

***In vitro* gas production of fibrous substrates with the inclusion of yeast**

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The *in vitro* gas production technique was applied for studying the effect of the yeast species and its inclusion dosage on ruminal fermentation of fibrous substrates. Two experiments were executed. In the first, the effect of yeast inclusion of different species on *in vitro* gas production of star grass (*Cynodon nlemfuensis*) was determined and in the second, two levels (5 and 10 mg of DM/mL) of the yeast strain showing the best performance in the first experiment were included for confirming its effect on gas production of maize stubble. Controls without yeast and a blank with the non-inoculated culture were included. As result, all strains had a gas production higher than the control, except in that including strain 15. The best performance was in the one in which strain 27 was incorporated. Strains of *S. cerevisiae* had an intermediate performance regarding the rest. The inclusion of 10 mg DM/mL of the strain 27 (*Pichia guillermondii*) stimulated *in vitro* gas production of the maize stubble until 48 h of fermentation. It is concluded that the species and strain of yeasts, as well as their inclusion dosage, have determinant effect on *in vitro* gas production of different fibrous substrates. This reasserts the importance of selecting the adequate strains for their utilization as additive in diets for ruminants, according to the feed intended to be used.

Key words: yeasts, *Pichia guillermondii*, rumen, gas production

The addition of yeasts as additive in ruminant diets has favorable effects on microbial population and on the ruminal fermentative indicators. Consequently, health and productivity of animals are improved (Stella *et al.* 2007 and Doleža *et al.* 2011). There are multiple products in the world market in which commercial strains of *Saccharomyces cerevisiae* yeast are employed as activators of ruminal fermentation (Chaucheyras *et al.* 2004). However, there are not plenty studies showing the utilization of other yeast genera for these purposes.

Nonetheless, the strain of *Issatchenkia orientalis* dy 252 has been indicated as a potential candidate that could be used as additive (Lee *et al.* 2002). The goodness of yeast genera different from *Saccharomyces* are known as activators of the ruminal fermentative processes (Galindo *et al.* 2008). In previous studies, Marrero *et al.* (2013) demonstrated that yeast strains of *Candida* genus species increased *in vitro* gas production of *C. nlemfuensis* when 20 mg DM/mL were included. These backgrounds justify the realization of studies for using yeasts of different species as additive activator of ruminal fermentation. Thus, the objective of this research was to study the effect of the yeast species and its inclusion dosage on *in vitro* gas production of two fibrous substrates.

Materials and Methods

The first experiment was carried out at the laboratory of the Department of Biophysiological Sciences of the Institute of Animal Science, in Cuba and the second at

the laboratories of Animal Nutrition of the Faculty of Zootechny of the Autonomous University of Chihuahua, Mexico.

The *in vitro* gas production technique (Theodorou *et al.* 1994) was used. In the first experiment the effect of the inclusion of yeasts of different species on *in vitro* gas production of star grass (*Cynodon nlemfuensis*) was determined. Previous results of Marrero *et al.* (2013) with the same yeast strains were taken into account. In addition, the utilization of a dosage of 10 g DM/animal was assessed, since it is the one generally included in ruminant diets (Rojo *et al.* 2000, Combillas *et al.* 2002 and Beauchemin *et al.* 2003). In the second experiment the *in vitro* gas production of other fibrous substrate (maize stubble) was studied with two levels of yeast strain. This strain had better performance in the first experiment.

Yeast strains. For the study two reference strains of *Saccharomyces cerevisiae* were used: one from the collection of the Cuban Research Institute of Sugar Cane Derivatives (ICIDCA), Cuba and the other isolated from the product LUVUCCELL® SC (LLSC). The rest is called LEVICA and belongs to the yeast collection of the Institute of Animal Science, Cuba (RYCASI), with registration number 980 in the World Data Centre for Microorganisms (WDCM). These belong to the species *I. orientalis*, *P. guillermondii*, *R. mucilaginosa*, *C. tropicales* and *Yarrowia lipolitica* and their gene sequences are found in the Gen Bank (Marrero *et al.* 2013).

For the study, wedged yeast cultures were used for 24 h. From these, two loops were taken and dissolved in 50 mL of malt extract culture medium. For that, Erlenmeyers (100 mL) were used and incubated at 30 °C for 24 h. From these cultures, in each case, the amounts for the incubations were taken.

Experiment 1. The incubations were carried out in glass bottles of 100 mL, sealed with butyl and agraffe cap. These were placed in incubation bath at 39 °C. The gas accumulated was measured by the piston displacement of a 20 mL syringe, after puncturing the cap with a needle inserted at its point. Four bottles for each strain studied were placed with controls without yeast and blanks with the non-inoculated culture medium.

One mL of the yeast culture containing approximately 5 mg DM/mL, was used which were equivalent to 10 g DM/animal and a concentration of 10^7 live cells/mL. Incubations were made in 50 mL of a mixture of filtrated rumen liquid (FRL) and artificial saliva, in a 1:3 proportion. The substrate consisted of 0.5 g of star grass (*Cynodon nlemfuensis*), with a chemical composition of (% DM): 7.26; 74.57; 10.11; 0.42 and 0.18 for CP, NDF, ash, calcium and phosphorus, respectively. For the preparation of the substrate, samples were dried in an oven at 60 °C for 48 h. They were grinded until attaining particle size of 1 mm and were preserved in flasks within a drier until their use. The measurements of gas production were determined at 2, 4, 6, 8, 12, 16, 20 and 24 h.

Experiment 2. The study was conducted to demonstrate the effect of the inclusion of two levels (5 and 10 mg DM/mL) of the *P. guillermondii* yeast, LEVICA 27 strain on ruminal fermentation of maize stubble.

Incubations were conducted in 50 mL glass bottles sealed with butyl and agraffe cap. These were placed at a rate of four bottles per treatment in the Orbital Shaker with mechanical agitation (120 r.p.m.) and temperature of 39 °C. The accumulated gas was measured by means of a pressure transducer, mark FESTO. The volume of gas produced by the diet was estimated through the utilization of a linear regression equation between the volume and the pressure.

$$\text{Volume (mL)} = 2.4964 * \text{pressure}$$

The inclusion of the strain culture was at a rate of 5 and 10 mg DM /mL, though 1.5 and 3 mL of yeast culture, respectively were added according to the treatment in 24 mL of the fermentation medium, with equal concentration of live cells and proportion of the FRL mixture and artificial saliva than in experiment 1. Two controls without yeast for each level and a blank with the non-inoculated culture medium were included. Measurements of gas production were made at 2, 4, 8, 12, 24, 36 and 48 h.

Maize stubble (0.2 g) per bottle was employed. The chemical composition was (% DM): 95.41; 91.9; 2.52; 33.29 and 0.96 for DM, OM, CP, CF and EE, respectively. For the preparation of the substrate the same

procedure as in experiment 1 was followed.

Statistical analysis. The modeling of the accumulated gas production curves was carried out by the bi-compartmentalization model and adjusted by the Gompertz model. The statistical criteria taken into account were: coefficient of determination, significance of the adjustment and parameters and normality of the residues:

$$GP(a, b, c, t) = a \exp(-be^{-ct})$$

The homogeneity test of the curves was realized according to the methodology indicated by Jay *et al.* (2012 a, b). As selection test, the probability of fulfilling the null hypothesis, by the corrected Akaike information criterion (CAIC). Later, the post-hoc multiple comparison tests, the quadratic distance mean of the adjusted curves and Tukey's comparison test for experiment 1 were taken as bases. For experiment 2, the significant distance of Scheffé (Montgomery 2004) was applied, both with a 95 % confidence level.

Data were analysed according to Balzarini *et al.* (2012).

Results and Discussion

Figure 1 shows the performance of the accumulated gas production in time, according to the model of Gompertz. In table 1 are presented the results of the estimation of the gas production model. It must be highlighted that the value of the coefficient of determination was $R^2 = 0.867$, though results show the good adjustment of this model for this type of study (Thornley and France 2007).

All treatments had a superior performance than the control (table 2). The treatment of better performance was that including strain 27. *S. cerevisiae* strains had an intermediate performance regarding the rest. However, that of ICIDCA was superior to CLLSc.

In similar studies, Castillo (2009) indicated that the inclusion of 20 mg of DM/mL of yeast strains of different genera, isolated from the ruminal ecosystem increased the *in vitro* gas production, on fermenting alfalfa (*Medicago sativa*) hay. This coincides with the results of Marrero *et al.* (2013), on employing star grass (*Cynodon nlemfuensis*). In both cases, the stimulation was superior to that provoked by *S. cerevisiae* strain, included as standard.

If previous results are compared to those obtained in this study, regarding the strains attaining greater stimulation of gas production, the determinant effect of the inclusion dosage, can be demonstrated, since on decreasing this to 5 mg of DM/mL, results varied substantially. This confirmed what was expressed by Doležal *et al.* (2011) regarding the fact that the efficiency of the use of yeast cultures depends, among other factors, on the culture conditions, the concentration of live yeast cells (CFU) and the culture dosage that is included.

Studies developed in Mexico by Castillo (2009) demonstrated that from the 20 strains isolated, nine had

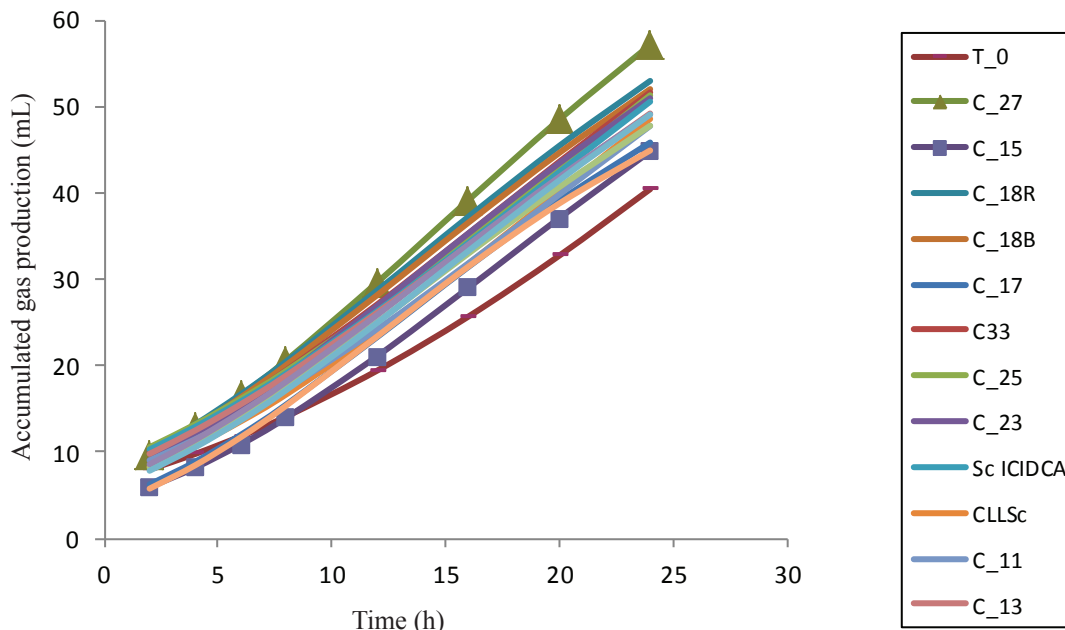


Figure 1. Effect of yeast inclusion on the accumulated gas production of *Cynodon nlemfuensis* according to Gompertz model

Table 1. Estimations of the parameters of the reduced model

Parameter	Est.	Typical error	Confidence interval at 95 %	
			Lower limit	Upper limit
a	90.15	8.41	73.65	106.66
c	0.0613	0.0052	0.0510	0.0716
b	2.653	0.061	2.532	2.773

Table 2. Comparison of the curves by the CAIC test and group formations by Tukey’s test

C11 (a)	C13 (b)	C15 (c)	C17 (d)	C22 (d)	C28 (be)	C18B (f)	C18R (f)	SE=0.12
C23 (beg)	C24 (abe)	C29 (ae)	C33 (fg)	C27 (h)	CLLS (ca)	C25 (bfg)	Sc ICIDCA (beg)	

Different letters represent significant differences at $P < 0.05$

similar performance in the *in vitro* gas production, when alfalfa hay was utilized as substrate of high ruminal digestibility. However, when these nine strains were included with oat straws, as poor digestibility substrates on *in vitro* ruminal fermentation, strain 15 had better performance in gas production, even until 48 h. This also evidences that the substrate or diet influences when yeasts are intended to be used as stimulators of the ruminal fermentative process.

From these conditions, experiment 2 was designed. In this the two dosages of yeast inclusion of best performance in experiment 1 (LEVICA 27) were compared and another substrate (maize stubble) was used. The fermentation time was extended until 48 h. In figure 2 are shown the regression curves of the *in vitro* gas production of that substrate, in which it can be seen that the asymptotes of the curves corresponding to the treatments including yeast tend to higher values than those of the controls. Treatment including 20 g of LEVICA 27 was the best performing.

The above mentioned is confirmed in the formation of

groups by the post hoc comparison (table 3). All seems indicating that when LEVICA 27 yeast was utilized as additive of a poor nutritive value diet, as maize stubble, a stimulation occurred of the populations in charge of degrading this fibrous substrate, that increased gas production.

In similar studies, Galindo *et al.* (2010) utilized dosages of 20 mg of DM/mL of strains of *Saccharomyces* and *Candida* genera. They obtained gas productions at 24 h of fermentation of 37.08 and 55.01 mL/g DM, respectively. In this case, the substrate used was star grass (*Cynodon nlemfuensis*) and the potentiality of the strains different to *Saccharomyces* was evidenced as stimulators of gas production in the fermentation process of fibrous substrates.

In this study it is important emphasizing that the stimulator effect of LEVICA 27 was also maintained for 48 h. This approach agrees with previous *in vivo* and *in vitro* studies in which the degrading rate of the cellulose and NDF was increased when yeasts were added (Olson *et al.* 1994 and Sullivan and Martin 1999). A positive

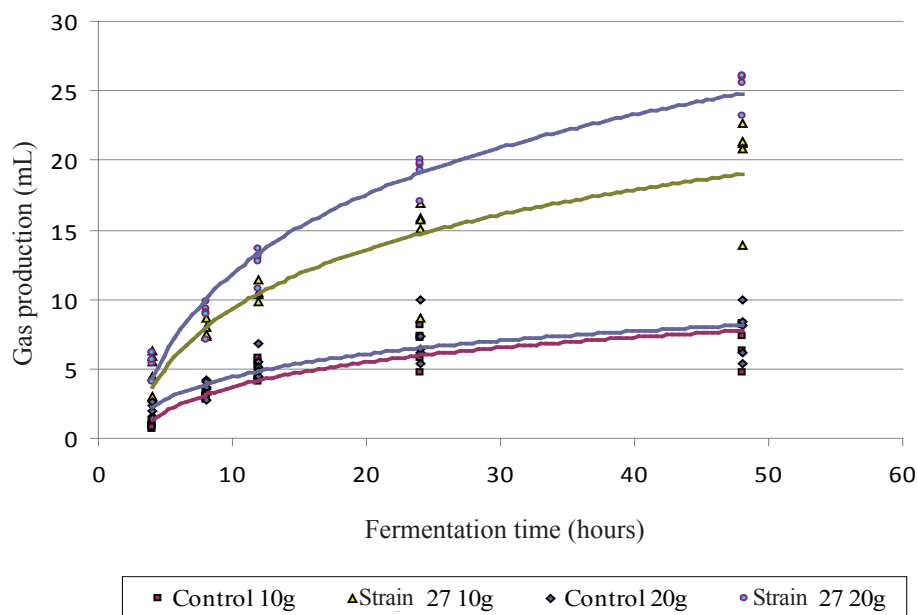


Figure 2. Effect of the inclusion of two levels of LEVICA 27 yeast on accumulated gas production of maize stubble according to Gompertz model.

Table 3. Comparison of the curves by CAIC test and group formations by Scheffé's test

Control 10g (a)	Strain 27 10g (b)	Control 20g (a)	Strai 27 20g (c)	SE=0.16
Different letters represent significant differences $P < 0.05$				

effect was also found of the addition of a yeast culture on gas production of straws of different cereals (Tang *et al.* 2008).

De Ondarza *et al.* (2011) stated that the increase of the fiber digestion and the pH control of the rumen provoked by yeast addition to the diets increases microbial yield, due to higher substrate availability and to the improvement in the function of the ruminal microbial population. These authors recommend new investigations aimed at designing diets optimizing the response to the inclusion of specific yeast strains.

Results exhibited corroborate that different species or different strains of a yeast species, as well as the dosage used, influence in an important way on the physiological response and thus, can have a repercussion on the productivity of the animal (Newbold 1996 and Enjalbert *et al.* 1999). In addition, they demonstrate that the selection of microorganisms to be used as microbial additives is a complex process. They also confirm that it is necessary securing the additives responding to the specific situations generated in dairy productions based on the use of fibrous diets of medium and poor quality. In the LEVICA 27 (*P. guillermondii*) strain, results are encouraging. Also, it has been demonstrated that a strain of the same species was capable of producing high levels of biomass and biomass-substrate yield, using molasses C as carbon and energy sources, in submerged cultures as in fermentation (Sánchez *et al.* 2007). This constitutes an advantage for obtaining it at large scale, at lower cost. In addition, it makes feasible the realization of *in vivo* physiological studies and the evaluation of the effect of

inclusion of this yeast species on milk production.

Results of this study allow concluding that the species and yeast strains, as well as its inclusion dosage had determinant effect on *in vitro* gas production of different fibrous substrates. This reaffirms the importance of selecting adequate strains for its use as additive in diets for ruminants, according to the feed intended to be used.

In vivo studies with LEVICA 27 (*P. guillermondii*) strain are recommended for confirming its potentiality as activator of ruminal fermentation.

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