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Efficiency of Lignocellulolytic Extracts from Thermotolerant Strain *Fomes* sp. EUM1: Stability and Digestibility of Agricultural Wastes

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ABSTRACT

Production of lignocellulolytic enzymes by the thermotolerant *Fomes* sp. EUM1 was determined in solid cultures using corn stover (CS) as a sole substrate or supplemented with 20 % wheat bran (CS+WB). This supplementation increased (P< 0.05) enzymatic activity per gram of initial dry matter (gdm) for xylanases and cellulases: 160 IU g dm^{-1} and 37 IU g dm^{-1}, respectively; while laccases reached a similar yield (3.3 IU g dm^{-1}) for both cultures. Nevertheless, laccases showed different stability patterns at 39°C and pH 6: half-life time ($t_{½}$) was doubled in extracts from CS+WB (23.5 h); whereas $t_{½}$ for the other enzymes from both cultures showed no difference. Both extracts by *Fomes* sp. EUM1 and a commercial enzymatic product were used on forages: corn stover, (CS), sugarcane bagasse (SCB), and alfalfa hay (AH). The fractional rate of gas production (FR; ml g^{-1} h^{-1}) increased (P< 0.05) at 9 hours in CS compared to the sample without enzymes. The use of any enzymes favoured higher maximum gas volume (*Vm*; h^{-1}) on SCB. The *in vitro* digestibility (IVD) of CS after using the commercial product was 12% higher, while our extracts from CS and CS+WB showed 16 and 21% improvements (P< 0.05), respectively, suggesting a higher specificity of these enzymes produced on the same substrate (CS). In addition to the proven stability, the versatility of extracts from CS and CS+WB was confirmed by the increase in IVD values for SCB (up to 100%) in relation to the control without enzymes.

Keywords: Enzymatic extracts, Fungal thermotolerance, *in vitro* gas production, Solid state cultures.

INTRODUCTION

Several species of white-rot fungi have been studied in both basic and applied sciences. These Basidiomycetes can efficiently grow on lignocellulosic substrate due to their ability to produce hydrolytic and oxidative enzymes (Elisashvili *et al*., 2009; Da Silva *et al*., 2005). These fungi can improve the degradation of fibrous complexes, for instance, some strains of *Trametes versicolor, Bjerkandera adusta*, *Fomes fomentarius* and *Pleurotus ostreatus*, all of which alter cell wall components and increase the quality of lignocellulosic substrates as ruminant feeds (Graciano *et al*., 2009; Peláez *et al*., 2008); in the Middle East sheep is one of species that has relatively high biodiversity (Esmailizadeh *et al*., 2011).

In particular, there is still a limited description of simultaneous production of hydrolytic and oxidative enzymatic extracts

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by the genus of white-rot *Fomes*, even though the use of exogenous lignocellulosic enzymes represent a biological method which favours the use of forages in ruminant diets, the celluloytic activity of ruminal bacteria is complemented, thus improving fibre digestibility (Colombatto and Beauchemin, 2003). The thermotolerant strain *Fomes* sp. EUM1 basidiomycetes could be an advantage in SSF since this strain is able to grow at temperatures up to 40°C on corn stover-based media (Ordaz *et al*., 2011).

Enzyme production depends on several factors such as the microbial source, culture conditions, type of substrate, and physical properties of the growth material (Membrillo *et al*., 2008). Several studies have focused on the use of agro-industrial wastes as substrates for microbial growth for production of enzymes in solid cultures as a first step towards the use of additives in ruminant nutrition, and as a contribution towards solving environmental problems (Levin *et al*., 2008).

Furthermore, stability of enzymatic extracts is usually the limiting factor in technology transfer processes. Then, once competitive production levels are achieved, assessment of enzymatic stability under operational conditions is also required. For instance, in the pretreatment of forages, *in vitro* gas production is commonly compared among treatments after the use of different exogenous enzymes.

Sugarcane bagasse is the main lignocellulosic waste generated in countries like Mexico, with an annual production of 380 million tons (Sánchez, 2009). This sugar industry by-product represents a potential energy source in animal nutrition (Santiago-Hernandez *et al*., 2007). Similarly, annual production of corn stover is 191 million tons (Sánchez, 2009). In terms of nutritional features, these two agricultural wastes show a low digestibility due to the presence of high content of lignin (Graminha *et al*. 2008). This is a common feature found in agricultural waste products worldwide, including wheat, barley, sugar cane, corn, and rice straws; specifically, in Iran there are some important efforts to find sustainable uses for these byproducts (Najafi *et al.,* 2009). On the other hand, wheat bran is widely used as a substrate inducer in the production of enzymes by basidiomycetes (Papinutti and Lechner, 2008).

The aim of this research was to produce lignocellulolytic enzyme extracts by *Fomes* sp. EUM1 under solid state fermentation (SSF) using corn stover (CS) as a substrate either alone or supplemented with 20% wheat bran (CS+WB). The stability patterns from these extracts were determined and the *in vitro* ruminal fermentation profiles were then compared for these extracts on corn stover, alfalfa hay, and sugarcane bagasse, which are all used as substrates in ruminant nutrition.

MATERIALS AND METHODS

Propagation of *Fomes* sp. EUM1

The isolate *Fomes* sp. EUM1 belongs to the fungal collection of the Universidad Autónoma Metropolitana and was recently identified and characterized as a thermotolerant isolate (Ordaz *et al*., 2011). This fungus was routinely propagated in a medium containing malt extract (40 g l^{-1}), yeast extract (3 g l^{-1}) and bacteriological agar (15 g l^{-1}) on Petri dishes for 7 days at 35 °C. These cultures were used as inoculants for the solid cultures, as described below.

Conditions for Solid Culture and Enzyme Extraction

The production kinetics for the cellulases, xylanases, and laccases were obtained in solid state fermentation cultures (SSF) on corn stover (CS) and corn stover supplemented with wheat bran (80:20 p/p) (CS+WB). The initial humidity for the SSF was adjusted to 80% and cultures were kept for 8 days at 35°C. The corn stover was

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sieved (> 0.81 mm and < 4.06 mm, according to Membrillo *et al*. (2008). The cultures were grown in 250 ml Erlenmeyer flasks with 3 g of substrate previously sterilized at 121ºC for 40 minutes. Each flask was inoculated with a mycelium suspension obtained by mixing in a vortex 5 discs of mycelium-agar (6 mm Ø) from the Petri dishes described above in 5 ml of sterilized distilled water (Sainos *et al*., 2006).

Culture samples were analysed for 8 days, obtaining the enzymatic extracts from each flask, which were placed in an ice bath on top of a stirrer. Seventy millilitres of citrate buffer (50 mM, pH 6) and a magnetic stirrer were added to each flask and agitated for 30 minutes. Next, the contents of each flask were filtered through a Whatman® No. 1 paper and the filtered matter was centrifuged (4°C, 10,000 rpm, 15 minutes). The supernatant was considered as the enzymatic crude extract (ECE) and used for the enzymatic determinations. Each enzyme determination was performed in triplicate.

Determination of Enzymatic Activity

Xylanase and cellulase activities were determined using the method described by Miller (1959) based on the quantification of reducing sugars using the reagent 3,5-dinitrosalicylic acid (DNS). Birchwood xylan (Sigma®) (0.5 %) was used as a substrate for the xylanases, previously dissolved in sodium citrate buffer (50 mM, pH 5.3). The substrate for cellulase activity was carboxymethylcellulose (Sigma-Aldrich) (1%), dissolved in citrate buffer (50 mM, pH 5). The reactions were performed as described by Loera and Córdova (2003) using xylose and glucose in a standard curve for xylanases and cellulases, respectively. Absorbance was read using a spectrophotometer (DU649 Beckman®) at 640 nm. The enzyme activities were reported as international units (IU), where 1 IU was defined as the amount of enzymes releasing 1 µm of reducing sugars (xylose or glucose) per minute under the assay conditions.

Laccase activity was determined according to Bourbonnais *et al*., (1997) using ABTS (2,2-azinobis-3-ethylbenzthiazolinesulphonic acid) as a substrate (0.1 M), dissolved in citrate buffer (50 mM, pH 5), and the absorbance was read at 420 nm every 10 seconds for 1.5 minutes. Laccase activity was reported as international units (IU), where 1 IU was defined as the amount of enzyme producing 1 µm of oxidized ABTS per min under the assay conditions. All enzymatic activities are reported as international units per initial gram of dry matter (IU g dm^{-1}).

Enzymes Stability Kinetics

The stability tests were performed with the ECE obtained from 6-day-old cultures of *Fomes* sp. EUM1, from either corn stover (CS) or corn stover supplemented with wheat bran (CS+WB). The recovery of ECE was performed as described above. Then, the ECE was diluted (1:1) by the addition of a citrate buffer (25 mM, pH 6) and incubated at 39°C which are common working conditions for these enzymes (Ramírez *et al*., 2005). Samples for enzymatic activity were analyzed during the incubation period (0 to 24 hours), and residual activity data were adjusted to a first order decay model in order to determine the half-life time ($t_{½}$) of each enzyme.

Forages Treatments on Three Different Substrates

The effects of ECE and a commercial enzymatic product (Fibrozyme®) on the digestibility and ruminal fermentation of the alfalfa hay, corn stover, and sugarcane bagasse forages were evaluated by *in vitro* gas production (Menke and Steingass, 1988). The commercial enzymatic product was prepared by dissolving 3 g l^{-1} in a citrate buffer (50 mM, pH 6). The ECE were characterized in terms of the activities of cellulases, xylanases and lacasses and was

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standardized to the xylanolytic activity of the commercial enzymatic product (15.4 IU ml^{-1}). For the *in vitro* ruminal fermentation test, the forages were treated for 22 hours either with the commercial enzymatic extract (l ml g^{-1}) or our ECE, obtained from CS (7.4 ml g^{-1}) and form CS+WB cultures (5.3 ml g^{-1}), then the forages were dried at 50°C for 48 hours. The control samples (using distilled water without enzymes) were treated similarly.

In vitro gas Production and Digestibility of Dry Matter

Ruminal liquid (RL) was obtained from two rumen-cannulated sheep with an average weight of 53 kg±6.8. Extraction of the ruminal liquid was performed in the morning (7:00 am) before feeding (alfalfa hay). The RL was mixed and filtered through four layers of cloth and, then, under constant CO_2 bubbling, a reduced mineral substance (RMS) was added in a 1:9 (v:v) proportion at 39°C. The RMS solution was prepared in two steps: (1) Mineral solution I: 1 l of K_2HPO_4 at 0.45 g l^{-1}, (2) Mineral solution II: KH_2PO_4 at 0.45 g l^{-1}; $(NH_4)_2SO_4$ at 0.45 g l^{-1}; $NaCl_2$ at 0.9 g l^{-1}; $MgSO_4$ at 0.18 g l^{-1}, $CaCl_2$ at 0.075 g l^{-1}; and two drops of rezarsurine (1% p/v). These two mineral solutions were mixed (1:1, v/v). Finally, 50 ml of a reducing solution was added to one litre of this mixture. The last 50 ml of the reducing solution contained NaOH (0.08 g), NaS (0.5 g) and cysteine (0.5 g). This final mix was considered as the ruminal inoculate.

Later, 90 ml of the ruminal inoculate were added to the amber-coloured serological bottles (125 ml) containing 0.5 g of dry forage, under a steady stream of CO_2. The gas pressure produced by the substrate fermentation was recorded periodically (from 0 to 72 hours of incubation) using a manometer (0 to 1 kg cm^{-2}). The pressure from every one bottle was completely released after each reading. All samples were incubated in duplicate in three series. After 72 hours of incubation, the forage residue was filtered using a previously dried and weighed Whatman No. 40 paper, which was then dried until constant weight at 65°C for 48 hours, and the weight of the dry matter was registered in order to determine *in vitro* digestibility (IVD) of the forage dry matter (DM). The pressure readings (kg cm^{-2}) were converted to gas volumes (ml) by means of a regression equation (Y= X/0.0238, where *Y* and *X* represent gas volume and pressure, respectively). This relationship between pressure and gas volume was obtained by injecting a previously known volume of gas into a 100 ml serological bottle and recording the pressure generated by this volume.

Once the gas volume was determined at each sampling time, the fractional rate (FR) of gas production was calculated from the gas volume divided by the dry matter (DM) and the time of that particular sample. The accumulated gas volume (*Va*) was calculated as the sum of the gas volume for each interval. From the accumulated gas volume, the variables of maximum volume (*Vm*; ml g^{-1} DM), gas production rate (*S*; ml h^{-1}) and lag time (*L*; h) were calculated in the gas production kinetics according to the logistical model described by Schofield and Pell (1995) using the SAS statistical program.

$$Va = \frac{Vm}{(1+e^{2-4S(t-L)})} \qquad (1)$$

Where, *Va* = Accumulated gas volume (mL g^{-1} DM); *Vm*= Maximum gas volume (mL g^{-1} DM); *S*= Gas production rate (mL h^{-1}); *L*= Lag time phase (h), *t*= Time (h).

Statistical Analysis

The statistical analysis of each parameter (*Vm*, *S*, *L* and IVD) was performed using the PROC GLM and the SAS software (SAS, 1994). Statistical analysis was also completed for FR after 9 hours of fermentation. All analyzed data were obtained from three independent

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experimental units and statistical differences were stated at $P \leq 0.05$.

RESULTS AND DISCUSSION

Lignocellulolytic Enzyme Production Profiles

The xylanolytic activity in the enzymatic extracts of *Fomes* sp. EUM1 reached maximal production on day 5 with values of 78 IU g dm^{-1} and 160 IU g dm^{-1} for CS and CS+WB, respectively. Thus, supplementation with WB showed a 136% increase ($P< 0.05$) in production compared to CS (Figure 1). In both treatments, production decreased on day 7, which may be attributed to the possible production of proteases (Papinutti *et al*., 2008).

The xylanolytic activity (160 IU g dm^{-1}) obtained by *Fomes* sp. EUM1 in this study was similar to that reported previously by Márquez-Araque *et al*. (2007) in a solid state culture on sugarcane bagasse (147 IU g $dm^{-1)}$; however, this value was obtained after 14 days of culture, whereas in our research the production peak was reached after 5 days using corn stover, thus, the productivity (IU g dm^{-1} d^{-1}) was improved 3 times. Accordingly, cell wall of corn stover is a better substrate for this fungus. Corn stover and sugarcane bagasse are the main wastes generated in many regions worldwide. This fact highlights the potential production of lignocellulolytic enzymes from basidiomycetes (Rodrigues *et al*., 2008), which in turn can also diminish anti-nutritional factors in forages with high lignin contents (Llewellyn *et al*., 2010).

Maximum cellulase production in both treatments was also reached on day 5 (Figure 2): 33 IU g dm^{-1} for CS and 37 IU g dm^{-1} for CS+WB, with no significant differences between these cultures. In a recent work by Márquez-Araque *et al*. (2007) using sugarcane bagasse, the production of cellulases (8.51 IU g dm^{-1}) was lower than that found in our study (33 IU g dm^{-1}). These differences can be attributed to the type of substrate used, particle size, fibre structure, porous space, humidity of the culture, pH, and the stage of development of the culture (Membrillo *et al*., 2011).

The laccase activity produced on CS+WB was higher ($P< 0.05$) than on CS alone, even after day 5 and until day 7 (Figure 3), whereas the production peak of laccases from CS was delayed until day 6, although this activity remained at the same level until day 8. These findings are in agreement with Safari-Sinegani *et al*. (2006) and Elisashvili *et al*. (2009) since the type of substrate affects strongly the production of lignolytic enzymes. Therefore, more knowledge is needed on the effect of complex carbon sources during enzyme production, evaluating different raw materials as growth substrate under state solid fermentation conditions as well as culture temperature when using a thermotolerant fungal strain such as *Fomes* sp. EUM1 (Ordaz *et al*., 2011).

The addition of wheat bran (WB) to corn stover increased ($P< 0.05$) production of the three enzymes evaluated. Wheat bran is a widely-used substrate in solid state fermentation for the production of cellulolytic enzymes by *Trichoderma viridae* and

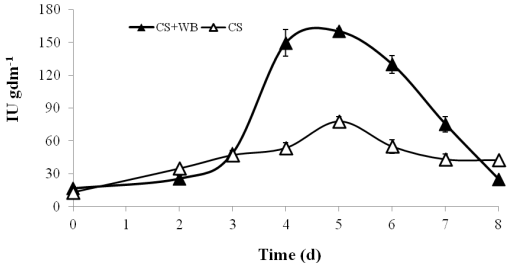

Figure 1. Production of xylanases on sole corn stover (CS) or supplemented with 20% wheat bran (CS+WB).

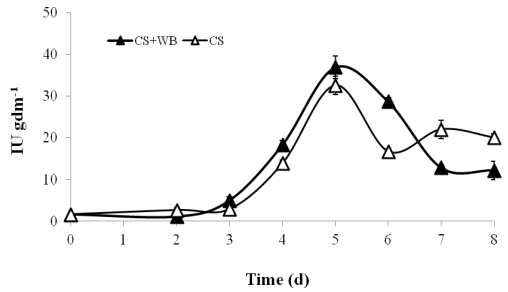

Figure 2. Production of cellulases on sole corn stover (CS) or supplemented with 20% wheat bran (CS+WB).

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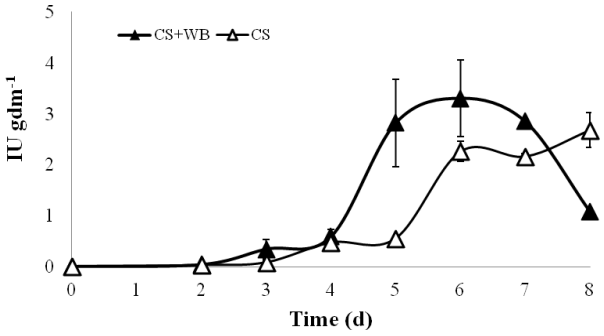

Figure 3. Production of laccases in both substrates on sole corn stover (CS) or supplemented with 20% wheat bran (CS+WB).

xylanolytic enzymes by *Aspergillus niger* (Gisell *et al*., 2008). Also, WB induced the production of xylanases and cellulases, even in the early stages of culture (72 hours). This effect of WB as a supplement was corroborated in the present study, since the production of xylanases doubled on day 5. While for laccases the major effect was in the increase of stability (Table 1). In a similar report, *Pleurotus ostreatus* cultivated in a mixture of sugarcane bagasse and wheat bran (5:1) reached a laccase production of 0.52 IU g^{-1} on day 9 of culture (Suguimoto *et al*., 2001), these values are lower than those obtained in our studies using CS+WB (80:20 p/p) as a substrate.

Enzymatic Stability

The average half-life time ($t_{½}$) for xylanases was 4.15 and 4.97 hours for extracts from CS and CS+WB, respectively (Table 1). Similarly, cellulase stability did not differ substantially: $t_{½}$= 4.39 h for CS+WB and $t_{½}$= 5.22 h for CS. On the other hand, the $t_{½}$ for laccases obtained from CS+WB was 23.5 h (P< 0.05), which was significantly superior to laccases from CS ($t_{½}$= 12.28 h). This result showed that addition of WB increased both laccase production and stability.

Production of stable enzymes is highly appreciated for practical applications in cases such as pre-treatment of forages, since the half-life time can be an indicator for recommending specific usages (Graminha *et al.,* 2008); in particular, here we tested these extracts at 39°C, which is a frequent air temperature in tropical regions. The recent description of *Fomes* sp. EUM1 as a thermotolerant strain (Ordaz *et al*., 2011) suggests that this fungus could produce stable fibrolytic enzymes. In fact, stability of the cellulases and xylanases found in our study are similar to those of the commercial extract Fibrozyme, with $t_{½}$ of 5.7 and 4.02 hours as reported by Pinos *et al*. (2001) and Ramírez *et al*. (2005), respectability.

On the other hand, laccases of *Fomes sclerodermeus,* cultivated on wheat bran, maintained 75% activity for 24 hours at pH 6 and 40°C (Rodrigues *et al*., 2008). These data are in agreement with our findings for laccases obtained with CS+WB ($t_{½}$= 23.5 h), corresponding to a 100% increase compared

Table 1. Stability, expressed as half-live time ($t_{½}$, h), of the enzymatic extracts produced by *Fomes* sp. EUM1.

Treatment	Celullases	Xylanases	Laccases
Corn Stover	5.22 ± 0.82[a]	4.15 ± 0.27[a]	12.28 ± 0.84[a]
Corn Stover+Wheat Bran	4.39 ± 0.49[a]	4.97 ± 0.7[a]	23.51 ± 1.08[b]

[a-b] Means in the same column with different superscripts differ (P< 0.05).

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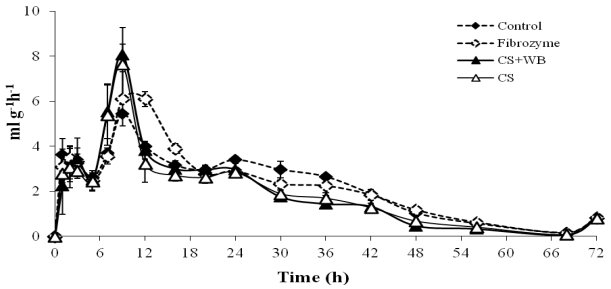

Figure 4. Fractional rate of gas production (*in vitro*) by the different enzymatic extracts on corn stover.

to the laccases obtained in CS; thus, WB induced the production of more resistant iso-enzymes.

The fact that *Fomes* sp. EUM1 extracts featured a comparable $t_{½}$ for xylanases and cellulases, with an improved $t_{½}$ for laccases from CS+WB, suggests that these extracts can be used in the pre-treatment of forages before animal feeding, since the fibrolytic enzymes have a major effect in the first 12 hours (Pinos *et al*., 2002), improving the bioavailability of nutrients and fibre digestibility (Graminha *et al*., 2008). The increased stability of laccases from CS+WB might be related to specific induction of isoenzymes as a result of components found in the wheat bran, since lignocellulolytic substrate composition determine the proportion of specific isoenzymes patterns in white rot fungi (Pappinutti and Lechner, 2008; Elisashvili *et al.,* 2008).

In vitro Gas Production

The fractional rate (FR) of gas production measured by the *in vitro* fermentation of corn stover at 9 hours (5.5 ml g^{-1} h^{-1}) without enzymes (control sample) was lower ($P< 0.05$) than that obtained for the corn stover incubated with extracts obtained from either CS (7.6 ml g^{-1} h^{-1}) or CS+WB (8 ml g^{-1} h^{-1}). However, these values were similar ($P> 0.05$) to those from the commercial extract (6.1 ml g^{-1} h^{-1}) (Figure 4). This suggested a greater specificity of the extracts when applied to the same substrate that the enzymes originated from, which in turn accelerated the generation of readily fermentable carbohydrate residues, oligosaccharides or soluble sugars. A positive effect on FR by enzymatic treatment was also observed in alfalfa hay and sugarcane bagasse, where commercial enzymatic product was superior in comparison to the extracts obtained by *Fomes* sp. EUM1, supporting the theory that the specificity of enzymatic extracts plays a major role in the treatment of substrates.

The pre-treatment of fodders with enzymatic extracts of *Fomes* sp. EUM1 may encourage its stability since contact between

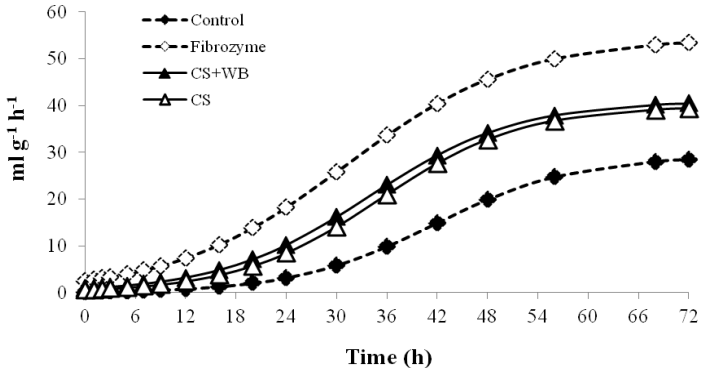

Figure 5. Gas production (*in vitro*) by the different enzymatic extracts on sugarcane bagasse.

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the enzymes and substrate protects the enzymes from proteolysis in the ruminal environment (Márquez-Araque *et al.,* 2010). Furthermore, the pre-treatment of plant fibres under aerobic conditions is necessary for the formation of radical hydroxyls, favouring hydrolysis of the plant walls (Yang *et al*., 2004). The effect of enzymatic extracts depends on the sources of the enzymes (microorganisms and culture conditions), since the inducement of different isoforms of the same enzymatic family, with different catalytic and stability properties, depends on these conditions.

Table 2 shows the accumulated gas production and the gas production kinetics (*Vm*, *S* and *L*) during *in vitro* ruminal forage fermentation. The *Vm* values for the control samples (without enzymes) showed the following ranking: alfalfa hay> corn stover> sugarcane bagasse. When alfalfa hay was treated with the commercial extract or extracts from *Fomes* sp. EUM1 (CS or CS+WB), fermentation of the substrate, measured as *Vm,* was reduced (P< 0.05), a result similar to that obtained for corn stover.

In contrast, the enzymatic extracts of *Fomes* sp. EUM1 and the commercial extract increased (P< 0.05) the fermentation of sugarcane bagasse compared to the control sample (Figure 5). In the case of alfalfa and corn stover, even when *Vm* did not increase with any of the enzymatic treatments, the lag time (L) was reduced with enzymatic treatment. Sugarcane bagasse without enzymes was the forage material that displayed the greatest lag time (24.9 hours), which was reduced (P< 0.05) by over 58% (10.5 hours) with the commercial extract; similarly, the *Fomes* sp. EUM1 extracts caused a significant decrease in *L*: 18 and 16 hours for CS and CS+WB extracts, respectively (Table 2).

Interestingly, the positive effect of wheat bran on the production of enzymes was also observed when applied to alfalfa hay, since only the extracts from CS+WB decreased (P< 0.05) lag time by 36%. In fact, the *L* values did not differ for the other enzymatic and control treatments. On the contrary, in corn stover, the enzymatic fermentation treatments did not show significant differences in *L* (P> 0.05) compared to the control sample (Table 2).

Similar studies have not found any effect with the use of enzymes on the *in vitro* gas production for alfalfa hay and corn silage fermentation; however, there was an effect when the substrate was more fibrous (oat straw), similar to corn stover. Our results agree with these reports, since the effect

Table 2. Gas production and *in vitro* digestibility of the forages with different enzymatic treatments.

Forage	Treatment	*Vm* (ml g^{-1} MS)	*L* (h)	*S* (ml h^{-1})	IVD %
Alfalfa hay	Control	179.4 ± 9.50^{a}	5.3 ± 0.10^{c}	0.030 ± 0.004^{a}	61.9 ± 1.87^{a}
	Fibrozyme	148.1 ± 11.53^{b}	5.0 ± 1.31^{c}	0.030 ± 0.001^{a}	63.24 ± 1.33^{a}
	CS	$135.8 \pm 3.70^{b,c}$	4.4 ± 1.24^{c}	0.029 ± 0.001^{a}	65.55 ± 1.83^{a}
	CS+WB	141.0 ± 9.84^{b}	3.2 ± 0.93^{c}	0.030 ± 0.001^{a}	61.84 ± 1.42^{a}
Corn stover	Control	140.4 ± 2.15^{b}	4.0 ± 0.89^{c}	0.030 ± 0.001^{a}	42.31 ± 1.15^{c}
	Fibrozyme	139.4 ± 2.95^{b}	3.6 ± 0.71^{c}	0.033 ± 0.003^{a}	$47.40 \pm 1.2^{b,c}$
	CS	117.0 ± 6.27^{c}	2.7 ± 0.72^{c}	0.035 ± 0.005^{a}	49.36 ± 1.98^{b}
	CS+WB	118.5 ± 5.91^{c}	2.9 ± 0.51^{c}	0.039 ± 0.003^{a}	51.15 ± 1.11^{b}
Sugarcane bagasse	Control	29.2 ± 2.68^{e}	24.9 ± 7.81^{a}	0.029 ± 086^{a}	15.24 ± 1.63^{e}
	Fibrozyme	54.4 ± 1.51^{d}	10.5 ± 4.01^{b}	0.024 ± 0.002^{a}	33.42 ± 6.10^{d}
	CS	$39.8 \pm 4.49^{d,e}$	18.2 ± 7.70^{b}	0.029 ± 0.008^{a}	30.96 ± 1.82^{d}
	CS+WB	$40.0 \pm 3.70^{d,e}$	16.1 ± 6.86^{b}	0.028 ± 0.009^{a}	28.22 ± 0.71^{d}

[a-e] Means in the same column with different superscripts differ (P< 0.05)

CS= Enzymes obtained with corn stover, CS+WB= Enzymes obtained with corn stover supplemented with wheat bran.

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found for the enzymatic extracts of *Fomes* sp. EUM1 and Fibrozyme® on *Vm* and IVD depended on the type of fodder and differed with the level of enzyme addition (Eun and Beauchemin, 2007; Jalilvand *et al*., 2008).

Apparent Digestibility of Dry Matter *in vitro*

Contrary to fermentation, measured as *Vm*, digestibility (IVD) improved after use of the enzymes, especially with the enzymes obtained by *Fomes* sp. EUM1 since IVD in corn stover increased from 42.3% up to 50%. Likewise, IVD in sugarcane bagasse improved from 15.2% up to 30%. All of these values were comparable to those obtained with the commercial extract (Table 2).

The addition of products containing polysaccharidases from *Trichoderma longibrachiatum* or *Penicillum funiculosum* increased the *in vitro* ruminal digestibility of alfalfa hay and corn silage fibre (Eun and Beauchemin, 2007). However, this effect was directly related to the dosage used, and this could be associated with the fact that no effect was found on the *Vm* in alfalfa hay and corn stover since the enzymes were used at a single dose (Figure 5). These same authors recommended the use of a combination of enzymatic extracts, an aspect that can be accomplished by using crude enzyme extracts of *Fomes* sp. EUM1, which is also able to produce enzyme complexes such as polysaccharidases and laccases. This is also supported by the fact that xylanases may be ineffective if they are not accompanied by other enzymes capable of cleaving the cross linkages in fibre (Grabber *et al*., 2002; Mondher and Narayan, 2006).

Similarly, there are works describing that the enzymatic extracts produced by *Trichoderma reesei* improved the *in vitro* digestibility of alfalfa hay. However, the effect of the enzymes depended on the substrate they were used on (Llewellyn *et al*., 2010). This was the case with the extracts produced by *Fomes* sp. EUM1, which improved both the fermentation (*Vm*) and digestibility (IVD) values for sugarcane bagasse. However, in the case of corn stover, only the IVD improved, whereas for alfalfa hay there were no effects on these variables.

The extracts of *Fomes* sp. EUM1 obtained from corn stover showed a greater specificity in the digestibility of dry matter than Fibrozyme®, when used on corn stover (Table 2). In future work, it would be useful to evaluate the effect of the crude enzymatic extracts of *Fomes* sp. EUM1, especially from CS+WB, during *in vivo* tests using sugarcane bagasse or corn stover even more concentrated as a protein source, in addition to an analysis of the extracts containing laccases on forages with different lignin contents.

In conclusion, the addition of wheat bran to corn stover substrate fostered xylanase production without affecting stability of these enzymes. Furthermore, the supplementation of wheat bran also doubled laccase stability. Compared to a commercial preparation, these extracts also showed a better specificity directly on corn stover (higher FR at 9 hours and higher IVD). The versatility of these extracts was proven on sugarcane bagasse (improvement in both *Vm* and IVD). Thus, the extracts of the thermotolerant fungus *Fomes* sp. EUM1 are a viable alternative for the treatment of lignocellulosic wastes. Future work could focus on biochemical analysis in order to probe whether new isoenzymes are expressed as a result of supplementation, in addition to *in vivo* evaluation of these extracts.

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کارآیی لیگنوسلولولیتیک عصاره گیری شده از ریسه ها ی مقاوم به گرما .*Fomes* sp EUM1 : پایداری و گوارش پذیری ضایعات کشاورزی

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چکیده

در این تحقیق، تولید آنزیم های لیگنو سلولولیتیک توسط ریسه های مقاوم به گرمای .*Fomes* sp EUM1در یک محیط کشت جامد و با استفاده از کلش ذرت (CS) به عنوان تنها بستره یا همراه با ۲۰٪ مکمل سبوس گندم (CS+WB) بررسی شد. افزودن این مکمل، فعالیت آنزیمی در هر گرم ماده خشک اولیه (gdm)را در مورد xylanases وسلولازها به ترتیب به ۱۶۰IU gdm^{-1} و 37 IU gdm^{-1}افزایش داد ($P < 0.05$) در حالی که برای لاکاز ها (laccases) مقدار آن در هر دو محیط کشت ، مشابه و برابر 3.3 IU gdm^{-1}بود. با این وجود، لاکازها از نظر پایداری تفاوتهایی را در شرایط pH 6و39 °C نشان دادند: نیمه عمر ($t_{½}$)آنها در عصاره گرفته شده از CS+WB در ۲۳.۵ ساعت دو برابر شد در حالیکه نیمه عمر برای آنزیم های دیگر از هردو محیط کشت هیچ تفاوتی نشان نداد.عصاره گرفته شده از*Fomes* sp. EUM1 و یک محصول آنزیمی تجارتی روی سه علوفه شامل کلش ذرت (CS)، باگاس نیشکر (SCB) و یونجه علوفه ای(AH) به کار رفت. نرخ جزیی تولید گاز(FR; ml g^{-1} h^{-1}) درCS بعد از ۹ ساعت بیشتر از نمونه بدون آنزیم بود($P < 0.05$) کار برد هر کدام از آنزیم ها منجر به افزایش مقدار گاز بیشینه (Vm; h^{-1})در SCB شد. بعد از کار برد آنزیم تجارتی ، گوارش پذیری درون شیشه ای CS و CS+WB ۱۲٪ بالا رفت در حالیکه که عصاره هایی را که ما از CSو CS+WB گرفته بودیم به ترتیب افزایش ۱۶٪ و ۲۱٪ را نشان داد ($P < 0.05$) که اشاره به اختصاصی بودن بیشتر این آنزیم های تولیدی روی بستره یکسان (CS) دارد. همچنین، نتایج به دست آمده علاوه بر اثبات پایداری آنزیم ها، تنوع عصاره های به دست آمده از CS CS+WB را هم به میانجی افزایش ارقام IVD(تا حد ۱۰۰٪) برای SCB در مقایسه با تیمار شاهد بدون آنزیم ، نشان داد.

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