**Effects of *Saccharomyces cerevisiae* at direct addition or pre-incubation on *in vitro* gas production kinetics and degradability of four fibrous feeds**

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**Abstract**

The objective of this study was to evaluate the effects of *Saccharomyces cerevisiae* on *in vitro* gas production (GP) kinetics and degradability of corn stover, oat straw, sugarcane bagasse and sorghum straw. Feedstuffs were incubated with different doses of yeast [0, 4, 8 and 12 mg/g dry matter (DM)] at direct addition or 72 h pre-incubation of *S. cerevisiae*. GP and methane were determined and contents of CH₄, DM and NDFD. Overall, the effect of dose varied among different feedstuffs and different application methods. Results suggested that the direct addition of *S. cerevisiae* could support and improve ruminal fermentation of low-quality forages at 4 to 12 g/kg DM.

**Introduction**

Direct-fed microbial offer a great potential for manipulation of ruminal fermentation and *Saccharomyces cerevisiae* is an especially attractive organism. *S. cerevisiae* addition was reported to increase nutritional value of poor quality forages. *S. cerevisiae* have the ability to increase dry matter (DM) and neutral detergent fibre (NDF) digestion (Carro et al., 1992), increase initial rates of fibre digestion (Williams et al., 1991). Numerous studies (Kumar et al., 2013; Pinoche et al., 2013) documented positive effects of yeast, not only on the rumen environment, but also on the improvement of microbial activities. *S. cerevisiae* supplementation leads to increase in the number of total anaerobic and cellulolytic bacteria (Newbold et al., 1996; Jousan, 2001). *Saccharomyces cerevisiae* can provide the rumen with important nutrients and nutritional cofactors in addition to vitamins such as biotin and thiamine, which reported to be required for microbial growth and activity (Callaway and Martin, 1997; Mao et al., 2013).

Many reports illustrated that administration of yeast is paralleled with increased gas production (GP). Many studies suggested that *S. cerevisiae* might stimulate the acetogens to compete or co-metabolise hydrogen with methanogens, thereby reducing CH₄ emissions (Iriostov et al., 2013). However, others reported increased CH₄ production (Martin et al., 1989; Martin and Nisbet, 1990), while Mathieu et al. (1996) reported no effects. These conflicting results on CH₄ production are likely due to strain difference of *S. cerevisiae* and type of diets (Patra, 2012). Supplementing diets with *S. cerevisiae* were shown to increase total volatile fatty acids (VFA) and propionic acid production (Mao et al., 2013). *S. cerevisiae* can enhance fungal colonisation of plant cell walls leading for increased DM and NDF digestion (Patra, 2012), increased initial rate of fibre digestion (Williams et al., 1991), improved *in situ* crude protein (CP) and NDF degradation.

**Materials and methods**

**Fibrous feed species and yeast product levels**

Three individual samples of each of the fibrous feeds corn stover, oat straw, sugarcane bagasse, and sorghum straw were randomly and manually harvested in triplicate from different sites in the State of Mexico. Samples were dried at 60°C for 48 h in a forced air oven.
to constant weight, ground in a Wiley mill to pass a 1 mm sieve and stored in plastic bags for subsequent determination of chemical components and in vitro GP. Four levels of commercial yeast product (Saccharomyces cerevisiae ATCC 1077, LEVUCELL® SC20; Lallemand, Montréal, QC, Canada) contain 1×10¹⁰ per gram yeast product. Doses of yeast were (g/kg DM): control (0 mg), low (4 mg), medium (8 mg) and high (12 mg). Feed samples were incubated with yeast doses that were added into the bottles immediately before incubation (direct method) or 72 h pre-incubated at room temperature. Stock solution of each yeast product doses was prepared before treatments in distilled water in order to get the suitable doses in each 1 mL of the stock solution.

**In vitro incubations**

Rumen inoculum was collected from two Brown Swiss cows (400 to 450 kg body weight) fitted with permanent rumen cannula. Cows were fed ad libitum a total mixed ration made up of 50:50 commercial concentrate (PURINA®, St. Louis, MO, USA) containing (g/kg DM): alfalfa hay was formulated to meet all of their nutrient requirements (National Research Council, 2001). Fresh water was available to cows at all times during the rumen inoculum collection phase.

Ruminal contents from each cow was obtained before the morning feeding, flushed with CO₂ then mixed and strained through four layers of cheesecloth into a flask with O₂ free headspace. Samples (1 g) of each feed were weighed into 120 mL serum bottles with appropriate addition of S. cerevisiae doses (g/kg DM). Consequently, 10 mL of particle free ruminal fluid was added to each bottle followed by 40 mL of the buffer solution according to Goering and Van Soest (1970), with no tryptophane case added, in a 1:4 (v/v) proportion.

During incubations, it was used 4 feedstuffs of 3 individual samples of each with the 4 doses of S. cerevisiae in 2 application methods (direct addition or 72 h pre-treatments) of S. cerevisiae and 4 bottles (replicates) were used for each incubated sample during 3 runs of incubation. Once all bottles were filled, they were immediately closed with rubber stoppers, shaken and placed in the incubator at 39°C. The volume of gas produced was recorded at times of 2, 4, 6, 8, 10, 12, 14, 24, 30, 48, 54 and 72 h of incubation using the pressure reading technique (Extech instruments, Waltham, MA, USA) of Theodorou et al. (1994). At the end of incubation (i.e. 72 h), bottles were uncapped, pH was measured using a pH meter (Conductronic pH15; Conductronic, Puebla, Mexico) and the contents of each bottle were filtered to obtain the non-fermented residue for determination of degraded substrate. After recording the final gas volume (i.e., 72 h), 2 mL of NaOH (10 M) were added to each bottles and gas pressure was determined immediately. Mixing of the contents with NaOH allowed absorption of CO₂, with the gas volume remaining in the head space of bottles considered to be CH₄ (Demeyer et al., 1988).

**Degradability and sample analysis**

At the end of incubation (i.e., 72 h), the contents of each serum bottle were filtered under vacuum through glass crucibles with a sintered filter (coarse porosity no. 1, pore size 100 to 160 μm; Pyrex, Stone, UK). Fermentation residues were dried at 105°C overnight to estimate DM disappearance with loss in weight after drying being the measure of undegradable DM. The NDF and ADF were calculated in the residues after incubation (DMD) determinations for establishing NDF and ADF degradability. Neutral detergent fibre was assayed without use of an alpha amylase but with sodium sulfite in the NDF. Both NDF and ADF are expressed without residual ash. Neutral detergent fibre and ADF were also determined in the residues samples after incubations for NDF and ADF degradability. Samples of the feeds were analysed for DM (#934.01), ash (#942.05), nitrogen (#954.01) and ether extract (#920.39) according to AOAC (1997). The NDF (Van Soest et al., 1991), ADF, and lignin (AOAC, 1997; #973.18) analyses used an ANKOM200 Fibre Analyser Unit (ANKOM Technology Corp., Macedon, NY, USA).

**Calculations and statistical analyses**

All the calculations were mentioned and described before in Salem (2012) as in the following.

Kinetic parameters of GP were estimated (mL/g DM) by fitted data in the NLN option of SAS (2002) according to France et al. (2000) as:

\[ A=b\times(1-e^{-0.1-1}) \]

where A is the volume of GP at time t; b is the asymptotic GP (mL/g DM); c is the rate of GP (/h); and L (h) is the discrete lag time prior to gas production.

Metabolisable energy (ME; MJ/kg DM) and in vitro organic matter digestibility (OMD; g/kg OM) were estimated according to Menke et al. (1979) as:

\[ \text{ME}=2.20+0.136 \text{GP (mL/0.5 g DM)}+0.057 \text{CP (g/kg DM)} \]

\[ \text{OMD}=148.8+8.89 \text{GP}+4.5 \text{CP} \]

where GP is net GP in mL from 200 mg of dry sample after 24 h of incubation.

The partitioning factor at 24 h of incubation (PF₂₄; a measure of fermentation efficiency) was calculated as the ratio of DMD in vitro (mg) to the volume (mL) of GP at 72 h (i.e., DMD Total gas production (GP₉₆) according to Blümml et al. (1997). Gas yield (GY₉₆) was calculated as the volume of gas (mL gas/g DM) produced after 24 h of incubation divided by the amount of DMD (g) as:

\[ \text{Gas yield (GY₉₆)}=\text{mL gas/g DM/g DMD} \]

Short chain fatty acid (SCFA) concentrations were calculated according to Getachew et al. (2002) as:

\[ \text{SCFA (mmol/200 mg DM)}=0.0222 \text{GP-0.00425} \]

where GP is the 24 h net gas production (mL/200 mg DM).

The experimental design for the in vitro ruminal GP, degradability and fermentation parameters analysis was a completely random design considering, as fixed factors, type of forage (S) and S. cerevisiae level (C) in the linear model (Steel et al., 1997) within each method (M) of application (direct or pre-incubation). Data of each of the three runs within the same sample were averaged prior to statistical analysis. Mean values of each individual sample within each species (three samples of each) were used as the experimental unit. The statistical model was:

\[ Y_{ijklm}=\mu+S_j+C_k+(S\times C)_{jk}+(S\times M)_{jk}+(M\times C)_{jk}+(S\times M\times C)_{jk}+\epsilon_{ijklm} \]

where Y_{ijkl} is every observation of the i th fibrous species (S) when incubated in the j th yeast (C; S. cerevisiae); μ is the general mean; S_j (j=1-4) is the feed effect; C_k (j=1-2) is the yeast dose effect (j=1-4); M_j is the application method (j=1-2), (S\times C)_{jk} is the interaction between feed and yeast dose; (S\times M)_{jk} is the interaction between feed and application methods; (S\times M\times C)_{jk} is the interaction between the three variable study (feed, yeast and application method); and \epsilon_{ijklm} is experimental error. Linear and quadratic polynomial contrasts were used to examine responses of feeds to increasing addition levels of the S. cerevisiae.

**Results**

The chemical composition varied between
Table 1. Chemical composition of the four fibrous feeds.

| Species             | OM, g/kg DM | CP, g/kg DM | ADF, g/kg DM | NDF, g/kg DM |
|---------------------|-------------|-------------|--------------|--------------|
| Corn stover         | 959.7       | 62.9ª       | 274.4ª       | 476.7ª       |
| Oat straw           | 923.6       | 37.2b       | 380.0ª       | 537.8ª       |
| Sugarcane bagasse   | 982.0       | 25.7c       | 324.4ª       | 458.9ª       |
| Sorghum straw       | 944.3       | 40.6ª       | 377.8ª       | 536.7ª       |
| SEM                 | 42.3        | 9.3         | 28.1         | 37.1         |

**Effect of fibrous species**

Gas production parameters were varied (P<0.001) between different fibrous species. Corn stover and oat straw improved the asymptotic gas production with increasing of the initial delay before GP beginning followed by sorghum straw and sugarcane bagasses which had the rate of GP increased. During the first 14 h of incubation, sugarcane bagasses improved (P<0.001) in vitro GP followed by corn stover where oat straw had the lowest (P<0.001). After 14 h of incubation, corn stover and sugarcane bagasses improved (P<0.001) in vitro GP followed by oat straw, while sorghum straw had the lowest (P<0.001). After 24 h of incubation only corn stover showed a gas production higher (P<0.001) than oat and sorghum straw (P<0.001), while sugarcane bagasses did not improve gas production compared to the straw (Table 2). Oat straw, sugarcane bagasses, and sorghum straw had both ruminal pH (P=0.006) and GY24 (P<0.001) increased with lowering (P<0.001) PF24 value compared to corn stover. Corn stover had DMD, OM, ME, SCFA, and PF24 values improved (P<0.001). On the contrary, sorghum straw had the highest (P<0.001) values of ME, NDFD, and ADFD compared to the other feeds. Sugarcane bagasses had the lowest values (P<0.001) of OM, NDFD, OMD, and SCFA production compared to other feeds (Table 3).

**Effect of application methods**

Direct addition of *S. cerevisiae* improved (P<0.001) the rate of GP with lowering (P<0.001) the initial delay before GP beginning. The in vitro GP was also improved (P<0.001) compared to the 72 h pre-incubation method (Table 2).

The direct addition of *S. cerevisiae* also improved CH4 (P=0.047), DMD (P=0.005), NDFD (P=0.005), ADFD (P=0.020), (P<0.001) compared to the other feeds. SCFA production improved (P<0.001) with lowering (P<0.001) NDFD and ME, where oat straw had the lowest (P<0.001) SCFA production compared to other feeds (Table 3).
OMD, SCFA, and GY24, with lowering (P<0.001) PF24 compared to the 72 h pre-incubation method. The method of application did not affect (P>0.05) both of the asymptotic gas production and ruminal pH (Table 3).

**Effect of yeast doses**

Both low and high doses of *S. cerevisiae* improved the asymptotic GP, with increasing *S. cerevisiae* doses, the rate of GP (linear, P=0.007; quadratic, P=0.006), the initial delay before GP beginning (quadratic, P<0.001), and in vitro GP (linear, quadratic, P=0.001) during the period before the first 24 h. After 24 h and up to 72 h of incubation, the highest dose of *S. cerevisiae* had the highest values of DMD, ME, OMD, and SCFA (Table 3). No effects (P>0.05) of *S. cerevisiae* doses on ruminal pH, CH4, and ADFD; however, addition of yeast caused a lowered (linear, P=0.005) values for NDFD (Table 3).

**Discussion**

**Chemical composition**

The chemical composition varied between the four fibrous feeds used in our study. These variations arise from variation in the genotype of the crops, differences between production environments, and from the interaction between environment and genotypes (Welch, 1995; Denić et al., 2011). Environmental differences will include variation in climate, the soil and agronomic practice, together with variations raised from different harvesting conditions, and post harvesting treatments (Welch, 1995; Elghandour et al., 2013). There is usually an inverse relationship between the CP and crude fibre content in a given forage species, and this has been revalidated in this study.

**Gas production, rumen fermentation and degradability**

The responses to *S. cerevisiae* are fibrous species type, forage composition, application methods and dose-dependent in addition to interactions among yeast and diet (Patra, 2012). Gas production from different fibrous species depends on its chemical composition. In our study, during the first 24 h of incubation, sugarcane bagasse and corn stover produced more GP than sorghum and oat straw compared to the period after 24 h up to 72 where they produced more gases. The production of gases from tested roughages depends on portentous and fibrous contents of feeds (Paya et al., 2007). Higher GP during the first period of fermentation of both sugarcane bagasse and corn stover refers to high content of highly fermentable constituents than sorghum and oat straw. Conversely, the fermentation process of sorghum and oat straw refers to their content of low fermentable constituents. Gas production depends on nutrient availability for rumen microorganisms (Mahala and Fadel Elseed, 2007). Fermenta-

Table 3. In vitro rumen fermentation profile of four low quality roughages as affected by the direct addition or 72 h pre-incubation with different levels of *Saccharomyces cerevisiae*.

| Effect of S | pH | CH4 | DMD | NDFD | ADFD | ME | OMD | SCFA | PF24 | GY24 |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| DM | mL/g | mg/g | mg/g | mg/g | MJ/kg | g/kg | mmol/g | mg | mL | gas/g |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Corn stover | 6.78 | 18.7 | 326.1 | 256.7 | 264.6 | 6.84 | 459 | 3.58 | 2.03 | 497.4 |
| Oat straw | 6.98 | 17.1 | 285.0 | 276.0 | 363.0 | 6.12 | 413 | 3.82 | 2.24 | 450.1 |
| Sugarcane bagass | 6.97 | 6.8 | 272.4 | 217.2 | 310.1 | 6.88 | 405 | 3.86 | 2.00 | 511.4 |
| Sorghum straw | 7.00 | 25.2 | 270.9 | 291.1 | 360.7 | 6.27 | 422 | 3.04 | 1.99 | 517.1 |
| LSD | 0.153 | 2.12 | 4.96 | 7.00 | 4.78 | 0.116 | 10.9 | 0.117 | 24.42 |
| P | 0.0006 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 |

**Effect of application M**

| Effect of Y product, mg/g DM | pH | CH4 | DMD | NDFD | ADFD | ME | OMD | SCFA | PF24 | GY24 |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Direct | 6.91 | 17.5 | 290.4 | 263.2 | 326.1 | 6.56 | 440 | 3.32 | 1.96 | 527.1 |
| Pre-incubation | 6.96 | 16.4 | 286.7 | 257.3 | 323.1 | 6.09 | 410 | 2.94 | 2.18 | 465.9 |
| LSD | 0.082 | 1.14 | 2.52 | 3.75 | 2.60 | 0.089 | 5.83 | 0.073 | 0.063 | 13.11 |
| P | 0.257 | 0.0467 | 0.0046 | 0.0045 | 0.0204 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 |

**Effect of Y product, mg/g DM**

| Interactions | SxM | SxY | MxY | SxMxY |
| --- | --- | --- | --- | --- |
| 0 | 0.1869 | 0.2509 | <0.0001 | <0.0001 |
| 4 | 0.9837 | <0.0001 | <0.0001 | <0.0001 |
| 8 | 1.2887 | <0.0001 | <0.0001 | <0.0001 |
| Linear | 0.4184 | 0.1536 | 0.0001 | 0.0001 |
| Quadratic | 0.7069 | 0.1493 | <0.0001 | <0.0001 |

CH4, methane emission; DM, dry matter; DMD, dry matter degraded substrate; NDFD, neutral detergent fibre degradability; ADFD, acid detergent fibre; ME, metabolisable energy; OMD, organic matter digestibility; SCFA, short chain fatty acids; PF24, partitioning factor at 24 h of incubation; GY24, gas yield at 24 h; S, species; LSD, least significant difference; M, method; Y, yeast product. **Different superscripts following means in the same row indicate differences at P<0.05.**
tion of dietary carbohydrates to acetate, propionate and butyrate produces gases in the rumen which are mainly composed of hydrogen, carbon dioxide and methane. However, fermentability of protein produces relatively small GP compared to carbohydrate fermentation (Makkar et al., 1995). This can explain how S. cerevisiae addition could improve GP at the time it reduced NDFD. All depends on the chemical composition of fermented feeds. Availability of nutrients for rumen microorganisms will stimulate the degradability of different nutrients (Paya et al., 2007). It is very important to stress that not only has the addition of S. cerevisiae the ability to improve GP, but it can also make qualitative changes in GP thereby reducing its negative effect on the environment. S. cerevisiae has the ability to decrease methane and ammonia production, and to improve fermentation efficiency to contribute to a reduction in greenhouse gas emissions (Hristov et al., 2013). Moreover, decreasing protein degradation and ammonia production in the rumen (Mao et al., 2013) has the ability to decrease the overall nitrogen excretion by the animal, which would contribute to decreased ammonia emissions from cattle manure.

There are a few data in the literature regarding the effect of S. cerevisiae method of application on in vitro gas kinetics and fermentation profile. It has been found that the method of the S. cerevisiae product application depends on number of live or metabolically active S. cerevisiae that will stimulate rumen fermentation (Dawson et al., 1998). Direct application of S. cerevisiae ensures the viability of S. cerevisiae cells so an improvement occur for in vitro GP and fermentation kinetics and profile compared with pre-incubation method. Pre-incubation of S. cerevisiae with different fibrous feeds may negatively affect the fermentation process which reflected on low fermentability for all fibrous species. Doreau and Jouany (1998) stated that direct-fed microbial are often recommended in various European countries than other administrations methods. Elam et al. (2003) hypothesised that the initial advantages of direct-fed microbial involve a favourable alteration of the gastrointestinal micro-flora and that over time that innate immunological mechanisms of control animals provide this same function.

Improved GP with increasing S. cerevisiae doses reflects the enhanced ruminal environment. Paulus et al. (2012) and Mao et al. (2013) documented the positive effects of S. cerevisiae on ruminal fermentation and microbial activities. A number of specific hypothetical biochemical mechanisms have been developed to explain the stimulatory effects of S. cerevisiae in the rumen (Chevaux and Fabre, 2007). Some of these mechanisms have been based on the ability of yeast to provide important nutrients or nutritional cofactors that stimulate microbial activities (Callaway and Martin, 1997). Another suggested the ability of S. cerevisiae to scavenges excess oxygen creating a more optimal environment for rumen anaerobic bacteria (Newbold et al., 1996; Jouany, 2001). Others studies suggested that S. cerevisiae supplementation could provide vitamins such as biotin and thiamine, which are reported to be required for microbial growth and activity (Akin and Borneman, 1990). In addition, others suggested that S. cerevisiae can provide a focal point for the development of a stable microbial consortium (Jouany, 2001). In this model, the S. cerevisiae cells provide a site for metabolic exchanges and an environment that promotes the growth of beneficial microorganisms around substrates.

One possible explanation for the varied response with a different level of S. cerevisiae in this study is at least partially due to the nature of the in vitro procedure. For the in vitro model, the substrate amount relative to the rumen liquid volume is much less than in the rumen of a cow (<1 vs 12%). Therefore, when a rumen modulator like S. cerevisiae is supplemented at a different rate, it could change the fermentation rate and cause different substrate depletion, resulting in different response as the fermentation length is changed (Mao et al., 2013). Lila et al. (2004) found variable effects of S. cerevisiae on ruminal fermentation when different substrates were used in vitro.

Decreased lag time with S. cerevisiae addition can be illustrated based on two basic mechanisms. The first mode of yeast action reported by Newbold et al. (1996) is the respiratory activity that scavenges O2, which is toxic to anaerobic bacteria and causes inhibition of adhesion of cellulolytic bacteria to cellulose, and this peak in O2 concentration occurs at approximately the time of feeding (initial time). The second mode is that S. cerevisiae contains small peptides and other nutrients that required to predominant ruminal cellulolytic bacteria to initiate growth (Callaway and Martin, 1997).

Addition of S. cerevisiae increased SCFA production on forage substrates (Mao et al., 2013). Increased SCFA production and ME are associated with high activities of microbes in the rumen. S. cerevisiae produces growth factors for microbial growth that can stimulate rumen microbial growth and activity (Chiquette, 2009). In addition to the ability of S. cerevisiae to provide conducive conditions to microbial growth in a way that is capable of using O2 in the rumen so that the conditions of an aerobic rumen awake (Mosoni et al., 2007). S. cerevisiae. Newbold et al. (1996), for example, used this mode of action to explain a 35% increase in total bacterial counts with S. cerevisiae in vitro.

Addition of S. cerevisiae lowered PF24 values. A lower PF24 would reflect lower conversion of degraded substrate into microbial biomass and vice versa (Harikrishna et al., 2012). Ruminal pH was not changed during fermentation processes. Several studies have suggested that S. cerevisiae moderate the ruminal pH by increasing lactate utilisation making pH relatively more stable and meet the needs of rumen microbes to perform its activity (Paulus et al., 2012).

Conclusions

The responses to supplemental S. cerevisiae varied among the fibrous species tested, the results of this study suggest that the addition of S. cerevisiae can support ruminal fermentation of low-quality forages. In general, S. cerevisiae added at 4 to 12 g/kg DM showed the greatest responses in most variables tested.

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