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Effect of the addition of cell wall degrading enzymes on fermentation kinetics of perennial ryegrass silage

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SUMMARY

Two studies were undertaken to evaluate the effects of cellulase and endoxylanase enzymes on the chemical composition and the fermentation characteristics of grass silage, using the gas production technique. Perennial ryegrass was ensiled in 1 litre glass containers for 90 days with cellulase (0·2 g/kg grass on fresh weight basis) and endoxylanase in the concentrations of 0·05 g/kg grass (Endox 0·05) and 0·2 g/kg grass (Endox 0·2). Subsequently, dried samples, ground through a 1 mm screen, were used in chemical analysis and gas production measurements. In Expt 1, the enzyme treatment significantly decreased silage NDF ($P < 0·001$), ADF ($P < 0·001$) and acetic acid ($P < 0·01$) concentrations and increased lactic acid ($P < 0·001$) production. In Expt 2, lower concentrations of NDF ($P < 0·001$) and ADF ($P < 0·001$) in treated silages resulted in increased sugar concentration ($P < 0·001$). In this experiment, butyric acid was detected. Addition of cellulase and Endox 0·05 enzymes did not alter silage digestibility. In both studies, cellulase and Endox 0·2 treatments tended to increase the rate of gas production within 10 hours of inoculation with rumen fluid whereas Endox 0·05 had no effect. The volume of gas produced was however greater for the untreated silage than for the enzyme-treated silage samples after 48 h of incubation. The strategy of applying cellulase and endoxylanase to the herbage in the ensiling process proved to be effective in modifying the chemical composition and increasing sugar concentration and the rate of gas production of the silages. Further research on the factors determining enzyme effectiveness is therefore suggested to elucidate the mechanisms leading to higher utilization of the released sugars.

INTRODUCTION

The mode of action of cell wall degrading enzymes during the ensiling process is the release of fermentable sugars from the structural polysaccharides which provides extra substrate for the indigenous microbial population, resulting in the production of lactic acid and consequently reducing the risk of clostridial fermentation (Van Vuuren *et al*. 1989). Also, the use of cell wall degrading enzymes as silage additives predigests plant cell walls which may increase the extent and rate of degradation in the rumen and, as a result, improve digestibility and nutritive value (McHan 1986). Chamberlain & Robertson (1992) and Selmer-Olsen *et al*. (1993*a*) observed decreases in NDF and ADF concentrations in grass silages due to enzyme treatment. Enzyme treated silages are also characterized by low pH (Fredeen & McQueen 1993; Chen *et al*. 1994), less acetate (Narasimhalu *et al*. 1992; Stokes & Chen 1994) and increased lactic acid concentration (Stokes 1992). However, the effects of cellulolytic preparations during ensiling upon rumen fermentation are more contradictory. Although the addition of cellulolytic enzymes to the herbage has been correlated to increased amounts of organic matter digested in the rumen (McHan 1986; Chamberlain & Robertson 1992), others have reported no significant effects of enzyme treatment (Kennedy 1988; Van Vuuren *et al*. 1989; Jaakkola 1990; Kung *et al*. 1991; Sheperd & Kung 1996). Most of those studies have been based on time course experiments using either *in sacco* or *in vitro* techniques which measure the organic matter residue after filtration through nylon gauze, paper filter or filtering crucibles.

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Table 1. *Chemical composition of the silages* (*Expt 1*; *g/kg*, *unless otherwise stated*)

Treatment	DM	OM	CP	Ammonia N (g/kg nitrogen)	Sugars	NDF	ADF	pH	Lactic acid	Acetic acid	Ethanol	Digestibility
Untreated	249	870	150	98	20	414	252	4·09	104	37·7	12·4	750
Cellulase	241	862	156	86	46	272	164	3·80	144	32·3	27·4	752
Endox 0·05	242	868	152	84	52	322	240	3·90	117	31·4	11·7	753
Endox 0·2	243	866	157	79	59	280	207	3·90	122	33·4	20·2	744
S.E. (4 D.F.)	1·1	2·5	1·6	11·7	6·8	1·7	2·4	0·004	1·4	0·64	3·43	10·6

DM, dry matter; OM, organic matter; CP, crude protein; NDF, neutral detergent fibre; ADF, acid detergent fibre.

It has been shown that more direct information and accurate data on the kinetics of fermentation can be provided by the gas production technique, especially when an automated system is used (Pell & Schofield 1993; Theodorou *et al*. 1994; Cone *et al*. 1996). This study investigated the efficacy of adding cell wall degrading enzymes on silage composition and rumen fermentation patterns as determined with fully automated gas production equipment (Cone *et al*. 1994, 1996).

MATERIALS AND METHODS

Enzyme preparations

Two different enzyme preparations, provided from Gist-brocades b.v., Delft, The Netherlands, were used: solid cellulase preparation from *Trichoderma viridae* (Cellulase) and Endoxylanase (Endox) with an activity of 158000 EXU/g powder, containing respectively 130 mg/g and 93 mg/g of protein (Engelen *et al*. 1996). The cellulase was used in a concentration of 0·2 g protein/kg grass (fresh weight) and the endoxylanase in the concentrations of 0·2 g protein/kg grass (Endox 0·2) and 0·05 g protein/kg grass (Endox 0·05). The enzyme concentration was calculated on a fresh weight basis.

Silage samples

Herbage from predominantly perennial ryegrass (*Lolium perenne*) swards grown in 1991 was cut at a yield of about 2000 kg/ha. The third regrowth, ensiled on 17 July, was wilted for 1 h on a black cloth to approximately 20% dry matter. In Expt 2, the fourth regrowth was ensiled on 14 August and shaded on the field for 6 days prior to harvesting to obtain samples with low sugar concentration. These samples, although not wilted, were ensiled at 28% dry matter. Nitrogen fertilizer was applied shortly after cutting at the rate of 30 kg N/ha, therefore totalling 150 kg N/ha per year.

For each treatment, duplicate 1·5 kg samples of herbage were chopped with a paper guillotine, to produce a chop length of 1–2 cm, and sprayed (25 ml/kg herbage) with the enzyme solution with a pressure sprayer in a concrete mixer of 138 litres. The untreated herbage received water applied at the rate of 25 ml/kg herbage. Samples were treated with 0·2 g protein of cellulase/kg herbage and 0·05 and 0·2 g protein of enzyme Endox/kg herbage. The herbage was ensiled (in duplicate) in 1 litre laboratory silos and stored for 3 months at room temperature before opening for further analysis. Mud was also applied (25 ml/kg) to supply Clostridial spores, therefore providing a greater challenge to the silage fermentation.

Sample preparation

Silage samples were dried in an air forced oven at 70 °C for 24 h, ground through a 1 mm screen and stored in air-tight glass flasks at room temperature. For gas production measurements, the duplicate silo samples were pooled.

Preparation of buffered rumen fluid (*BRF*)

Rumen fluid was obtained from two rumen fistulated sheep kept on a daily ration of 800 g hay and 200 g commercial concentrate offered in two equal meals at 08·00 and 16·00 h. Rumen fluid was filtered over two layers of cheese cloth. Filtered rumen fluid was mixed (1:2 v/v) with an anaerobic buffer/mineral solution containing per litre: 8·75 g $NaHCO_3$, 1·00 g NH_4HCO_3, 1·43 g Na_2HPO_4, 1·55 g KH_2PO_4, 0·15 g $MgSO_4 \cdot 7H_2O$, 0·52 g NaS, 0·0017 g $CaCl_2 \cdot 2H_2O$, 0·015 g $MnCl_2 \cdot 4H_2O$, 0·002 g $CoCl_3 \cdot 6H_2O$, 0·012 g $FeCl_3 \cdot 6H_2O$ and 0·125 g resazurin (Beuvink & Spoelstra 1992). All handling was done under continuous flushing with oxygen-free CO_2.

Determination of gas production kinetics

Gas production upon incubation of samples with buffered rumen fluid was monitored for 48 h as described by Cone *et al*. (1996). Samples were

incubated in duplicate in two separate series performed on different days. In each series, a blank (rumen fluid without sample) was run in duplicate.

Chemical analyses

Silage pH and concentrations of ethanol, lactic acid, acetic acid and butyric acid were determined in the aqueous silage extract. Ethanol, lactic acid, acetic acid and butyric acid concentrations were determined by gas chromatography from a 4 ml sample of centrifuged (20 min at 8000 ***g***) extract preserved with 0·8 ml of a 5% phosphoric acid solution. Lactic acid concentration in centrifuged extracts was determined as acetaldehyde by gas chromatography. The concentrations of oven-dry matter, ash, crude protein and sugars were determined by routine methods (Spoelstra 1983). Ammonia was estimated in a centrifuged extract preserved with 1 ml 1N H_2SO_4 per ml and stored at -24 °C until analysis according to a modified Bertholet method (Searle 1984) on an autoanalyser. Neutral detergent fibre (NDF) and acid detergent fibre (ADF) were determined according to Robertson & Van Soest (1981). The *in vitro* organic matter digestibility was measured by the Tilley & Terry (1963) method.

Dry matter was corrected for loss of volatile fermentation products assuming that ethanol, acetic acid, propionic acid and butyric acid were completely volatilized during oven drying. All of the silage components were expressed on this corrected dry matter basis.

Statistical procedures

The effect of the addition of enzymes and the influence of ensiling conditions (stage of maturity) on silage composition and total gas production was determined by Fisher's Protected LSD analysis (Wilkinson *et al.* 1996).

RESULTS

The chemical composition of the silages of the first and second studies is presented in Tables 1 and 2, respectively. Enzyme treatment decreased pH ($P < 0{\cdot}001$) and the concentrations of DM ($P < 0{\cdot}05$), NDF ($P < 0{\cdot}001$), ADF ($P < 0{\cdot}001$) and acetic acid ($P < 0{\cdot}01$) and increased the concentrations of lactic acid ($P < 0{\cdot}001$) and crude protein ($P < 0{\cdot}05$). Cellulase increased ethanol concentration ($P < 0{\cdot}05$) whereas Endox 0·05 and 0·2 had no effect ($P > 0{\cdot}05$). Enzyme treatment had no effect ($P > 0{\cdot}05$) on the sugar concentration or digestibility relative to the untreated silage.

The untreated silage in the second study was well preserved as measured by pH and the concentration of ammonia nitrogen. Treatment with enzymes decreased the concentrations of NDF ($P < 0{\cdot}001$) and

Table 2. *Chemical composition of the silages (Expt 2; g/kg, unless otherwise stated)*

Treatment	DM	OM	CP	Ammonia N (g/kg nitrogen)	Sugars	NDF	ADF	pH	Lactic acid	Acetic acid	Butyric acid	Ethanol	Digestibility
Untreated	236	863	101	145	32	480	295	4·00	100	4·0	18·5	18·2	634
Cellulase	233	860	114	129	64	391	230	4·00	111	5·5	20·9	21·9	638
Endox 0·05	231	865	115	135	45	426	280	4·24	96	8·5	25·0	23·3	643
Endox 0·2	233	865	117	129	88	377	246	4·20	110	13·0	21·3	22·3	663
S.E. (4 D.F.)	1·8	2·7	1·9	10·2	4·0	3·2	1·2	0·013	9·8	0·50	0·63	1·26	5·8

DM, dry matter; OM, organic matter; CP, crude protein; NDF, neutral detergent fibre; ADF, acid detergent fibre.

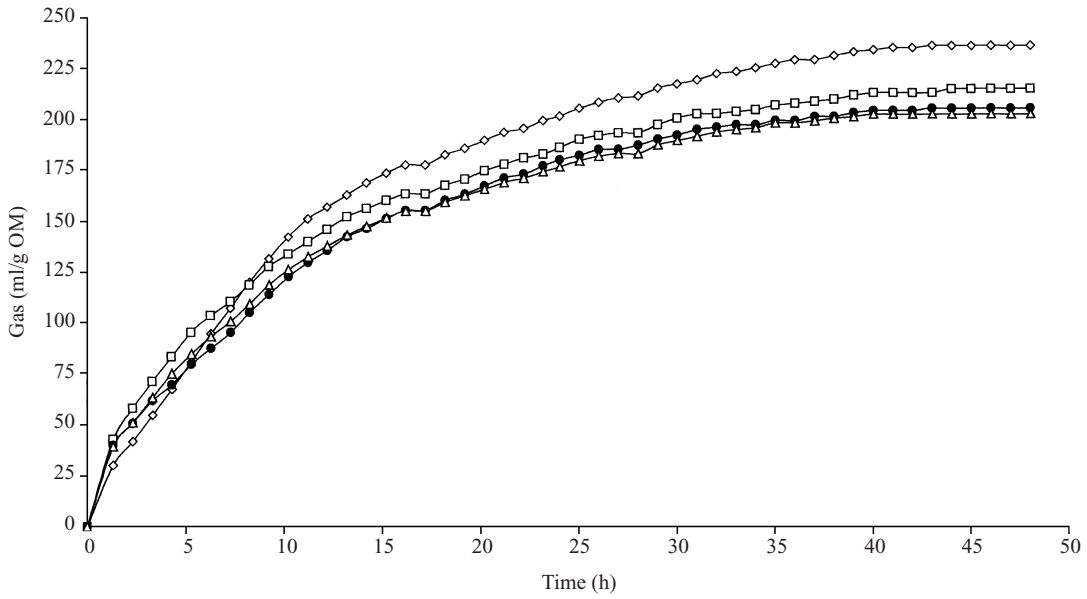

Fig. 1. Cumulative gas production (Expt 1). Untreated (◇), Cellulase (□), Endox 0·05 (●), Endox 0·2 (△).

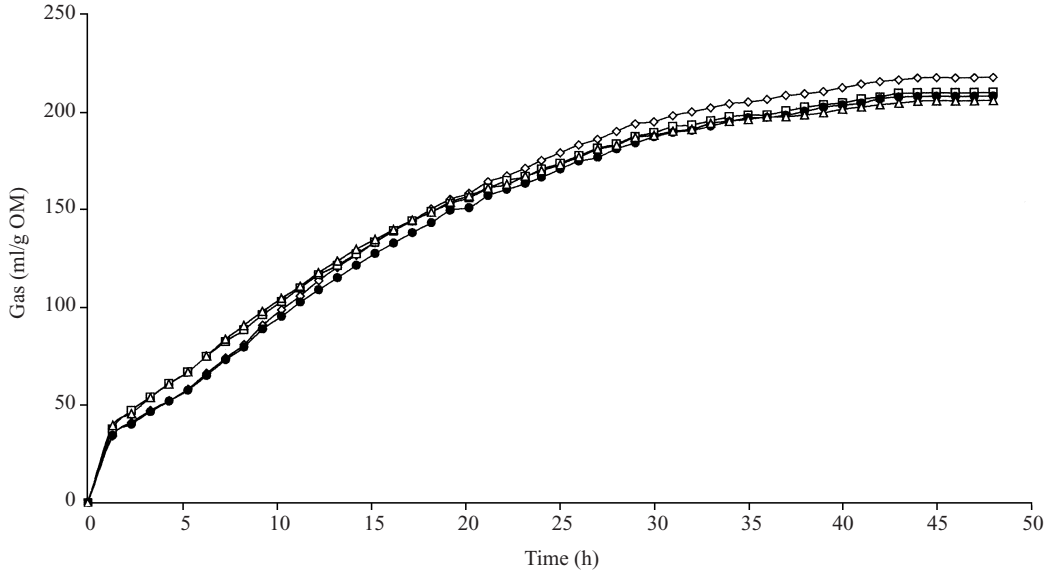

Fig. 2. Cumulative gas production (Expt 2). Untreated (◇), Cellulase (□), Endox 0·05 (●), Endox 0·2 (△).

ADF ($P < 0·001$). Relative to the untreated silage, Endox 0·05 and 0·2 treatments increased pH ($P < 0·001$) and concentrations of acetic acid ($P < 0·001$) and butyric acid ($P < 0·01$). Cellulase and Endox 0·2 increased sugar concentrations ($P < 0·01$) whereas Endox 0·05 had no effect ($P > 0·05$). Endox 0·2 increased digestibility relative to the untreated silage whereas cellulase and Endox 0·05 had no effect ($P > 0·05$). Treatment had no effect ($P > 0·05$) on the concentrations of dry matter, organic matter, crude protein, ammonia N, lactic acid and ethanol.

The effects of treatment on cumulative gas production for Expts 1 and 2 are presented in Figs 1 and 2, respectively. In the first experiment, after 8–10 h of fermentation, the volume of gas produced was greater for the untreated silage than for the enzyme-treated

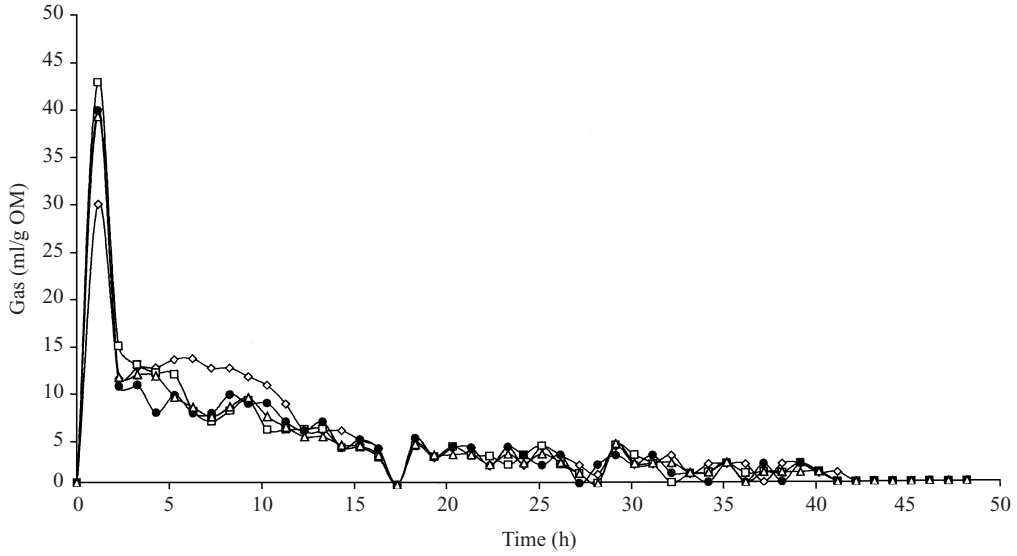

Fig. 3. Rate of gas production (Expt 1). Untreated (◇), Cellulase (□), Endox 0·05 (●), Endox 0·2 (△).

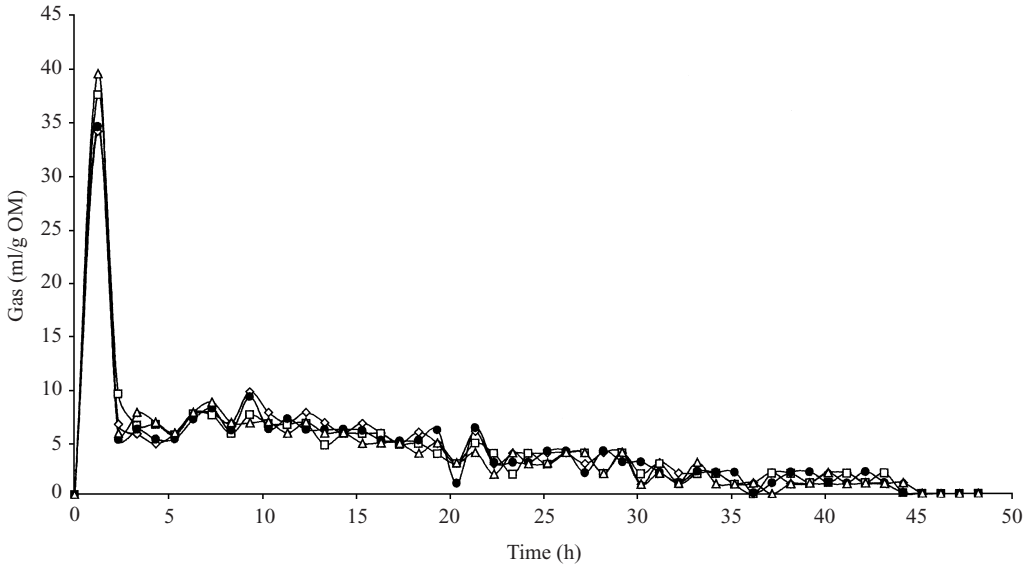

Fig. 4. Rate of gas production (Expt 2). Untreated (◇), Cellulase (□), Endox 0·05 (●), Endox 0·2 (△).

silage samples. Among the studied enzymes, the cellulase was the most effective leading to higher gas production during the whole fermentation procedure. However, in Expt 2, the gas production profiles were similar for the different substrates.

The effects of treatment on the rate of gas production in Expts 1 and 2 are presented in Figs 3 and 4, respectively. In both studies gas production reached a peak after 1–2 h with the untreated silage producing the lowest peak in both studies. Treating the herbage with enzyme tends to increase the rate of gas production within 10 h of inoculation with rumen fluid. This effect was, however, less evident in Expt 2.

DISCUSSION

In this study, the addition of cell wall degrading enzymes during the ensiling process induced differences between the chemical composition of treated and untreated ryegrass silage. As shown in

Tables 1 and 2, cellulase and endoxylanase mainly modified the concentration in cell wall components. While decreasing NDF and ADF in both experiments, the enzyme treatment was more effective in Expt 1, dealing with less mature ryegrass. These observations, which are in agreement with those of Beuvink & Spoelstra (1994) and Adogla-Bess & Owen (1995*a*,*b*), should be viewed as a result of the increase in lignin-polysaccharide complexes of plant cell walls which are refractory to enzyme hydrolysis. The increased production of lactic acid due to the enzyme treatment, as observed in Expt 1, as well as the appearance of butyric acid in Expt 2 also support that hypothesis. Notwithstanding the general increase in sugar concentration of the treated silages, it should therefore be assumed that lignification and higher lignification rate occurring in mature forages become the major constraint to the efficacy of the cell wall degrading enzymes in the ensiling process.

Differences between the gas production profiles of treated and untreated ryegrass silage were also observed. Although it has been shown that enzyme treatment favourably modifies the chemical composition of silages, the observed gas production profiles did not reflect this mode of action. Despite the tendency for higher rates of production when treated silages were the substrate, especially from less mature herbage, the total amount of gas produced did not reach the values observed in untreated samples. It seems unlikely that the extra sugars released by the action of cellulase and endoxylanase would have been completely available to microbial fermentation. McHan (1986), Van Vuuren *et al*. (1989), Jaakkola *et al*. (1992) and Selmer-Olsen *et al*. (1993*a*,*b*) showed an increase in the concentration of soluble cell wall fractions of the grass silage after enzyme treatment, but it is also recognized that the effectiveness of enzyme treatment is dependent on factors including plant species, maturity, chemical composition and ensiling conditions.

In our study, the efficacy of the enzyme treatments appeared to be reduced. This may also be a plausible explanation for our data not supporting the expected positive correlation between digestibility and enzyme addition in the ensiling process. These findings have also been reported by Adogla-Bess & Owen (1995*b*), Jaakkola (1990) and Jaakkola *et al*. (1992). However, applying cellulase and endoxylanase to the herbage in the ensiling process proved to be a valuable strategy for increasing sugar concentration and the rate of gas production of the silages. Since the gas production method has proved to be accurate and also allows the measurement of fermentation kinetics of soluble substrates, more research into the evaluation of factors determining enzyme efficacy would be helpful in elucidating the mechanisms leading to higher utilization of released sugars.

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