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EFFECT OF FIBROLITIC ENZYMES ON RUMEN MICROBIAL DEGRADATION OF SUGARCANE FIBER

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ABSTRACT

Aiming to study the limiting factors for degradation of sugarcane fiber to be used as cattle feed, sugarcane fibrous components were fractioned in neutral- and acid-detergent fibers (respectively, NDF and ADF). Whole sugarcane and its fibers were incubated with rumen bacteria, in presence or absence of fibrolytic enzymes (Fibrozyme, Alltech Inc.). Microbial growth and total bacteria count were determined, as well as growth rate, generation rate, lag time, and degradability. Results were analyzed in randomized block design, with a 2x3 factorial arrangement, with two doses of enzymes, and three substrates, using four replications for incubation. The highest microbial growth was observed for whole sugarcane, and the lowest for its fibers. The in vitro degradability was 0.701, 0.392 and 0.191, respectively for whole sugarcane, NDF and ADF (P<0.01). ADF fraction with the addition of fibrolytic enzyme had its degradability increased from 0.387 to 0.425 (P<0.01). Microbial growth was limited in fibrous fractions, possibly due to lignification grade of cell walls. The highest in vitro degradability of fibrous fractions is related to the presence of soluble sugars. Addition of fibrolytic enzymes increased the maximum microbial growth and ADF degradability, indicating that it could be a potential addictive to enhance diets containing sugarcane.

KEYWORDS: Cell wall, in vitro degradability, microbial growth.

ABSTRACT

EFEITO DE ENZIMAS FIBROLÍTICAS SOBRE A DEGRADAÇÃO MICROBIANA RUMINAL DA FIBRA DE CANA-DE-AÇÚCAR

Com o objetivo de estudar os limitantes da degradação da fibra da cana-de-açúcar para utilizá-la como alimento para bovinos, fracionaram-se os componentes fibrosos da cana-de-açúcar (variedade Mex69-290) em fibra em detergente neutro (FDN) e em fibra em detergente ácido (FDA). A cana integral e as fibras foram incubadas com bactérias ruminais, na presença ou ausência de enzimas fibrolíticas (Fibrozyme, Alltech Inc.). Determinaram-se o crescimento microbiano e as bactérias totais, assim como a taxa de crescimento, a taxa de geração, o tempo de colonização e a degradabilidade. Os resultados foram analisados em um delinea¬mento de blocos ao acaso, com arranjo fatorial 2 x 3, com duas doses de enzima e três substratos, utilizando quatro repetições de incubação. Observou-se o maior crescimento microbiano para cana integral e os menores para suas fibras. A degradabilidade in vitro foi de 0,701, 0,392 e 0,191, respectivamente para cana integral, FDN e FDA (P<0,01). A fração FDA com a adição das enzimas fibrolíticas teve sua degradabilidade aumentada de 0,387 para 0,425 (P<0,01). O crescimento microbiano foi limitado nas frações fibrosas, possivelmente pelo grau de lignificação das paredes celulares. A maior degradabilidade in vitro das frações fibrosas está associada à presença de açúcares solúveis. A adição de enzimas fibrolíticas aumentou o crescimento microbiano máximo e a digestibilidade da FDA, indicando que estas podem ser um potencial aditivo para melhorar o aproveitamento de dietas com cana-de-açúcar.

PALAVRAS-CHAVES: Crescimento microbiano, degradabilidade in vitro, parede celular.

INTRODUCTION

The main limitations of using sugarcane (Saccharum officinarum L.) in ruminants feeding are the low digestibility of its cell wall, the high index of sugars and the molecular structure of cell walls (LOPEZ et al., 2003; ARANDA et al., 2004). GÓMEZ-VAZQUEZ et al. (2003) noticed a linear response in the digestibility of the neutral detergent fiber (NDF) and in the weight gain of steers fed stargrass pasture and sugarcane, by receiving increasing doses of an exogenous fibrolytic enzyme. The objective of this research was to study the effect of the addition of exogenous fibrolytic enzymes on the microbian growth and ruminal fermentation of the sugarcane, under the hypoyhesis that fibrolytic enzymes may increment the digestibility of sugarcane cell walls, as well as improve the ruminal fermentation efficiency and stimulate microbian growth.

MATERIAL AND METHODS

In vitro incubations of whole sugarcane, variety Mex69-290 (Chart 1), were carried out, as well as the incubation of its fibrous fractions with neutral and acid detergent fiber (respectively, NDF and ADF) (VAN SOEST et al., 1991), with or without the addition of the fibrolytic enzyme Fibrozyme© (Alltech Inc.), at the dose of 100 mg/g of incubated substrate (PINOS, 1999).

CHART 1. Sugarcane composition, variety Mex69-290, with twelve months of vegetation

Composition	Rate (%)
Dry Matter (DM)	31,90
Crude Protein (CP)	2,53
Cellular content	39,50
Neutral detergent fiber (NDF)	60,50
Hemicellulose	15,64
Acid detergent fiber (ADF)	44,86
Acid detergent lignin (ADL)	6,72
°Brix	16,40

Growth rate was calculated based on the natural logarithm regretion of the concentration of bacteria in function of time and generation time by means of the relation 0.693/k, being k the specific growth rate of the maintenance coefficient of the inverse relation of bacteria concentration, on time zero (PIRT, 1982). Colonization time (lag phase) was estimated as the inverse of the growth values by extrapolation of time zero (ZWIETERING et al., 1991). Parameters were estimated by regression (DRAPPER & SMITH, 1981).

A total of 150 mL of anaerobic medium (Chart 2), 1 mL of ruminal liquid, and 100 mg of substrate incubated in nylon bags (4 x 5 cm, for 24 hours, at constant temperature of 39 °C) were put in a 250-mL-capacity Erlenmeyer flasks. For that, ten replications for sample were used, with and without the addition of librolytic enzyme. At the end of the incubation, the bags dried at 65 °C, until they reached constant weight. The difference between the initial and final weight of the samples was considered degraded material and the degradability was calculated as the ratio between the degraded material and the initial weight.

According to the methodology described, the samples were incubated with and without the fibrolytic enzyme, with three replicates, repeated in five essays. Microbial growth was determined in 30-minute intervals during the first ten hours, with a final reading at the 25th hour, by the use of a spectrophotometer (Spectronic 20, Bausch and Lomb), adjusted to 600 nm, according to RUSSEL & DOMBROWSKI (1980). Total bacteria count was carried out, to transform the values of optical density into bacteria concentration, aiming at characterizing the growth curves (MIRANDA, 1998).

The results were analyzed according to a randomized block design (STEEL & TORRIE, 1980), with a 2 x 3 factorial arrangement, with two enzyme contents and three substrates (whole sugarcane and its fractions – NDF and ADF). Four incubation repetitions were performed (CLARY et al., 1988). The following statistical model was used: $Y_{ijk} = \mu + B_j + S_i + C_k + S_i*C_k + B_j*S_i*C_k + \varepsilon_{ijk}$, being Y_{ijk} the variable response; μ, the general average; B_j, block effect; S_i, substrate effect; C_k, effect of enzyme addition; S_i*C_k and $B_j*S_i*C_k$, interactions; and ε_{ijk}, residual error of the model. The procedure GLM of SAS (1985) was utilized, considering block interations x treatments as

the experimental error. The averages were compared by Tukey test (STEEL & TORRIE, 1980).

CHART 2. Composition of the anaerobic medium used to estimate total and cellulolytic bacteria.

Composition	Medium GCA-FR	Medium PW-FR
	Quantity (/100 mL)	
Yeast extract	0,1	0,1
Trypticase-peptone	0,2	0,2
Glucose	60	--
Cellubiose	60	--
Starch	60	--
Mineral solution I[a]	5,0	5,0
Mineral solution II[b]	5,0	5,0
Clarified ruminal fluid	30,0	30,0
Cysteine-sulfide solution[c]	2,0	2,0
Sodium carbonate solution 8%	5,0	5,0
Resazurin 0,1%	0,1	0,1
Distilled water	52,6	52,6
Whatman Paper nº 1	---	One layer

[a] 6 g K_2HPO_4/1000 mL,.
[b] 6.0 g KH_2PO_4,/1000 mL; 6.0 g $(NH_4)_2$ SO_4,/1000 mL; 12 g NaCl,/1000 mL; 2.45 g $MgSO_4$,/1000 mL and 1.6 g $CaCl_2H_2O$/1000 mL.
[c] 2.5 g L-cisteína (15 mL de NaOH 2N; 2.5 g $Na_2S\cdot 9H_2O$,)
GCA-FR = glucose, cellubiose, starch and ruminal fluid
WP-RF = Whatman paper and ruminal fluid.

RESULTS AND DISCUSSION

Ruminal bacteria growth according to the studied substrate is presented in Table 1 and Figure 1. Although there is a tendency for a greater microbian growth in whole sugarcane, the statistical differences regarding the substrate were observed from 3.5 hours. At the first hours of incubation, there was no significant difference, possibly due to the great absorbance variability, coinciding with the observations by MERTENS (1993), who pointed out that in vitro researches present high variability at the first stage. The higher microbian growth of the whole sugarcane is due to the presence of soluble sugars of easy degradation (BANDA & VALDEZ, 1976), and the lower growth in NDF is associated to the high livel of lignin (AMJED et al., 1992).

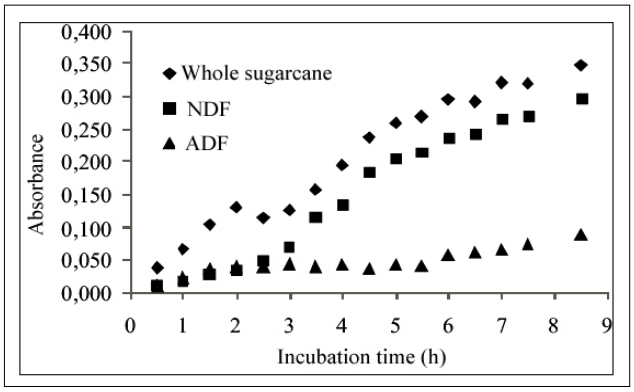

FIGURE 1. Ruminal bacteria growth incubated with sugarcane and its fibrous fractions.

The response of the microbian growth to the addition of enzymes is presented in Figures 2, 3 and 4, being one figure for each substrate. In Table 2 the main effects are presented, and a higher microbian growth may be observed as a response to the enzyme addition. There is a positive response to the addition of fibrolytic enzymes, indicating an increase of availability of metabolites for microbian growth. The biggest difference was observed in NDF fraction (Figure 4), which could be associated to the greater degradability due to the enzymatic activity on lignocellulosic connections and possibly to other indirect effects. It is possible that the addition of exogenous enzymes might increment the potentially degradable fraction of the cellulose contained in the ADF (AKIN, 1986).

In Table 3 the parameters for microbian growth in sugarcane are presented. Despite the differences presented in Table 2, statistical differences were not detected for the parameters of microbian growth in sugarcane and its fibrous fractions. This problem was detected in first-order kinetic analysis. MENDOZA et al. (1995) suggest that the data should be analyzed per incubation time, considering that differences in some incubation times may exist, and the linearization with the natural logarithm alters the residual error of the model, which does not allow the statistical detection of some biologically important differences.

TABLE 1. Microbian growth (optical density) of ruminal microorganisms incubated in sugarcane and its fibrous fractions (NDF and ADF).

Time (h)	Substrate			A.S.E.
	Sugarcane	NDF	ADF	
0,5	0,060	0,037	0,040	0,034
1,0	0,087	0,052	0,054	0,040
1,5	-0,129	0,068	0,074	0,058
2,0	0,156	0,094	0,097	0,070
2,5	0,148	0,099	0,088	0,059
3,0	0,159	0,118	0,095	0,066
3,5	0,200[a]	0,155[ab]	0,111[b]	0,063
4,0	0,240[a]	0,169[a]	0,121[b]	0,057
4,5	0,265[a]	0,221[a]	0,146[b]	0,048
5,0	0,293[a]	0,245[a]	0,159[b]	0,055
5,5	0,307[a]	0,252[a]	0,162[b]	0,046
6,0	0,333[a]	0,271[b]	0,182	0,048
6,5	0,342[a]	0,294[a]	0,203	0,055
7,0	0,361[a]	0,301[a]	0,212	0,050
7,5	0,367[a]	0,308[a]	0,216	0,051
8,5	0,391[a]	0,321[a]	0,242	0,059

A.S.E. = average standard error
[ab] Averages with distinct supersciptions, in the lines, differ among each other (Tukey test; P<0.05).

TABLE 2. Effect of the addition of fibrolytic enzymes on the microbian growth (optical density) by incubating ruminal microorganisms with sugarcane.

Time (h)	Treatments		A.S.E.
	Without enzymes	With enzymes	
0,5	0,026[b]	0,070[a]	0,034
1,0	0,042[b]	0,093[a]	0,040
1,5	0,066[b]	0,124[a]	0,058
2,0	0,081[b]	0,162[a]	0,070
2,5	0,077[b]	0,158[a]	0,059
3,0	0,091[b]	0,170[a]	0,066
3,5	0,118[b]	0,206[a]	0,063
4,0	0,142[b]	0,229[a]	0,057
4,5	0,176[b]	0,266[a]	0,048
5,0	0,194[b]	0,290[a]	0,055
5,5	0,200[b]	0,301[a]	0,046
6,0	0,223[b]	0,323[a]	0,048
6,5	0,232[b]	0,348[a]	0,055
7,0	0,246[b]	0,359[a]	0,050
7,5	0,249[b]	0,368[a]	0,051
8,5	0,273[b]	0,385[a]	0,059

A.S.E. = average standard error
[ab] Averages with distinct supersciptions, in the lines, differ among each other (Tukey test; P<0.05).

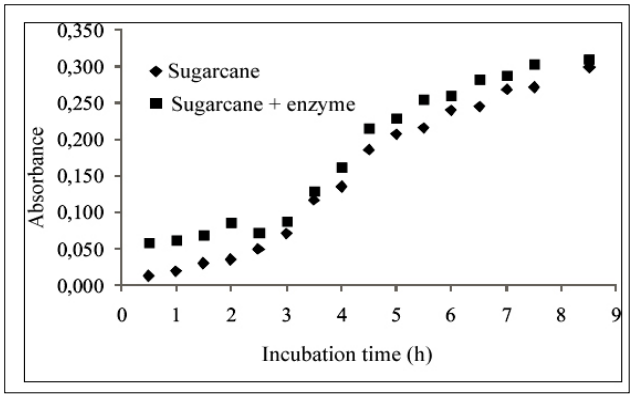

FIGURE 2. Ruminal bacteria growth incubated with sugarcane with or without exogenous fibrolytic enzymes.

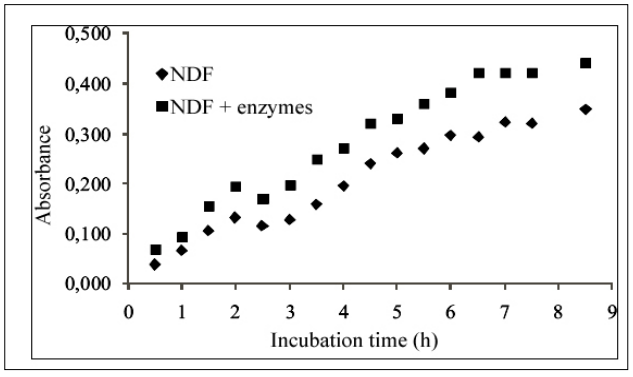

FIGURE 3. Ruminal bacteria growth incubated with sugarcane NDF with or without exogenous fibrolytic enzymes.

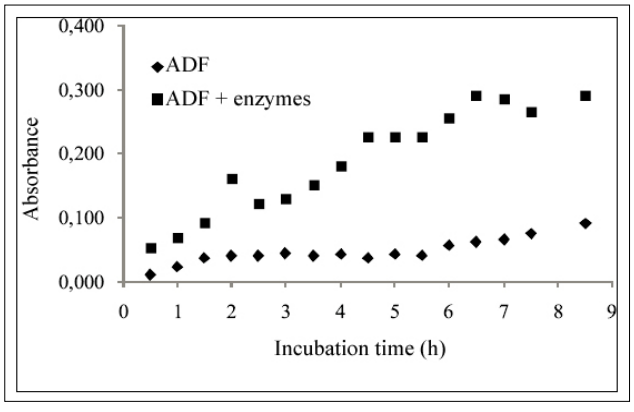

FIGURE 4. Ruminal bacteria growth incubated with sugarcane ADF with or without exogenous fibrolytic enzymes.

Growth and generation rates were similar for whole sugarcane and NDF and ADF fractions (Table 3). Growth rates observed in this experiment are smaller than the ones reported for mix cultures in lignocellulosic substrates (1.13/h) as the rests of the corn culture (MIRANDA et al., 1999). Cellulolytic microorganisms, F. succinogenes, R. flavefaciens and R. albus, have the ability to degrade cellulose with the rate between 0.05 and 0.10/h (WEIMER, 1996). It is possible to infer that the growth rate is influenced by fiber lignification (Chart 1), and it might be the main factor for the use of sugarcane, making necessary to search alternatives to increase the digestibility of these fractions with physical, chemical, enzymatic and biological treatments. Colonization time tended to be higher in whole sugarcane (Table 3). Values of 2.5 to 3.0 h of colonization time in incubations with rests of corn culture with 70% of NDF were reported (MIRANDA et al., 1999), which are smaller than the ones observed in this sutdy. Lag phase in sugarcane ADF confirms the importance of lignin in the food digestion.

TABELA 3. Characteristics of the microbian growth and in vitro degradability of sugarcane and its fibrous fractions.

Variables*	Substrate			A.S.E.
	Sugarcane	NDF	ADF	
K	0,214	0,330	0,267	0,11
M	0,860	3,680	0,581	5,62
Lag	0,46	3,18	5,06	5,73
Ymax	3,11	1,68	2,17	1,39
$k_{0.5}$	3,53	2,50	2,98	1,14
IVDDM	0,701[a]	0,302[b]	0,191[c]	0,038

* k: growth specific rate (/h); m: maintenance coefficient; Ymax: maximum growth; Lag: colonization time (h); k0.5: generation time (/h); IVDDM: in vitro degradability of DM.
[abc] Averages with distinct supersciptions, in the lines, differ among each other (Tukey test; P<0.05).

The in vitro degradability of sugarcane and its fibrous fractions reflects the microbian activity and points out to limitations inherent of the cell wall for animal digestion. The greater degradability of sugarcane is due to the concentration of soluble sugars in the cellular content (BANDA & VALDEZ, 1976; AROEIRA et al., 1993a; AROEIRA et al., 1993b).

Statistical differences were observed only in the

degradability. Sugarcane showed the highest one and was followed by NDF and ADF. The in vitro degradability of ADF observed in this study is within the interval of values reported (PATE, 1977) and it coincides with the potentially degradable NDF reported for sugarcane byproducts (AMJED et al., 1992) associated to the negative linear relation between in vitro degradability of NDF and the NDF:lignin proportion (PATE, 1977). The smaller degradability of ADF confirms the importance of lignin as a limiting factor in the use of sugarcane by ruminal microorganisms.

In Table 4, the main effects of fibrolytic enzymes on the parameters of microbian growth and in vitro degradability are presented. The enzyme addition increased the degradability of the substrates in 3.81 percentual units, what may be explained by the highest maximum microbian growth, without affecting other growth paramenters. Increase in the in vitro degradability of DM, NDF and ADF of grasses and legumes with the addition of the enzyme Fibrozyme© has been reported (FENG et al., 1996; TRICARICO et al., 1998; PINOS et al., 2001; PINOS et al., 2002a; PINOS et al., 2002b) as well as in vivo digestibility (KRAUSE et al., 1988; BEAUCHEMIN et al., 1998). Nevertheless, results are very variable, possibly due to the enzyme:substrate ratio and enzyme degradation by ruminal proteases (HARRIS, 1998).

The enzymes used are a combination of cellulases and hemicellulases produced by fungi, protected by glucosilation, and the posibility to remain reactive for around 12 hours in the rumen is estimated (HARRIS, 1998; LYONS, 1998). PINOS (1999) studied the ruminal in vitro composition and degradation of the enzyme Fibrozyme and reported that the complex has 93.6% of DM, 45.8% of NDF, 32.5% of ADF and 9.5% of ashes, complex average degradation time of 57 hours, with higher ammonia nitrogen liberation after 24 hours.

The use of exogenous cellulolytic enzymes to increment the digestibility of the sugarcane fiber is a viable alternative, as the results by GÓMEZ-VÁSQUEZ et al. (2003) show. Besides, some studies with temperate climate grasses and legumes show that the application of fibrolytic enzymes may improve the digestibility and growth of steers in 30% (BEAUCHEMIN et al., 1996) and milk production in 10% BEAUCHEMIN et al., 1998; KUNG et al., 1998; YANG et al., 1998). The knowledge of the structure of the cell wall of sugarcane and industrial enzymes make possible to develop new alternative treatments to improve the use of cellulose and hemicellulose by ruminants.

TABLE 4. Effect of the addition of fibrolytic enzymes on the microbian growth and on sugarcane in vitro degradability.

Variables	Treatment		A.S.E.
	Without enzymes	With enzymes	
K	0,317	0,228	0,11
M	2,97	0,647	5,62
Lag	3,340	8,314	5,73
Ymáx	1,549[b]	3,038[a]	1,39
$k_{0.5}$	2,736	3,253	1,13
IVDDM	0,387[a]	0,425[b]	0,021

* k: growth specific rate (/h); m: maintenance coefficient; Ymax: maximum growth; Lag: colonization time (h); k0.5: generation time (/h); IVDDM: in vitro degradability of DM.
[ab] Averages with distinct supersciptions, in the lines, differ among each other (Tukey test; P<0.05).

CONCLUSIONS

Microbian growth from sugarcane degradation is limited by fibrous fractions, possibly due to the level of lignification of cell walls. Higher sugarcane in vitro degradability in relation to its fibrous fractions is associated with the presence of soluble sugars.

The addition of fibrolytic enzymes increased maximum bacterial growth and ADF degradability. Thus, it is possible to consider the use of fibrolytic enzymes as a potential additive to improve the use of diets with sugarcane.

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