Improving the nutritive value of ensiled green rice straw 2- *In vitro* gas production

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Abstract: Fresh green rice straw of Sakha 101 variety treated for silage making with *Cellulomonas* sp. as cellulolytic bacteria (CB) and *Lactobacillus* sp. as lactic acid bacteria (LAB) as follows, 1) control, 2) lactic acid bacteria (LAB) at 10^8 cfu/g, 3) cellulolytic bacteria (CB) at 10^8 cfu/g and 4) LAB+CB at 10^8 cfu/g per type of bacteria. The different treatments were addition with or without 5% molasses and ensiled for 60 days. Bacterial inoculants and molasses addition increased *in vitro* gas production volume, gas production fractions (*a&b*) and gas production rate (*c*) and CB more effective than LAB and the LAB+CB combination had the higher values. Gas production from the fermentation of soluble fraction (GPSF) and insoluble fraction (GPNSF), short chain fatty acids concentration (SCFA), DM intake (DMI), organic matter digestibility (OMD), metabilizable (ME) and net energy (NE) and *in vitro* DM degradability (IVDMD) increased significantly ($P<0.05$) with bacterial inoculants and molasses addition and the LAB+CB had the higher values.

[Elmenofy, E.K; M.I. Bassiouni; E.B. Belal; H.M.A. Gaafar; E.M. Abdel-Raouf and S.A. Mahmoud. **Improving the nutritive value of ensiled green rice straw 2- *In vitro* gas production.** *Nat Sci* 2012;10(12):86-91]. (ISSN: 1545-0740). http://www.sciencepub.net/nature. 13

Keywords: green rice straw silage, bacterial inoculants, *in vitro* gas production.

1. Introduction

The *in vitro* gas production technique can be used to determine the nutritive value of the roughages and to identify differences among their potential digestibility and energy contents (Sallam, 2005).

Microbial fermentation of feeds produces carbon dioxide, methane and short chain volatile fatty acids (VFA). Gas measured by IVGPT is produced directly from fermentation or indirectly by these VFA reacting with bicarbonate included in the incubation medium. Gas production profiles produced by IVGPT has been shown to have good relationships with VFA produced in the rumen (Blummel and Ørskov, 1993; Brown *et al*., 2002; Rymer and Givens, 2002), as well as neutral detergent fiber (NDF) (Herrero and Jessop, 1996) and dry matter (DM) disappearance (Prasad *et al*., 1994).

Menke and Steingass (1988) reported a strong correlation between metabolizable energy (ME) values measured *in vivo* and predicted from 24 h *in vitro* gas production and chemical composition of feeds. The *in vitro* gas production method has also been widely used to evaluate the energy value of several classes of feeds (Getachew *et al*., 1998), particularly straws (Makkar *et al*., 1999), agro-industrial by-products (Krishna and Gunther, 1987), compound feeds (Aiple *et al*., 1996) and various tropical feeds (Krishnamoorthy *et al*., 1995).

Incubation of feedstuff with buffered rumen fluid *in vitro*, the carbohydrates are fermented to short chain fatty acids (SCFA), gases mainly CO and CH and microbial cells. Gas production is basically the result of fermentation of carbohydrates to acetate, propionate and butyrate. Gas production from protein fermentation is relatively small as compared to carbohydrate fermentation while, contribution of fat to gas production is negligible (Beuvink and Spoelstra, 1992 and Blummel and Ørskov, 1993).

The objective of this study was to investigate the effect of lactic acid and cellulolytic bacteria inoculants with molasses suppementation on *in vitro* gas production of green rice straw silage.

2. Materials and methods

The current work was carried out at the Department of Animal Production, Faculty of Agriculture, Kafrelsheikh University during years 2010 and 2011. The experiment was done in factorial design (4 x 2) to study the effect of bacteria inoculants and molasses additive on silage quality characteristics and chemical composition of green rice straw silage (Sakha 101 variety).

Fresh green rice straw of Sakha 101 variety was taken immediately after harvesting grains, chopped handly to 10 – 15 cm of length. The moisture content of fresh rice straw was adjusted to the normal range for making silage (65–75%) by adding water. Rice straw was treated with 10^8 or 10^6 cfu/g from *Cellulomonas* sp. as cellulolytic bacteria (CB) and *Lactobacillus* sp. as lactic acid bacteria (LAB) for high and low bacterial count. Two

bacterial strains were obtained from Dr. Elsayed Belal, associate professor of Agricultural microbiology, Dep. of Agric. Botany, Fac. of Agriculture, Kafrelsheikh University. *Cellulomonas* sp. as cellulolytic bacteria was grown on nutrient broth medium (contents per liter, 5 gm glucose, 5 gm yeast extract, 5 gm peptone and 5 gm sodium chloride) and *Lactobacillus* sp. was grown on MRS medium (contents per liter, peptone 10g, beef extract, 10g, yeast extract 5g, glucose 20g, Dipotassium phosphate 2g, Sodium acetate 5g, Diamonium citrate 2g, Magnesium sulphate 2g, tween 80 1g). Green rice straw sprinkled by molasses and bacterial inoculants and ensiled in plastic buckets with about 2 kg of weight capacity in triplicates for each treatment and pressed by hand to exclude the air from the bucket silos. The buckets were tightly sealed after good pressing to get anaerobic conditions and ensiled 60 days at room temperature.

The experimental treatments were done as follows, 1) control, 2) lactic acid bacteria (LAB) at 10^8 cfu/g, 3) cellulolytic bacteria (CB) at 10^8 cfu/g and 4) LAB + CB at 10^8 cfu/g. The different treatments were supplemented with or without 5% sugar beet molasses during silage making. Representative samples of rice straw silage were taken at opening time and dried in a forced air oven at 60 ^oC for 48 hours.

In vitro gas production was undertaken according to the procedure described by Menke and Steingass (1988). Samples (100 mg) of the air-dry feedstuffs were accurately weighted into 50 ml calibrated glass syringe fitted with plungers (fig 5). The buffer solution was used in vitro gas production defined as MB9 (Onodera and Handerson, 1980). The buffer consisted of 2.8 g NaCl; 0.1 g CaCl2; 0.1 MgSO4.7H2O; 2.0 g KH2PO4; 6.0 g Na2HPO4 which dissolved in distilled water and mad up to 1 L. Then the pH adjusted at 6.8 and CO2 flushed for 15 min.

Rumen contents (50% solid: 50% liquid, Bueno *et al*., 2005) were collected from three rumen cannulated sheep which were fed with rice straw *ad lib* and commercial concentrate mixture. The rumen contents were collected were before the morning feeding of the animals. Liquids and solids were placed in pre-warmed (39 ^oC) insulated flasks and transported under anaerobic conditions to the laboratory. The rumen contents were squeezed through four layers of cheese-cloth and kept in a water bath at 39 ^oC with CO_2 saturation until inoculation took place. The buffer and inoculan (2:1 v/v) were mixed and kept in a water bath at 39 ^oC with CO_2 saturation, (Sallam, 2005; Soliva *et al*., 2005 and Nasser *et al*., 2006)

Buffered rumen fluid (15ml) is pipetted into each syringe, containing the feed samples, and the syringes are immediately placed into the water bath at 39oC. Three runs were performed for each experiment. Syringes of each run included two syringes contain only buffered rumen fluid are incubated and considered as the blank. The syringes are gently shaken every 2h, and the incubation terminated after recording the 96 h gas volume. The gas production was recorded after 3. 6. 9. 12. 24. 48. 72 and 96 h of incubation. Total gas values are corrected for the blank incubation and reported gas values are expressed per 200 mg of DM.

Fermentation kinetics were described according to Ørskov and McDonald (1979) as:

$$Y = a + b(1 - e^{-ct})$$

where Y is gas production (ml/g OM) at time t, *a* is gas production from the immediately soluble fraction, *b* is gas production from the insoluble fraction, and *c* is gas production rate constant for fraction *b*.

As a new approach to evaluate feeds from those parameters, gas production caused by fermentation of the soluble fraction (GPSF) was estimated by gas produced after 3 hr (GP3) of incubation. Gas production caused by fermentation of the insoluble fraction (GPNSF) could be estimated from the gas production between 3 hr (GP3) and 24 hr (GP24) of incubation according to Van Gelder *et al*. (2005) as follows:

GPSF= Gas 3hr * 0.99 - 3
GPNSF= 1.02*(Gas 24hr – Gas 3hr) + 2
Where : Gas 3hr is 3hr net gas production (ml/200mg DM), Gas 24hr is 24 hr net gas production (ml/200mg DM), GPSF is gas production from soluble fraction (ml/ g DM) and GPNSF is gas production from non-soluble fraction (ml/ g DM).

The energy values were calculated from the amount of gas produced at 24 hr of incubation with supplementary analyses of crude protein, ash and crude fat. This approach was developed by the research group in Hohenheim (Germany) and is based upon extensive *in vitro* incubation of feedstuffs (Menke *et al*., 1979 and Menke and Steingass, 1988).

ME (Mcal/kg DM) = (2.2 + 0.136*GP + 0.057*CP)/4.186
NE (Mcal/kg DM) = (2.2 + 0.136*GP + 0.057*CP + 0.149*EE)*2.2/ 14.64

Where: ME is the metabolizable energy (Mcal/kg DM), GP is 24 hr net gas production (ml/200 mg DM), CP is crude protein (% of DM) and EE is either extract (% of DM).

OMD (%) = 14.88 + 0.889*GP + 0.45*CP + 0.0651*A

Where: OMD is organic matter digestibility (%), GP is 24 hr net gas production (ml/200mg DM), CP is crude protein (% of DM), A is ash (% of DM).
Short chain fatty acids (SCFA) were calculated according to the Getachew *et al*. (2005) as follow:
SCFA = (- 0.00425 + 0.0222*GP 24hr)*100

Where: GP is 24 h net gas production from the soluble fraction (ml)

Dry matter intake (DMI) was calculated according to Blummel and Ørskove (1993) as follow:

DMI = 1.66 + 0.49 * (*a*) + 0.0297 * (*b*) – 4 * (*c*)
Where: *a* = the gas production from the soluble fraction (ml), *b* = the gas production from the insoluble fraction (ml), *c* = the gas production rate (ml / hr).

The residual solutions were filtered into preweighed Gooch filter crucibles, dried at 105 ^{o}C for 24 hour, and weighed for the determination of *in vitro* DM degradability (IVDMD).

The data were subjected to statistical analysis using factorial models procedure adapted by SPSS for windows (2008) for user's guide. Duncan test within program SPSS was done to determine the degree of significance between the means (Duncan, 1955).

3. Results

Cumulative gas production:

The effect of bacterial inoculants and molasses addition *in vitro* on cumulative gas production of rice straw silage are presented in Table (1). The *in vitro* gas production during the different incubation times revealed that cellulolytic bacteria (CB) was more effective than lactic acid bacteria (LAB) and the combination between them (LAB+CB) showed higher gas production (P<0.05). Molasses addition led to significant (P<0.05) increase *in vitro* gas production during different incubation times. Gas production of the different rice straw silage treatments was fast until 48 hours of incubation and slow thereafter from 48-96 hours of incubation.

The effect of bacterial inoculants and molasses addition on fractions and rate of gas production of rice straw silage are shown in Table (2). The gas production from the immediately soluble fraction (*a*), the gas production from the insoluble fraction (*b*) and the gas production rate constant for the insoluble fraction (*c*) values of rice straw silage increased significantly (P<0.05) with bacterial inoculants and molasses addition. The LAB+CB with molasses addition had the higher *a, b* and *c* values.

Gas production from the fermentation of soluble and insoluble fractions:

The gas production from the fermentation of soluble fraction (GPSF) and insoluble fraction (GPNSF) of rice straw silage as affected by bacterial inoculants and molasses addition are presented in Table (3). The GPSF and GPNSF increased significantly (P<0.05) with bacterial inoculants and molasses addition. The gas production of rice straw silage from the insoluble fraction was 5-10 times higher than the gas production from the soluble fraction.

Short chain fatty acids (SCFA):

The concentration of short chain fatty acids (SCFA) of *in vitro* fermented rice straw silage as affected by bacterial inoculants and molasses addition are shown in Table (3). The concentration of SCFA increased significantly (P<0.05) with bacterial inoculants. The CB increased the SCFA concentration more than LAB and the combination between them LAB+CB recorded the higher SCFA concentration. Moreover, the SCFA concentration increased significantly (P<0.05) with molasses addition.

Dry matter intake (DMI):

From the results in Table (4), we believe that *in vitro* gas production of rice straw silage are valuable predictors of the voluntary intake potential when fed alone or in mixed rations. The DM intake of rice straw silage increased significantly (P<0.05) with bacterial inoculants. The DM intake of rice straw silage treated by CB was higher than silage treated by LAB and the LAB+CB combination treated silage recorded the higher DM intake. These results may be attributed to that cellulolytic bacteria was more effective in fiber degradability than lactic acid bacteria. The potential DM intake of rice straw silage increased significantly (P<0.05) with molasses addition.

Organic matter digestibility (OMD):

Based on the strong relationship between measured digestibility and that predicted from gas production, regression equations have been developed and the method has been standardized. As presented in Table (4) the OMD of rice straw silage increased significantly (P<0.05) with bacterial inoculants. Inoculated rice straw silage by cellulolytic bacteria increased OMD more than the lactic acid bacteria, while the combination between then showed the higher OMD. Molasses supplemented rice straw silage significantly increased (P<0.05) OMD.

Metabolizable energy (ME) and net energy (NE):

The predicted metabolizable energy (ME, Mcal/kg DM) and net energy (NE, Mcal/kg DM) from gas production for rice straw silage are presented in

Table (4). The predicted ME and NE contents of rice straw silage increased significantly (P<0.05) with bacterial inoculants. The ME and NE contents of CB treated silage were higher than those LAB treated silage and silage treated with LAB+CB revealed the higher ME and NE contents. Also, the predicted ME and NE contents of rice straw silage increased significantly (P<0.05) with molasses addition.

***In vitro* DM degradability (IVDMD):**

The effect of bacterial inoculants and molasses addition on *in vitro* DM degradability of rice straw silage at 96 hours of incubation are shown in Table (4). Bacterial inoculants resulted in significant (P<0.05) increase in IVDMD. The CB was more effective in IVDMD than LAB and the LAB+CB combination revealed the higher IVDMD. The IVDMD increased significantly (P<0.05) with molasses supplemented silage.

4. Discussions

Incubation of feedstuff with buffered rumen fluid *in vitro*, the carbohydrates are fermented to short chain fatty acids (SCFA), gases mainly CO and CH and microbial cells. Gas production is basically the result of fermentation of carbohydrates to acetate, propionate and butyrate. Gas production from protein fermentation is relatively small as compared to carbohydrate fermentation while, contribution of fat to gas production is negligible (Beuvink and Spoelstra, 1992 and Blummel and Ørskov, 1993). Biological inoculants increased *in vitro* gas production of sugar beet pulp silage (Kilic and Saricicek, 2011). Sallam (2005) found that rice straw showed medium gas volume (*b*) and lower rate of gas production (*c*). Garcıa-Rodriguez *et al.* (2005) reported that differences in parameters *B* and *c* between silages indicate different fermentation patterns.

Incubation of feedstuff with buffered rumen fluid *in vitro*, the carbohydrates are fermented to short chain fatty acids (SCFA), gases mainly CO and CH (Beuvink and Spoelstra, 1992 and Blummel and Ørskov, 1993). The degradability measurement accounts for feed conversion into all products of microbial degradation and synthesis, essentially microbial biomass, short chain fatty acids (SCFA) and gases, whereas the gas volume measurement reflects feed conversion into SCFA and gases (Grings *et al*., 2005).

Forage intake is mainly restricted by low digestibility, where the content of the cell wall constituents have the greatest impact on digestibility (Blummel & Becker, 1997; Mould, 2003). Several authors have found high correlations between *in vitro* GP studies and DMI of forages (Blummel & Becker, 1997; Hetta *et al*., 2007).

Using the *in vitro* gas measurement and chemical composition in multiple regression equation, Menke *et al*. (1979), McLeod and Minson (1971) and Van Soest (1994) found a high precision in prediction of *in vivo* OMD.

There was a positive correlation between metabolizable energy calculated from 24 hours *in vitro* gas production together with CP and fat content with metabolizable energy value of conventional feeds measured *in vivo* (Menke and Steingass, 1988). The *in vitro* gas production method has also been widely used to evaluate the energy value of several classes of feeds (Getachew *et al*., 1998), particularly straws (Makkar *et al*., 1999).

Some LAB inoculants applied at ensiling or added directly to the rumen fluid had the potential to increase the *in vitro* DMD (Weinberg *et al*., 2007). Biological inoculants increased IVDMD of sugar beet pulp silage (Kilic and Saricicek, 2011).

Table 1: Effect of bacterial inoculant and molasses addition on the cumulative gas production during different incubation time (ml/200 mg DM).

Item	Incubation time (hour)							
	3	6	9	12	24	48	72	96
Bacterial inoculant								
Control	5.33^{c}	8.79^{c}	13.48^{c}	18.75^{c}	25.89^{c}	35.32^{c}	38.62^{c}	40.49^{c}
LAB	6.33^{bc}	10.45^{bc}	16.01^{bc}	22.28^{bc}	30.76^{bc}	41.97^{bc}	45.89^{bc}	48.11^{bc}
CB	7.03^{ab}	11.59^{ab}	17.77^{ab}	24.72^{ab}	34.14^{ab}	46.57^{ab}	50.92^{ab}	53.38^{ab}
LAB+CB	7.87^{a}	12.99^{a}	19.92^{a}	27.71^{a}	38.25^{a}	52.19^{a}	57.07^{a}	59.82^{a}
SEM	0.27	0.45	0.69	0.96	1.32	1.80	1.97	2.06
Molasses								
Without	5.78^{b}	9.53^{b}	14.61^{b}	20.33^{b}	28.07^{b}	38.29^{b}	41.87^{b}	43.89^{b}
with	7.50^{a}	12.38^{a}	18.98^{a}	26.40^{a}	36.45^{a}	49.73^{a}	54.38^{a}	57.00^{a}
SEM	0.27	0.45	0.69	0.96	1.32	1.80	1.97	2.06

a, b, c: values in the same column for each item with different superscripts differ significantly (P<0.05).

Table 2: Effect of inoculants, molasses addition on gas production fractions and gad production rate.

Item	*a* (ml/g DM)	*b* (ml/g DM)	*c* (ml/hour)
Bacterial inoculant			
Control	0.60^{c}	43.40^{c}	0.043^{b}
LAB	0.71^{bc}	47.96^{bc}	0.044^{ab}
CB	0.78^{ab}	51.11^{ab}	0.045^{ab}
LAB+CB	0.88^{a}	54.97^{a}	0.046^{a}
SEM	0.03	1.28	0.0005
Molasses			
Without	0.64^{b}	45.44^{b}	0.044
with	0.84^{a}	53.28^{a}	0.045
SEM	0.03	1.28	0.0005

a, b, c: values in the same column for each item with different superscripts differ significantly (P<0.05).

Table 3: Effect of inoculants, molasses addition on gas production caused by fermentation of the soluble (GPSF) and insoluble (GPNSF) fractions and short chain fatty acids (SCFA).

Item	GPSF (ml/g DM)	GPNSF (ml/g DM)	SCFA (mM)
Bacterial inoculant			
Control	2.28^{c}	22.98^{c}	57.90^{c}
LAB	3.27^{bc}	26.92^{bc}	68.72^{bc}
CB	3.96^{ab}	29.65^{ab}	76.20^{ab}
LAB+CB	4.80^{a}	32.99^{a}	85.35^{a}
SEM	0.26	1.07	2.93
Molasses			
Without	2.72^{b}	24.74^{b}	62.73^{b}
with	4.43^{a}	31.53^{a}	81.35^{a}
SEM	0.26	1.07	2.93

a, b, c: values in the same column for each item with different superscripts differ significantly (P<0.05).

Table 4: Effect of inoculants, molasses addition on dry matter intake (DMI), organic matter digestibility (OMD), metabolizable energy (ME) and *in vitro* DM degradability at 96 hours of incubation (IVDMD).

Item	DMI kg/day	OMD %	ME Mcal/kg DM	NE Mcal/kg DM	IVDMD %
Bacterial inoculant					
Control	3.05^{b}	42.08^{c}	1.46^{c}	0.97^{c}	69.89^{b}
LAB	3.23^{ab}	46.58^{bc}	1.63^{bc}	1.08^{bc}	74.49^{ab}
CB	3.35^{a}	49.54^{ab}	1.74^{ab}	1.15^{ab}	77.50^{a}
LAB+CB	3.51^{a}	53.49^{a}	1.88^{a}	1.24^{a}	81.55^{a}
SEM	0.06	1.29	0.05	0.03	1.41
Molasses					
Without	3.13^{b}	43.84^{b}	1.53^{b}	1.02^{b}	71.67^{b}
with	3.44^{a}	52.00^{a}	1.82^{a}	1.20^{a}	80.04^{a}
SEM	0.06	1.29	0.05	0.03	1.41

a, b, c: values in the same column for each item with different superscripts differ significantly (P<0.05).

Conclusion:

From these results it could be concluded that the combination of lactic acid bacteria and cellulolytic bacteria at 10^{8} cfu/g with 5% molasses addition showed the best results concerning *in vitro* gas production.

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10/3/2012