad spectrum of sugars and sugar acids, and the highly diverse microbial population is capable of fermenting nearly the entire range of these sugars. Gas accumulation in the head space is also favored by the IVR incubation conditions. In the SSF system, a N_2 head space was used, and most of the gas produced was dissolved in the liquid medium. In the IVR system, a bicarbonate buffer was used in concert with a CO_2 head space, reducing the ability of CO_2 to participate in bicarbonate formation. The primary gaseous product was methane (1 mol of methane produced by reduction of 1 mol of CO_2 with 4 mol of fermentatively produced H_2), which is poorly soluble in water. Moreover, in the IVR system, gas evolution also occurs indirectly (Beuvink and Spoelstra 1992) due to the production of fermentation acids (chiefly acetic, propionic and butyric) that reduce culture pH, resulting in CO_2 evolution due to a shift in the equilibrium of the bicarbonate buffer ($HCO_3^- + H^+ \rightarrow H_2CO_3 \rightarrow CO_2 + H_2O$). The higher yield of gas per gram biomass substrate makes the IVR assay more sensitive than the SSF assay if gas accumulation is the sole measure of fermentative activity.

The IVR method also has some disadvantages. Obviously, the method requires the availability of ruminal fluid, preferably from several animals to permit preparation of a composite sample that would minimize variation among donor inocula. The correlation between data from IVR and SSF also appears to depend somewhat on sample type. Regression of gas accumulation by mixed ruminal microbes against either ethanol production or gas production in the SSF system yielded equations whose goodness of fit varied among these C_4 grasses. The fit was better for eastern gamagrass than for big bluestem and switchgrass. These data suggest that differences exist in how effectively mixed ruminal microbes and fungal-derived enzymes can handle the challenges presented by unique chemical and structural features of individual forage species. Some samples of switchgrass contained compound(s) that inhibited yeast fermentation during SSF, but no such inhibition was noted in the IVR assay. Thus, use of the IVR assay as a primary screen would identify samples based on their inherent fermentability. Research needs to be conducted to determine if the inhibitors in these samples are neutralized in pretreatment processes.

The study described here was intended to test the feasibility of gas production as a rapid and convenient method of assessing biomass fermentability. In order to reduce the number of confounding factors, the biomass materials were not subjected to physical or chemical pretreatments beyond simple grinding. In bioconversion schemes, additional pretreatments are regarded as critical for increasing the extent of conversion of substrate in the SSF system (Lynd et al. 2002) The IVR method may also be amenable to use with pretreated substrates. Although pretreatments are typically not employed when biomass materials are fed to ruminants, such pretreatments almost invariably enhance in vitro ruminal digestibility, or in vivo animal performance. It remains to be determined how well the IVR and SSF methods would correlate on such pretreated materials. Taking these caveats into account, we conclude that, for some biomass materials, the IVR gas production assay may serve as a method that can be used by agronomists and breeders to develop and evaluate management practices and cultivars with improved feedstock conversion characteristics. However, final evaluations for ethanol production potential will still need to be made using SSF methods.

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References

Beuvink JMW, Spoelstra SF (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

Davies ZS, Mason D, Brooks AE, Griffith GW, Merry RW, Theodorou MK (2000) 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. Anim Feed Sci Technol 83:205–221

Dien BS, Bothast RJ, Iten LB, Barrios L, Eckhoff SR (2002) Fate of Bt protein and influence of corn hybrid on ethanol production. Cereal Chem 79:582–585

Goering HK, Van Soest PJ (1970) Forage fiber analysis: apparatus, reagents, procedures and some applications, Agriculture Handbook No. 379. Agricultural Research Service, United States Department of Agriculture. Washington

Lee MR, Brooks AE, Moorby JM, Humphreys MO, Theodorou MK, MacRae JC, Scollan ND (2002) In vitro investigation into the nutritive value of *Lolium perenne* bred for an elevated concentration of water-soluble carbohydrate and the added effect of sample processing: freeze-dried and ground vs frozen and thawed. Anim Res 51:269–277

Lynd LR, Weimer PJ, Pretorius IS, Van Zyl WH (2002) Microbial cellulose utilization: fundamentals and biotechnology. Microbiol Mol Biol Rev 66:506–577

Menke KH, Raab L, Salewski A, Steingass H, Fritz D, Scheider W (1979) An estimation of the digestibility and metabolizable energy content of ruminant feedstuffs from the gas production when they are incubated with rumen liquor in vitro. J Agric Sci 93:217–222

Mertens DR, Weimer PJ, Waghorn GC (1998) Inocula differences affect in vitro gas production kinetics. In: Deaville ER, Owen E, Adesogan AT, Huntington JG, Lawrence TLJ (eds) In vitro techniques for measuring nutrient supply to ruminants. Occasional Publication No.22, British Society for Animal Science, Edinburgh, UK, pp 95–97

Mourino F, Akkarawongsa R, Weimer PJ (2001) Initial pH as a determinant of cellulose digestion rate by mixed ruminal microorganisms in vitro. J Dairy Sci 84:848–859

Schofield P, Pell AN (1995) Validity of using accumulated gas pressure readings to measure forage digestion in vitro: a comparison involving three forages. J Dairy Sci 78:2230–2238

Shelton DR, Tiedje JM (1984) General method for determining anaerobic biodegradation potential. Appl Environ Microbiol 47:850–857

Theodorou MK, Williams BA, Dhanoa MS, McAllan AB, 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

United States Department of Energy (1995) SSF experimental protocols: cellulosic biomass hydrolysis and fermentation. Protocol LAP008. http://www.eere.energy.gov/biomass/analytical_procedures.html

Vogel KP, Brejda JJ, Walters DT, Buxton DR (2002) Switchgrass biomass production in the Midwest USA: harvest and nitrogen management. Agron J 94:413–419