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PRODUCTION, CHARACTERIZATION AND EVALUATION OF FIBROLYTIC ENZYMES ON DIGESTIBILITY OF FORAGE MAIZE

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ABSTRACT

The objectives of this research were to produce and characterize an enzyme complex (EC), using the fungus *Humicola grisea*, and evaluate its effect on true digestibility of forage maize dry matter. We observed that the fungus produced cellulase, β-glucosidase and xylanase enzymes. The cellulase and xylanase activities were high at the temperature of 50° C. The optimum temperature of β-glucosidase was between 50 and 60° C. The optimum pH of cellulase and xylanase enzyme was 6.0. As for β-glucosidase, the enzyme showed higher activity at pH 6.5. Cellulase remained stable for 60 minutes at 39° C. Xylanase and β-glucosidase maintained 99.2 and 88.2% of their activity at 50° C for 240 minutes, respectively. The treatments were as follows: control (10 mL of sterile water), level 1 (2.5 mL EC), level 2 (5.0 mL EC) and level 3 (10 mL EC). In the digestibility experiment, there was interaction between enzyme levels and time of incubation in the rumen. The addition of 10 mL of the fibrolytic enzymes improved the digestibility at 10.58; 12.52; 9.05 and 6.81% compared to control for 12; 24; 48 and 96 hours of incubation, respectively. The fungus *Humicola grisea* is an enzyme producer that is important in ruminant feed.

KEYWORDS: Ankom; bovine; cellulose; fungi; xylanase.

PRODUÇÃO, CARACTERIZAÇÃO E AVALIAÇÃO DE ENZIMAS FIBROLÍTICAS NA DIGESTIBILIDADE DA FORRAGEM DE MILHO

RESUMO

Os objetivos deste trabalho foram os de produzir e caracterizar um complexo enzimático (CE) de *Humicola grisea* e avaliar seu efeito na digestibilidade verdadeira da matéria seca de forragem de milho. Observou-se que o fungo produziu as enzimas celulases, xilanase e β-glicosidase. A caracterização bioquímica mostrou que a celulase e xilanase produzidas apresentaram maior atividade a 50°C. A temperatura ótima de β-glicosidase ficou entre 50 e 60°C. O pH ótimo de celulase e xilanase foi 6,0. Quanto à β-glicosidase, a enzima revelou maior atividade em pH 6,5. A celulase permaneceu estável após incubação por 60 minutos, a 39°C. As xilanase e β-glicosidase produzidas mantiveram 99,2 e 88,2 % de sua atividade, a 50°C, durante 240 minutos, respectivamente. Os tratamentos foram: controle (10 mL de água esterilizada); nível 1 (2,5 mL do CE); nível 2 (5,0 mL do CE) e nível 3 (10 mL do CE). No ensaio de digestibilidade, verificou-se interação entre níveis enzimáticos e períodos de incubação no rúmen. Para 12; 24; 48 e 96 horas de incubação, 10 mL do CE aumentou a digestibilidade em 10,58; 12,52; 9,05 e 6,81%, em relação ao controle. O fungo *Humicolagrisea* é produtor de enzimas de interesse na alimentação de ruminantes.

PALAVRAS-CHAVE: Ankom; bovino; celulase; fungo; xilanase.

INTRODUCTION

Ruminants present a diverse and sophisticated ecosystem to use fibrous carbohydrates of vegetable cellular wall, due to the symbiotic relation with the varied microbial population in the rumen.

Although rumen microorganisms can digest cellulose and other fibrous carbohydrates, factors related to the plant structure and composition, such as the physical-chemical interactions between the hemicellulose matrix and lignin, as well as aspects related to the animal, such as chewing, salivation and ruminal pH, may limit digestion of the food in the rumen (MARTINS et al., 2006), which leads to the necessity of developing new biotechnological animal feeding programs to maximize nutrient use.

The use of fibrolytic enzymes, produced by filamentous fungus culture, in ruminant's feeding has shown satisfying results, with an increase in dry matter and neutral detergent fiber digestibility, in milk production and milk fat content (SCHINGOETHE et al., 1999), as well as in weight gain in bovines (BEAUCHEMIN et al., 1995).

Although enzymes are efficient catalysts in biological systems, their activity is affected by factors such as pre-treatment of the food, pH and length of the gastrointestinal tract, hydration level and temperature of the animal body, susceptibility of exogenous enzymes to the attack of endogenous enzymes, product concentration due to enzymatic hydrolysis, endogenous enzyme concentration and type of ingredient used in the diet (ACAMOVIC & MCCLEARLY, 1996).

Research has shown that, based only on biochemical characterization, the estimation of exogenous enzymes potential to improve the use of the diet by the animal is not possible. Therefore, in vitro essays are used to evaluate the efficiency of different groups of enzymes on the digestibility of diets offered to ruminants (COLOMBATTO et al., 2003).

This study was carried out with the purpose of producing and characterizing a fibrolytic enzymes complex, using the fungi *Humicola grisea*, and evaluating its effect on dry matter digestibility of corn forage.

MATERIAL AND METHODS

The experiments were carried out at the Enzymology and Digestive Physiology Laboratories of the Biological Sciences Institute (ICB II) of the Universidade Federal de Goiás (UFG), in Goiânia – GO, from March 2006 to October 2007.

For the production of the enzymatic complex (EC), we used the fungi *Humicola grisea* var. *thermoidea*, isolated from composting at Universidade Federal de Viçosa (MG). This microorganism has the capacity of producing thermostable enzymes, such as cellulase and xylanase.

The fungi *H. grisea* was cultivated in oatmeal agar medium (4.0% of Quaker oatmeal for children, 1.5% of agar and distilled water) at 42 ºC for four days, and then kept at room temperature for another three days. The Petri dishes with the fungus were stored at 4 ºC.

The production of hydrolytic enzymes was carried out in liquid medium, by inoculating ten culture dishes (5 mm), containing fungus spores, by 1 L Erlenmeyer flask with 250 mL of induction medium (corn forage: 5 g/L; yeast extract 3 g/L; ammonium sulphate: 1.4 g/L; $CaCl_2.6H_2O$: 0.3 g/L; magnesium sulphate: 0.3 g/L; trace element of $CuSO_4$ and $FeSO_4$). The flasks were incubated in a rotary shaker (Controlled Environment Incubator Shaker, Brunswick Scientific Co. Inc., U.S.A) at 42 ºC and speed of 120 rpm. After 96 hours of cultivation, the enzymatic complex was filtered and aliquots were collected and centrifuged at 4000 rpm for 10 minutes. The supernatant was kept at 4 ºC for further enzymatic trials.

Cellulase activity in filter paper (FPase) was determined by a 1.0 x 6.0 cm strip of Whatman filter paper No. 1 (50 mg) as substrate. In the test tube containing the paper strap, 150 µL of the enzymatic extract and 350 µL of the citrate-phosphate buffer 50 $mmol.L^{-1}$ were added. The reaction mixture was incubated in double boiler at 40 °C for 60 minutes. The reaction was interrupted by the addition of 1 mL of 3.5-dinitrosalicylic acid (DNS). Subsequently, the samples were boiled for 10 minutes and cooled in ice bath.

The concentration of reducing sugar released was determined by spectrophotometry at 550 nm by DNS method, using glucose as pattern (MILLER, 1959). One unity of enzymatic activity (U) was defined as the necessary amount of enzyme to make one reducing sugar µmol per minute of reaction.

The xylanolytic activity was determined by the reducing sugar methodology (DNS – MILLER,

1959).

For the enzymatic essay we used 50 µL of the enzymatic extract, 100 µL of xylan (oat spelts - sigma) and 350 µL of citrate-phosphate buffer 50 mmol.L^{-1}. The reaction mixture was incubated at 50 °C for 30 minutes. After this period, we added 500 µL of DNS to the mixture, which was incubated for 10 minutes in boiling water, with further addition of 1000 µL of distilled water.

The concentration of reducing sugar released was determined by spectrophotometry at 540 nm, using xylose as pattern. The unity of enzymatic activity (U) was defined the necessary amount of enzyme to make one reducing sugar µmol per minute of reaction.

The β-glucosidase activity was determined using ρ-nitrophenyl-β-glycopyranoside (ρ-NPG) as substratum. The reaction mixture consisted of 50 µL of enzymatic extract, 350 µL of citrate-phosphate buffer 50 mmol.L^{-1} and 100 µL of substratum. The enzymatic essay was carried out within 10 minutes of incubation at 40 ºC, and the reaction was interrupted with the addition of 1 mL of sodium carbonate (0.5 M). The amount of ρ-nitrophenyl released was determined by spectrophotometry at 405 nm. The unity of enzymatic activity (U) was defined as the necessary amount of enzyme to make one ρ-nitrophenyl µmol per minute of reaction.

In this experiment, optimum pH trials were carried out to verify if the enzymes produced present activity within a range compatible with rumen conditions (5.0-7.0).

The enzyme activities of the celullolytic complex were evaluated in citrate-phosphate buffer 50 mmol.L^{-1} in the pH values of 5.0; 5.5; 6.0; 6.5 and 7.0. The tests of the enzymes total cellulose, β-glycosidase and xylanase, in different pHs, were carried out according to the enzymatic essay previously described.

The optimum temperature essays were also carried out to evaluate if the enzymes produced maintain activity within rumen temperature range (39-42º C). Total cellulose, β-glycosidase and xylanase were determined according to method described at the enzymatic essay item, by incubating the samples at temperatures of 30, 40, 50 and 60 ºC. The tests were carried out with citrate-phosphate buffer 50 mmol L^{-1}, pH 6.5.

At the thermostability trial, the enzymes total cellulase, xylanase and β-glycosidase were evaluated at the times 60, 120, 180 and 240 minutes, at 40 °C and pH 6.5. The tests were carried out with citrate-phosphate buffer 50 mmol L^{-1}.

In the in vitro true digestibility trial, we used the methodology described by TILLEY & TERRY (1963), modified for the rumen fermenter DAISY II, following the methodology presented at the equipment instructions (ANKOM® Technology) supplied by the manufacturer. In this trial, we evaluated in vitro true digestibility of dry matter (IVDDM) of corn forage, treated with different doses of the enzymatic complex. Corn forage used in this experiment was composed of 34.6; 7.9 and 49.1% dry matter, crude protein and neutral detergent fiber, respectively.

The following treatments were performed: Control (10 mL sterile water); level 1 (2.5 mL of EC); level 2 (5.0 mL EC) and level 3 (10 mL of EC). The levels 2.5 and 5.0 were completed to 10 mL with sterile water. The enzymatic doses were applied by sprinkle on 17 g of forage, in a uniform way.

The experiment was performed in random blocks, with four replications, in a 4x4 subdivided plot arrangement. The blocks (replications) were constituted of four rumens. The plots were constituted of corn forage treated with four enzyme levels and the subplots of four evaluation moments (incubation periods). The data obtained were submitted to analysis of variance and the means of the treatments were compared by Tukey test at 5% probability, with the aid of the software R (R DEVELOPMENT CORE TEAM, 2010).

A total of 34 nylon filter bags F57 - ANKOM® (32 treated samples, one white and one witness) were used per block. The samples were incubated for 12, 24, 48 and 96 hours at 39 ºC, in anaerobic medium. Subsequently, the bags were incubated in neutral detergent solution for determination of in vitro true digestibility.

During ruminal fluid collection, two crossbred steers (Brown Swiss x Jersey and Jersey x Girolando), with approximate weight of 370 and 327 kg, respectively, were used, and the ruminal fluid was mixed for the experiment. The animals were kept in paddocks and were acclimated to the diet for 14 days, before ruminal fluid collection, and had free access to water and mineral fluid. The diet (DM basis), supplied in the morning, consisted of 5 kg of Tifton 85 hay and 2 kg of corn forage.

The calculation of the IVDDM was carried out according to the following formula (ANKOM® technology): $IVDDM\% = 100 - ((W_3 - (W_1 * C_1)) * 100 / W_2)$, where:

W_1 = weight of the filter bag; W_2 = weight of the samples; W_3 = final weight of the filter bag after in vitro and sequential determination with NDF solution; C_1 = correction of the white filter

bag (final weight of the bag after greenhouse / initial weight of the filter bag).

RESULTS AND DISCUSSION

We observed that the enzymes cellulase and xylanase presented greater activity in pH 6.0. Regarding β-glycosidase, the activity of the enzyme was higher in pH 6.5 (Figure 1).

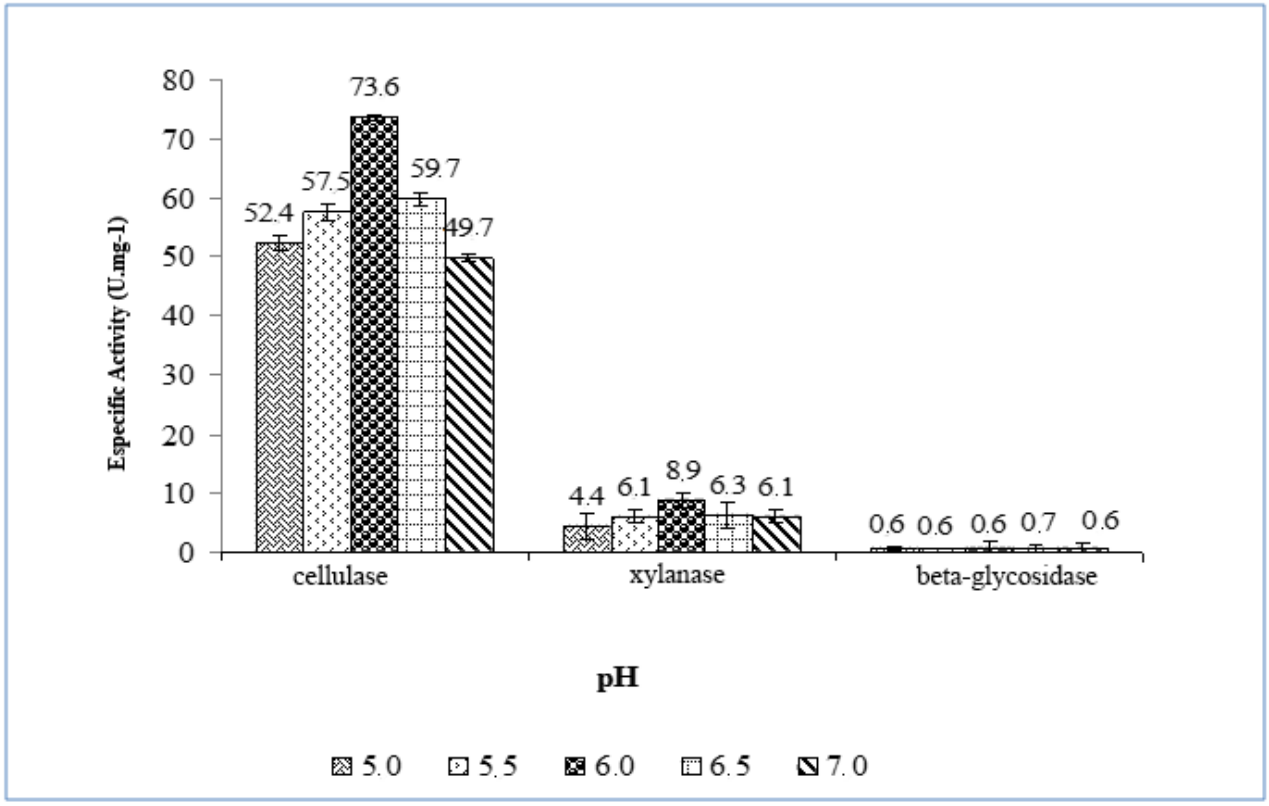

Figure 1. Effect of pH on enzyme activity of the enzymatic complex.

The optimum pH of cellulase, xylanase and β-glycosidase, widely discussed in literature, depends on the producer microorganism.

According to MAHESHWARI et al. (2000), the optimum activity of endo and exoglucanases of thermophilic fungi is between 5.0 and 6.0. SILVA et al. (2005) verified optimum value of 5.0 for CMCase of *T. aurantiacus*. Results obtained by EMTIAZI et al. (2000) showed that cellulase of *Cellulomonas sp* was more active in pH 6.0, which was also verified by LOWE et al. (1987) for cellulase of *Neocallimastix* sp.

According to KULKARNI et al. (1999) xylanase produced by fungi is usually stable in wide pH intervals (3.0-10.0), showing optimum pH within 4.0 and 7.0.

LUCENA NETO & FERREIRA FILHO (2004) found that xylanase of *H. grisea* was more active in pH intervals of 4.5-6.5, which corroborates the results obtained in this experiment.

KITPREECHAVANICH et al. (1984) and DUSTERHOFT et al. (1987) observed that optimum pH of xylanase of *H lanunginosa* and *H insolens* was 6.0.

FERREIRA FILHO (1996) verified greater activity of β-glycosidase of *H. grisea* in pH between 4.0 and 4.5. LIN et al. (1999), characterizing β-glycosidase of *T. lanuginosus*-SSBP, observed 6.0 as optimum pH. Most part of the already known fungal β-glycosidases presente ideal pH between 4.0 and 6.5.

We observed in this experiment that the enzymes remained stable in the range of 5.0-7.0, suggesting that these proteins may present activity in the rumen pH conditions of 5.0-7.0.

To be used in ruminants diet, enzymes have to be stable to temperature variations between 39 and 42 °C, since this is the temperature range in the rumen, the main digestion organ of ruminants. The optimum temperature found for the enzymes cellulase and xylanase was 50 ºC, the enzyme β-glyicosidase kept good activity in the range 50-60 ºC (Figure 2).

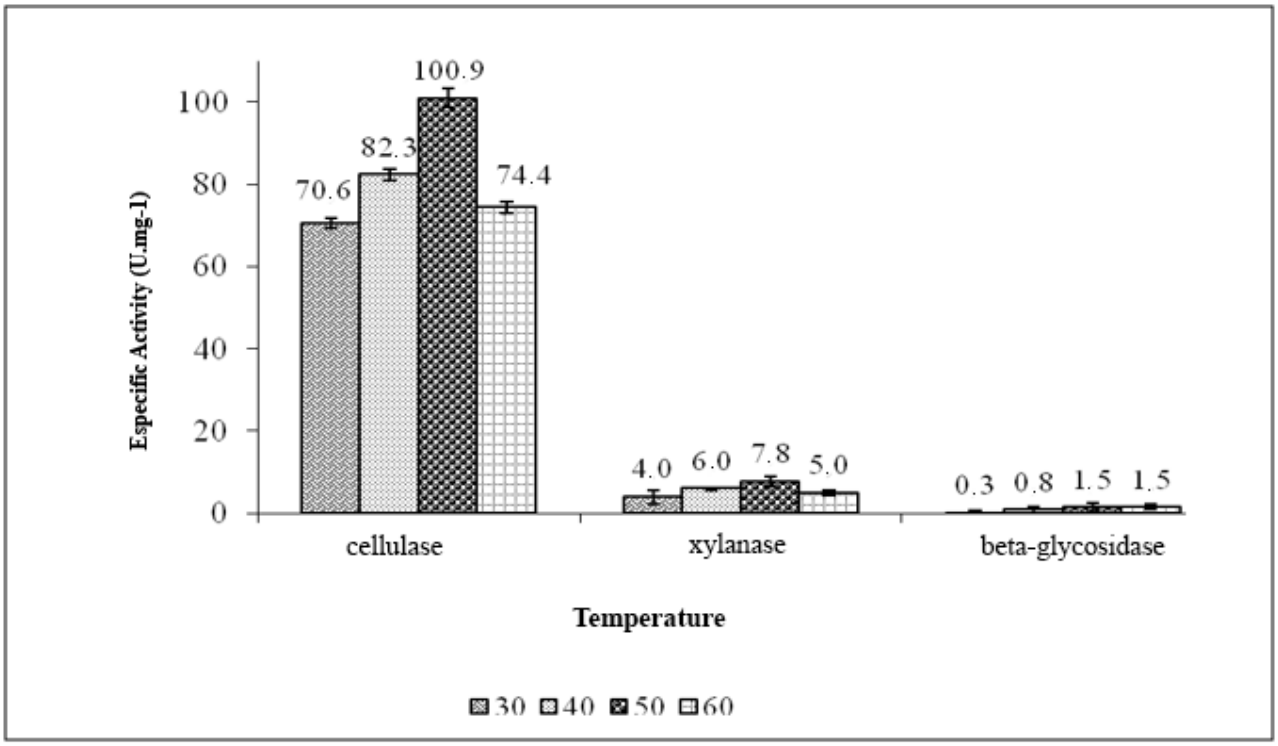

Figure 2. Effect of temperature on enzyme activity of the enzymatic complex.

According to MAHESHWARI et al. (2000), endoglucanases and exoglucanases of thermophilic fungi present good activity at temperatures ranging from 55 to 80 ºC and from 50 to 75 ºC, respectively. For β-1.4-exoglucanases and β-1.4-endoglucanases of *Humicola insolens*, the optimum temperature obtained was 50 ºC, remaining stable at 65 ºC; similar values were found for *Thermoascus aurantiacus*.

YOSHIOKA et al. (1982) verified that CMCase of *H. grisea* showed higher activity at temperature of 50 ºC. The researchers LOWE et al. (1987) and EMTIAZI et al. (2000), in studies on *Cellulomonas* sp and *Neocallimastix* sp, respectively, reported optimum cellulase activity at 45 ºC.

LUCENA-NETO & FERREIRA FILHO (2004), studied xylanase of *H. grisea*, and observed greater activity of the enzyme between 55 and 60 ºC. KITPREECHAVANICH et al. (1984) verified that xylanase of the fungus *H. lanunginosa* was more active within the range of 60-75 ºC, with maximum activity value at 65 ºC, similarly to waht was verified for *H. insolens* (DUSTERHOFT et al. 1997) and xylanase of *H. grisea* (MONTI et al., 1991). According to KULKARNI et al. (1999), the optimum temperature of fungi xylanase varies between 40 and 60 ºC.

Research about the fungi *H. grisea*, *H. lanunginosa* and *H. insolens* showed that β-glycosidase presented optimum activity at temperatures 50 to 60 ºC (MAHESHWARI et al., 2000). These results were similar to those obtained by FERREIRA FILHO (1996), who studied β-glycosidase of *H. grisea* variety *thermoidea*. LIN et al. (1999) observed that β-glycosidase of *Thermomyces lanuginosus-SSBP* presented optimum temperature of 65 °C.

In this experiment, we verified that enzimes of the EC of *H. grisea* showed specific activity within the rumen temperature range, which is inferior to the activity presented at the optimum temperatures of the enzymes.

In order to be used in animal feeding, enzymes must present wide range of thermostability to endure the internal temperature of the animals and the temperature variation during rations processing. The thermostability of the enzymes varies considerably due to its origin, and fungal enzymes present the highest thermostability (CASTRO & MENDES, 2004).

In this study, we observed that the cellulase produced remained stable for 240 minutes, presenting relative activity higher than 100%. The enzymes xylanase and β-glycosidase maintained 99.2 and 88.2% of the activity within 240 minutes, respectively (Figure 3).

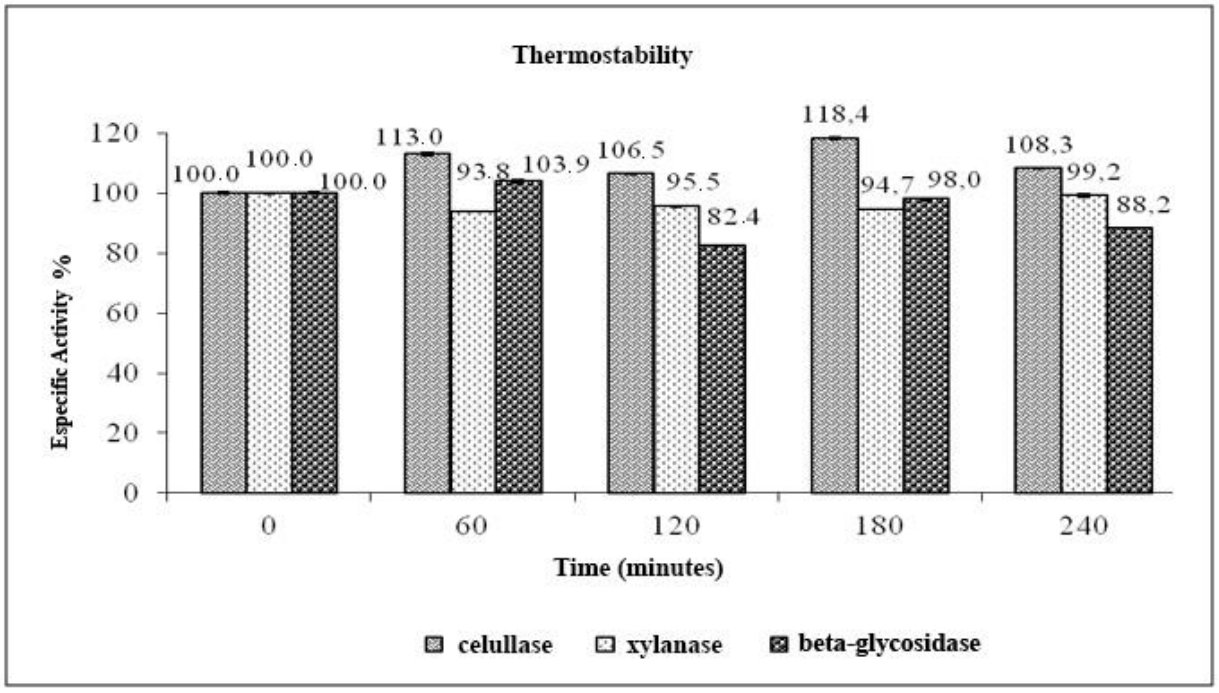

Figure 3. Stability of the enzymes of the enzymatic complex in different times.

OLIVEIRA et al. (2013) verified that endoglucanase produced by *H. grisea* maintained 88% of its activity after 240 minutes of incubation at 50, 60 and 70 °C. The temperatures of 50 and 60 °C increased the enzymatic activity.

DAMASO et al. (2002) verified that the xylanase produced by *T. lanuginosus* remained stable during the time interval of 0-400 minutes, at 50 ºC. LUCENA-NETO & FERREIRA FILHO (2004) verified that xylanase of *H. grisea* variety *thermoidea* presented thermostability at 60 ºC, with half-life of approximately 5.5 hours.

Xylanase purified by MONTI et al. (1991) kept good stability at temperatures between 40 and 60 ºC, presenting half-life of 20 minutes at 60 ºC. REIS et al. (2001) evaluated the thermostability of four fungal xylanases and verified that three of the enzymes maintained 100 to 80 % of activity at the temperature interval of 50 to 70 ºC, while the other enzyme maintained 40% of its activity at 50 °C.

In a study by FERREIRA FILHO (1996), the enzyme β-glycosidase produced by *H. grisea* variety *thermoidea* was thermostable at 60 ºC for one hour, presenting half-life of 15 minutes at 65 ºC. LIN et al. (1999) observed that β-glycosidase of *T. lanuginosus* maintained its total activity after 30 minutes of incubation at 50 ºC, but was inactivated at 70 ºC.

The values of IVDDM of corn forage are described on Table 1. There was interaction between the enzymatic levels and the periods of incubation in the rumen ($p<0.01$). We verified that, regarding the control treatment, the addition of 2.5 mL of enzymes improved the IVDDM in 4.88 and 6.87%, with 12 and 48 hours of ruminal incubation, respectively. For the 5.0 mL treatment, compared to control, we observed increase in the digestibility of 6.66; 7.72 and 5.42%, at the times 12; 24 and 48 hours of incubation in the rumen, respectively. Applying 10 mL of enzymes improved digestibility in 10.58; 12.52; 9.05 and 6.81% at 12; 24; 48 and 96 hours of permanence of the forage in the rumen, respectively, compared to control.

TABLE 1. Digestibility of corn forage in different enzyme levels and four incubation times in the rumen

Enzyme levels (mL)	Incubation time in the rumen (hours)			
	12	24	48	96
Control	80.09 cB	86. 50 cA	86.95 bA	90.05 bA
2.5	84.00 bB	84. 87 cB	92.92aA	90.20 bA
5.0	85.42 abB	93.18 bA	91.66 aA	91.98 bA
10.0	88. 56 aB	97.33 aA	94.82 aA	96.18 aA

Means followed by different letters (uppercase in the row and lowercase in the column) differ ($p<0.01$) by Tukey test. CV (subplot) % = 2.30; CV (plot) % = 2.30

The increase in IVDDM of corn forage is a result of the hydrolytic activity of the enzymes in the substratum. This information is consistent with the chemical evaluation of the enzymatic complex used in the experiments, which showed cellulase and xylanase activities.

The addition of fibrolytic enzymes to the forage, before the ingestion, alters the structure of the food, making them more susceptible to ruminal hydrolysis (NSEREKO et al. 2000). According to FONTES et al. (1995), the enzymes may be partially protected from ruminal degradation due to changes in their conformation. The alteration in the enzyme structure is caused by its strong connection with the substratum when it is incorporated into the food.

According to BEAUCHEMIN et al. (2003), there is evidence that exogenous enzymes cause hydrolysis of soluble carbohydrate during ruminal pre-incubation phase. Its direct application on the substratum favors the formation of a stable enzyme-substratum complex that increases the efficiency of the exogenous enzymes in the rumen.

GIRALDO et al. (2007), applied two cellulases produced by *Aspergillus niger* and *Trichoderma longibrachiatum* at the dose of 30 U/g of substratum (70% of grass hay and 30% of concentrate), and oserved higher disappearance of DM after 6 and 24 h, with no effects after 48 hours of incubation. LEWIS et al. (1996) did not observe increase in the IVDDM of grass hay at 8; 16 and 24 hours of incubation when fibrolytic enzymes were applied; however, they observed an increase after 32, 40 and 96 hours.

CYSNEIROS et al. (2006) did not verify effects of 5; 10 and 20 mg of fibrolytic enzymes per kg of natural matter on the ruminal disappearance of corn silage DM at 6, 24 and 96 hours. YANG et al. (1999) verified that the curves of ruminal digestibility of alfalfa were similar for control and enzymatic treatment. The disappearance was fast during the first 12 h of incubation, reaching a plateau close do 23 h.

According to DAWSON & TRICARICO (2007), a series of in vitro studies have shown that it is possible to use specific fibrolytic enzymes preparations to improve the processes associated to the digestion of food in the rumen. The response is usually evaluated by the increase in the initial disappearance rate of dry matter or the disappearance rate of the neutral detergent fiber of forage supplemented with enzymes. It is believed that exogenous enzymes can make the fiber soluble or more available to the microbial attack in the rumen. According to the same authors, the first 6-12 hours of the digestive process seem to be the more active period for the effects of exogenous enzymes. In most cases, fibrolytic enzymes do not have significant effects on fiber digestion after long periods of ruminal incubation.

Moreover, some variables associated to the use of exogenous enzymes in the diets of ruminants are related to the supplementation with insufficient or excessive amount of enzymes. The preparations of exogenous enzymes are usually used in low concentrations, in which, the enzymes do not seem to contribute to better fiber digestion in the rumen, even if the conditions of the ruminal environment are ideal for their activities.

Studies carried out by BEAUCHEMIN et al. (2003) showed that the optimum enzymatic level depends on the substratum. These authors reported different levels of enzymes application and different responses to alfalfa hay, corn silage, barley silage and corn grains. For alfalfa hay, reduction in the neutral detergent fiber content was observed, despite the addition of a low enzymatic level, without any alteration observed with high application levels. High amounts of enzymes connected to the substratum may limit the connection of rumen microorganisms to the food, limiting its digestibility (BEAUCHEMIN et al. 2003).

Fibrolytic enzymes are related to their capacity of improving initial degradation of structural carbohydrates of plants and complement the enzymatic activities associated to ruminal microorganisms. It is likely that exogenous enzymes act in the rumen right after the ingestion of the food and during a short period of time, before bacterial colonization of the food and beginning of the digestion. As a result, exogenous fibrolytic enzymes may complement enzymatic activities of the microorganisms in the rumen and allow better digestion of the substratum during the preliminary phases, which are critical to digestion. The global effects of supplementation with enzymes may result in their capacity to expose the slowly degraded substratum to microbial attack (DAWSON & TRICARICO 2007).

CONCLUSIONS

The fungus *Humicolagrisea* variety *thermoidea* is an important fibrolytic enzymes producer for ruminant feeding. The enzymes total cellulase, xylanase and β-glycosidase presented activities within the temperature and pH range observed in the rumen. The enzymes, at the three

tested levels, increased IVDDM of corn forage but they did not significantly affect digestion, after long periods of ruminal incubation.

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