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Chemical composition, *in vitro* gas production and energetic value of prickly pear fermented with and without *Kluyveromyces marxianus*

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ABSTRACT

The aim of this study was to characterize the chemical composition, *in vitro* gas production and energetic value of prickly pear during solid state fermentation (SSF) with *Kluyveromyces marxianus*. Prickly pear was incubated in SSF at 28°C for 0, 24, 48, 72, 96 and 120 h without and inoculation with *K. marxianus.* The fermented cactus pear samples were dried at 55°C for 24 h, to determine the percent of dry matter (DM), crude protein (CP), neutral detergent fibre (NDF) and acid detergent fibre (ADF). The volume of gas was recorded at 0, 3, 6, 9, 15, 24, 36, 48, 72 and 96 h and the parameters of *in vitro* gas production were obtained from the model: $A= b*(1-e^{-c(t-L)})$. *In vitro* gas production at 24 h was utilized for estimation of metabolizable energy (ME) and short chain fatty acid (SCFA). Data were analyzed as a completely randomized design with a 2x6 factorial arrangement of treatments. The SSF in presence of *K. marxianus* increases the CP, maximum gas production (**b**), constant rate of gas production (c), ME and SCFA of prickly pear (P<0.05) but decreases NDF and ADF contents (P<0.05). We conclude that SSF with *K. marxianus* significantly improvement nutritional quality of prickly pear and may even promote animal performance.

Key words: prickly pear, *Kluyveromyces marxianus,* solid state fermentation, nutritional quality, *in vitro* gas production

Introduction

Acacia and other shrubby plants also can be used to mitigate desertification, alleviating the effects of droughts, allowing soil fixation, and enhancing restoration of the vegetation and rehabilitation of rangelands (Amber et al., 2010; Rodriguez et al., 2014). For farmers in arid zones, cactus pear planting is one solution to alleviate the problem of recurrent drought. The prickly pear is well adapted for cultivation in semi-arid regions (Stintzing & Carle, 2005). The cladodes, also known as cactus pads, of the spineless cultivars can be used as livestock feed, but because of their low crude protein content of about 40 g/kg DM, they should be regarded as a cheap energy source rather than as a balanced fodder crop (Akanni et al., 2015). However, nutritive value of prickly pear could be improved by single cell protein production. Thus, the cladodes have potential of serving as lignocellulosic feedstock for microbial cultivation. The solid state fermentation (SSF) is a biotechnology may enhance the nutritional quality of prickly pear, because this technology is able to produce biomass from carbohydrates present in prickly pear (Peláez et al., 2011). Currently, yeasts like *Saccharomyces cereviseae* and some yeasts species of *Kluyveromyces*, are among the microorganisms that are frequently used in this process (Van Markis et al., 2006).

On the other hand, the relevance of evaluating the nutritional value of forage has an important contribution in the feeding of grazing cattle. The *in vitro* method of Tilley & Terry (1963), *in situ* method (Mehrez & Orskov, 1977) and enzymatic methods (Aufrere, 1982) have all been widely used to estimate the nutritional quality of forages. In present, the *in vitro* gas production method has also been widely used to evaluate the quality nutritional and energetic value of several classes of forages as straws (Makkar et al., 1999), agro-industrial by-products (Krishna & Günther, 1987) and grassland (Murillo et al 2011). Until now, there is little information about chemical composition, *in vitro* gas production and energetic value of prickly pear fermented with *Kluyveromyces marxianus.* Therefore, the aim of this study was to characterize the chemical composition, *in vitro* gas production and energetic value of prickly pear during solid state fermentation with *Kluyveromyces marxianus*.

Materials and Methods

Location

The present study was performed in the Postgraduate Laboratory of the Faculty of Medicine Veterinary and Husbandry of Juarez University at the Durango and Microbial Biotechnology Laboratory of the Graduate and Research Unit of the Technological Institute of Durango.

Yeast strain

The yeast strain *K. marxianus* ITD00262 was obtained from the collection of the Microbial Biotechnology Laboratory (Technological Institute of Durango). This strain was previously isolated by traditional agave fermentation and identified using restriction fragment length polymorphism and the Yeast-Id date base (Páez et al., 2013).

Physiological characterization

Cultures were obtained from cultures that had been inoculated on agar plates and incubated overnight at 28°C in 10 ml Yeast Nitrogen and Base (YNB) medium (containing 1.7% YNB w/w nitrogen, 2% lactose and 2% inulin) with 0.5% ammonium as the sole nitrogen source.

Cultivation

K. marxianus ITD00262 was grown in peptone, 2% dextrose and 1% yeast extract broth at pH 4.8 and 28°C for 12 h under agitation (120 rpm) to obtain an initial count of 10^8 cells/ml (Frazier & Westhoff, 1998).

Solid state fermentation

Fermentation was carried out on flasks with 250 g chopped cactus pear without the yeast and chopped cactus pear inoculated with 2.6×10^6 cells/g DM *K. marxianus* ITD00262. Each flask was incubated under statics conditions at 28°C for 0, 24, 48, 72, 96 and 120 h in an incubator. At each sampling time, three flasks were withdrawn from the incubator and the total contents of each flask was collected and individually homogenized.

Chemical analysis

The prickly pear samples and fermented prickly pear were dried at 55°C for 24 h in a forced air oven. All samples from all fermentation times were subjected to several nutritional analyses. Dry matter (DM) and crude protein (CP) concentration were determinate according to the AOAC (1994). Neutral detergent fibre (NDF) and acid detergent fibre (ADF) determined by procedures proposed by Van Soest et al., (1991). In *vitro* organic matter digestibility (IVOMD) and digestible organic matter (DOM) were determined through methods suggested by ANKOM (2008).

In vitro gas production

The *in vitro gas* production was carried out using method proposed for Menke & Steingass (1988). Approximately, 200 mg of samples fermented without and with *K. marxianus*, were ground to 1 mm and placed in triplicate in modules of ANKOM system. Buffer and mineral solutions were added in a 2:1 ratio to ruminal liquid collected from fistulated steers, which were fed with alfalfa hay. Forty milliliters of this mixture were introduced in each module for incubation. The volume gas was recorded at 0, 3, 6, 9, 15, 24, 36, 48, 72, and 96 h of incubation.

Model and calculations

To estimate kinetics parameters of *in vitro* gas production (GP), data were fitted using the NLIN procedure of SAS (2003), according to France et al. (2000) as: $A = b*(1-e^{-c(t-L)})$ where: A is the volume of GP (ml) at time *t; b* is the asymptotic GP (ml/g DM); *c* is the rate GP (ml/h) and *L* (h) is the discrete lag time prior to GP.

The metabolizable energy (ME) and short chain fatty acid (SCHFA) were calculated using equations of Menke & Steingass (1988) and Getachew et al. (2002) as:

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ME (MJ/Kg DM) = 1.06+0.1570 GP + 0.0084 CP + 0.022 CF – 0.0081 CA

SCFA (mmol/200 mg DM) = 0.0222 GP - 0.00425

where: GP is 24 h net gas production (ml/200 mg DM); CP, CF, and CA are crude protein, crude fat and crude ash (% DM), respectively.

Statistical analysis

Data were analyzed as a completely randomized design with a 2x6 factorial arrangement of treatments using MIXED procedure (SAS, 2003). The factors evaluated were prickly pear fermented with and without yeast and the different fermentation times in solid state (0, 24, 48, 72, 96 and 120 h). The model included the effects of the yeast, fermentation time and interactions among both factors. The comparison of means among yeast ('-' without; '+' with) and fermentation time was performed using the LSMEANS statement of SAS (2003).

Results and Discussion

The crude protein and digestible matter organic (DOM) contents of fermented prickly pear without and with *Kluyveromyces marxianus* is shown in Table 1. There were interaction between yeast and fermentation time on CP and DOM, contents (P<0.05). At 120 h of fermentation was obtained higher CP content in cactus pear in presence of yeast (P<0.01). To our knowledge, this study is the first to publish the CP values of prickly pear fermented with *K. marxianus*. In another study, Diaz (2011) reported an increment of 24.52% CP for prickly pear fermented with *K. lactis*, while Diaz et al., (2012) reported an increment of 19.36% CP for prickly fermented with *K. lactis*, urea, ammonium sulphate and minerals. These results were lower to register in this study. The general increase in CP may be explained by the growth of native microorganisms in prickly pear without inoculum. In comparison, the high increase obtained in prickly pear fermented with *K. marxianus* may be due to growth of yeasts during fermentation. The DOM content increased by 18.1% after 120 h fermentation in presence of *K. marxianus*. The neutral detergent fibre and acid detergent fibre contents of fermented prickly pear without and with *Kluyveromyces marxianus* is shown in Table 2.

Table 1. *Least square means for crude protein (CP) and digestible matter organic (DMO) contents of fermented prickly pear without and with Kluyveromyces marxianus.*

Fermentation time (h)	**CP (g/Kg, DM)**		**SEM**	**P<**
	- K. Marxianus	**+ K. Marxianus**		
0	38.5	78.2	0.77	**
24	73.5	95.1	0.77	**
48	59.5	98.9	0.77	**
72	74.2	118.5	0.77	**
96	46.5	146.5	0.77	**
120	42.5	171.4	0.77	**
Fermentation time (h)	**DOM (g/Kg, DM)**		**SEM**	**P<**
	- K. Marxianus	**+ K. Marxianus**		
0	505	643	1.1	*
24	486	614	1.1	**
48	531	686	1.1	**
72	564	703	1.1	**
96	575	733	1.1	**
120	628	742	1.1	**

K. marxianus ('-' without; '+' with); (P<0.05); SEM = standard error of mean; * P<0.05; ** P<0.01.

Table 2. *Least squares means for neutral detergent fibre (NDF) and acid detergent fibre (ADF) of fermented prickly pear without and with Kluyveromyces marxianus.*

Fermentation time (h)	**NDF (g/Kg, DM)**		**SEM**	**P<**
	- K. Marxianus	**+ K. Marxianus**		
0	278	348	0.18	*
24	370	363	0.18	*
48	242	212	0.18	*
72	240	196	0.18	*
96	291	172	0.18	**
120	283	150	0.18	**
Fermentation time (h)	**ADF (g/Kg, DM)**		**SEM**	**P<**
	- K. Marxianus	**+ K. Marxianus**		
0	155	131	0.91	*
24	215	150	0.91	*
48	180	137	0.91	*
72	158	114	0.91	**
96	192	110	0.91	*
120	201	108	0.91	**

K. marxianus ('-' without; '+' with); (P<0.05); SEM = standard error of mean; * P<0.05; ** P<0.01.

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There were interaction between yeast and fermentation time on NDF and ADF contents (P<0.05). Liekwise, at 120 h of fermentation were obtained lower NDF and ADF contents in presence of yeast (P<0.01). The NDF and ADF contents of prickly pear decreased by 47 and 46.2%, after 120 h SSF in presence of *K. marxianus*, respectively (P<0.01). Previous studies have not reported the concentration of these fractions in fermented prickly pear with *K. marxianus.* However, our results support an earlier study by Pinos et al. (2010), who reported 28.8% and 15.4% NDF and ADF, respectively, for prickly pear without fermentation. In fact, Van Soest (1982) considered that forages with a content lower at 40% of NDF to be of good nutritive quality. Therefore, the prickly pear after fermentation with *K. marxianus* may be considered as very good quality forage to livestock. The NDF and ADF are mainly composed by lignocellulosic material, which includes cellulose, hemicellulose and lignin (lignocellulosic complex) (Van Soest, 1994). The decreased in NDF and ADF fractions could be attributed to action synergistically among enzymes produced by yeast and native fungi of substrate. These enzymes degrade the plant cell wall, releasing sugars monomers that can be used as substrates for the metabolism of the microorganisms (Stricker et al., 2008). This phenomenon leads to glucose being released in a free form, which can enter the metabolism of the microorganism, providing its energy (Himmel et al., 2007). This could be explicated by DOM content obtained in present study which increased in presence of yeast (P<0.05). According to Van Soest (1994) the DOM is equivalent of the energy that is supplied by forages to ruminal microorganisms.

The *in vitro* gas production parameters of prickly pear fermented without and with *K. marxianus* are shown in Table 3. There were interactions yeast*fermentation times on **"b"** and **"c"** parameters (P<0.05). Nevertheless, there were no yeast*fermentation times interactions on the **Lag** phase. (P>0.05). The maximum of gas production **(b)** and constant rate of gas production **(c)** increased in presence of yeast through the fermentation times (P<0.05). The **"b"** values ranged of 5.6 ml to 29.8 ml from 24 at 120 h of fermentation in absence of yeast and 11.8 ml to 46.3 ml from 24 at 120 h of fermentation in presence of yeast. Likewise, the **"c"** values ranged from 3.0 to 3.5 ml/h in absence of yeast and 3.9 to 6.0 ml/h in presence of yeast. Until now, there is little information about the *in vitro* gas production of prickly pear fermented with *K. marxianus*. Nevertheless, the **"c"** values observed in prickly pear without the addition of yeast are similar to those reported by Cerrillo & Juarez (2004) in cladodes. Thus, the increase in the maximum of gas production **"b"** as well as rate of gas production **"c"** in presence of *K. marxianus* could possibly due to an increase in the available nutrient to ruminal microbiota especially nitrogen (Calabro et al., 2012). Likewise, in this study the decrease in NDF and ADF content with yeast addition suggest that *K marxianus* increases the availability of carbohydrate for rumen microorganisms (Arhab et al., 2009). When higher nitrogen content and is available in presence of sufficient carbohydrate, more amino acids are taken up into the bacterial cell, leading to greater microbial growth and consequently, increased fermentation activity with higher gas production.

Table 3. *Least squares means for In vitro gas production parameters of fermented prickly pear without and with Kluyveromyces marxianus.*

Fermentation time (h)	b (ml/g DM)		SEM	P<
	- K. Marxianus	+ K. Marxianus		
0	6.3	12.4	2.2	*
24	5.6	11.8	2.2	*
48	10.2	24.2	2.2	**
72	14.7	31.7	2.2	**
96	22.1	39.6	2.2	*
120	29.8	46.3	2.2	**
Fermentation time (h)	**c (ml/h)**		**SEM**	**P<**
	- K. Marxianus	**+ K. Marxianus**		
0	2.8	3.7	0.74	*
24	3.0	3.9	0.74	*
48	3.2	5.4	0.74	**
72	3.3	5.4	0.74	**
96	3.5	5.4	0.74	*
120	3.5	6.0	0.74	**

K. marxianus ('-' without; '+' with); (P<0.05); SEM = standard error of mean; * P<0.05; ** P<0.01.

The metabolizable energy (ME) and short chain fatty acid (SCFA) predicted from gas production of fermented prickly pear without and with *K. marxianus* are shown in Table 4. There were interactions yeast*fermentation times on ME and SCFA value (P<0.05). In this study, ME and SCFA predicted from gas production in prickly pear fermented in absence *K. marxianus* were lower as compared to prickly pear fermented in presence *K. marxianus* (P<0.05). This may due to a lower absolute gas production which is based mainly on carbohydrate fermentation (Sallam et al., 2007). In incubation of forages with rumen fluid *in vitro*, the carbohydrates are fermented mainly to short chain fatty acids as well as carbon dioxide and methane (Parnian et al., 2013). Therefore, in this study the increased of ME and SCFA could be attributed to decrease of fibre fraction in the prickly pear fermented with *K. marxianus*. The fibre forms a barrier for ruminal microorganisms that obstructs the optimal fermentation of the digestible organic matter of the substrates (Van Soest, 1994). In fact, there are a positive relationship between the digestible organic matter and production of SCFA and a negative relationship between OMD and ME and fibre fraction of the forages (Van Soest, 1994; Murillo et al., 2011). About 94% of the variation in the *in vitro* gas production is explained by SCFA produced, which mainly comes from carbohydrate fermentation (Njidda & Nasiru, 2010).

Table 4. *Least squares means for predicted metabolizable energy and short chain fatty acid of fermented prickly pear without and with K. marxianus from in vitro gas production.*

Fermentation time (h)	ME (Mcal/kg DM)		SEM	P<
	- K. Marxianus	+ K. Marxianus		
0	4.5	7.1	0.19	*
24	4.1	6.8	0.19	*
48	6.2	7.8	0.19	*
72	5.8	8.1	0.19	**
96	4.1	8.5	0.19	**
120	4.4	8.7	0.19	**
Fermentation time (h)	**SCFA (mmol/g DM)**		**SEM**	**P<**
	- K. Marxianus	**+ K. Marxianus**		
0	0.15	0.20	0.022	*
24	0.10	0.17	0.022	*
48	0.16	0.31	0.022	**
72	0.14	0.48	0.022	**
96	0.11	0.61	0.022	**
120	0.13	0.63	0.022	**

K. marxianus ('-' without; '+' with); (P<0.05); SEM = standard error of mean; * P<0.05; ** P<0.01.

Conclusion

This study confirmed that solid state fermentation with *K. marxianus* successfully increases the PC, *in vitro* gas production and energetic value (ME, SCFA) of prickly pear and decreases NDF content, which significantly improvement the nutritional quality of this forage. The fermented prickly pear obtained in this study represents a promising non-conventional alternative feed source for cattle. However, further studies are required to examine certain factors, such as use of some nutrients, which could improve *K. marxianus* performance during solid state fermentation. In addition, scale-up studies are required to develop a viable commercial process that could generate a low cost technology for using fermented prickly pear as high quality forage.

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