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Catalytic properties of xylanases produced by *Trichoderma piluliferum* and *Trichoderma viride* and their application as additives in bovine feeding

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ABSTRACT

This study investigated the xylanases produced by *Trichoderma piluliferum* and *T. viride*. These enzymes were characterized and used as additives in bovine diet. The fungus *T. piluliferum* was a more efficient producer of xylanase (301 U g^{-1} dry substrate) when grown on wheat bran, with 75 or 80% moisture for 96 h. The optimum pH of the enzyme produced by *T. piluliferum* was 4.5, obtaining as the optimum temperature 50 °C. The enzyme was stable at a wide pH range (3.0–10.0), and it remained stable for 1 h at 40 °C. The best culture parameters for the cultivation of *T. viride* (671 U g^{-1} dry substrate) were as follows: wheat bran as substrate, 65, 70 and 75% initial moisture, and 48 h cultivation time. The optimum pH and temperature for the xylanase produced by this fungus were 6.0 and 45 °C, respectively, and the enzyme was stable at a wide pH range (3.0–10.0), maintaining its activity after 1 h at 40 °C. Both the enzyme extracts produced were effective at increasing digestibility of Tifton 185 hay, sugarcane silage, and corn silage. The enzymatic extracts proved promising for xylooligosaccharides production and showed low cellulase activity, indicating potential for application in biobleaching processes of cellulose pulp and paper.

1. Introduction

Xylanases are enzymes of the hemicellulolytic complex that act on the central chain of hemicellulose, which is a constituent polysaccharide of the vegetal cell wall, thus reducing its degree of polymerization drastically. There are two types of xylanases: the endo-1,4-β-xylanases (EC 3.2.1.8) that act inside the xylan structure and release xylooligosaccharides (XOS) as the main product; and the β-D-xylosidases (EC 3.2.1.37) that externally hydrolyze the xylan chain and small XOS, thus releasing xylose (Keshwani and Cheng, 2009; Knob et al., 2014).

Xylanases have gained prominence in feed supplements for ruminant animals (Fagundes et al., 2008; Knob et al., 2014; Nogueira et al., 2013). These enzymes contribute to the digestibility of fibrous foods by increasing the availability of its compounds for ruminal microbiota, allowing the animal to effectively utilize the nutrients in foods that are difficult to degrade (Cysneiros et al., 2013; Nogueira et al., 2013).

Besides increasing food digestibility, the addition of enzymes in the diet of cattle can accelerate weaning and increase resistance against infections. These enzymes may also help decrease environmental impact by reducing methane production by the animals (Campestrini et al., 2005).

Despite the interesting action of these enzymes, some problems for large-scale production of xylanases, such as high production cost, must be overcome. With the aim of reducing these costs, several studies have evaluated the production of xylanases by solid-state cultivation (SSC) of filamentous fungi at agro-industrial residues (Colla et al., 2012; Costa et al., 2016; Sadaf and Khare, 2014; Terrasan and Carmona, 2015).

Recently, our research group isolated two fungal strains with considerable potential for xylanase production from soil samples collected from the Pantanal, Mato Grosso do Sul, Brazil. The strains were identified as *Trichoderma piluliferum* and *T. viride*. These strains had the ability to produce high concentrations of xylanase when cultivated in agro-industrial residues. This led us to the present study with the aim to

Abbreviations:

IVDMD	*In vitro* dry matter digestibility
NDF	Neutral detergent fiber
NDFD	*In vitro* NDF digestibility
ADF	Acid detergent fiber
XOS	Xylooligosaccharides
SSC	Solid-State Cultivation
CMC	Carboxymethyl cellulose
FPase	filter paper activity

produce xylanases in a low-cost medium for application in the diet of ruminant animal diet supplement. In this context, this work seeks to contribute actively to xylanases use in animal feed, presenting *in vitro* digestibility assays for several foods used in ruminant nutrition. This fact enhance the present article in relation to classical works of enzymatic production, permeating between basic and applied science.

2. Material and methods

2.1. Microorganisms

The filamentous fungi used in this study were isolated from soil samples from Pantanal, Mato Grosso do Sul, Brazil. The fungi were identified as *T. piluliferum* and *T. viride* by Micoteca URM (Universidade Federal de Pernambuco-UFPE). The microorganisms were maintained on Sabouraud Dextrose Agar at 4 °C.

2.2. Inoculum

The inoculum was prepared in 250 mL Erlenmeyer flasks containing 40 mL of Sabouraud Dextrose Agar slants and incubated for 72 h at 28 °C. The microbial suspension was obtained by adding 50 mL of nutrient solution containing 0.1% ammonium sulfate, 0.1% magnesium sulfate heptahydrate, and 0.1% ammonium nitrate (w/v), followed by gentle scraping of the medium surface. Inoculation of the microorganism was performed by transferring 5 mL of the microbial suspension to flasks containing the agro-industrial residues (Pereira et al., 2015).

2.3. Solid-state cultivation for xylanase production

Several agro-industrial residues such as wheat bran, soy bran, corn cob, corn straw, rice peel, and sugarcane bagasse were tested for the production of xylanase. All substrates were washed with distilled water and dried at 50 °C for 48 h. The fungus was cultivated in 250 mL Erlenmeyer flasks containing 5 g of substrate (agro-industrial residue) moistened with nutrient solution (described above). Initially, the moisture content was adjusted to 70% (w/v), the cultivation time was fixed at 96 h, and the temperature was maintained at 28 °C. The substrate optimal for enzyme production was selected to evaluate other culture parameters such as different proportions of sugarcane bagasse and wheat bran as substrate (5:0; 3.75:1.25; 2.5:2.5; 1.25:3.75; and 0:5), initial moisture (50–80%), and cultivation time (24–120 h). The optimized parameter in each step was adopted in subsequent cultivation. All assays were performed in triplicate, and the described values represent the respective averages.

2.4. Enzyme extraction

The enzymatic extract was obtained by adding 50 mL of distilled water in Erlenmeyer flasks containing the fermented residue. The flasks were agitated for 1 h at 150 rpm. The samples were filtered through a nylon cloth and subsequently centrifuged at $3000 \times g$ for 5 min. The supernatant was used in subsequent assays.

2.5. Determination of enzymatic activities

The activities of endoglucanase (or carboxymethyl cellulase; CMCase) and xylanase were quantified using 3% carboxymethyl cellulose (CMC - C5678; Sigma-Aldrich) and 1% xylan (Beechwood, Sigma-Aldrich), respectively. The filter paper activity (FPase) was quantified according to Ghose (1987), using a strip of Whatman No. 1 filter paper (1.0×6.0 cm) as substrate. The reducing sugars released were quantified using the DNS method (Miller, 1959). The activities of β-glucosidase and β-xylosidase were measured with the respective synthetic substrates (4 mM; p-NP-β-D-glucopyranoside and 4 mM p-NP-β-D-xylopyranoside) (Sigma-Aldrich) as described by Pereira et al. (2015). One unit of enzymatic activity was defined as the amount of enzyme required to release 1 μmol of product per min of reaction.

2.6. Effect of pH and temperature on the xylanolytic activity

The xylanase produced under optimized conditions was biochemically characterized. The optimal pH was determined by quantifying the enzymatic activity at 50 °C at different pH values (3.0–8.0) using 0.1 M citrate-phosphate buffer. The optimum temperature was determined by measuring the enzymatic activity from 30 °C to 70 °C at the optimal pH for enzyme activity. pH stability was determined by incubating the enzyme for 24 h at different pH values at 25 °C using 0.1 M citrate-phosphate (3.0–8.0) and 0.1 M glycine-NaOH (8.5–10.5) buffers. Thermostability was evaluated by incubating the enzyme for 1 h at different temperatures from 30 °C to 50 °C. Residual activities were measured under optimal enzyme conditions; the highest value of residual activity obtained after sample treatment was considered to be 100% of activity (Costa et al., 2016). For determination of the half-life (t½) of the enzymes, the produced extracts were incubated at 45 °C for different periods of time, and their enzymatic activity was tested.

2.7. Thin layer chromatography (TLC) of hydrolysis products

Chromatographic analysis of the reaction end products of xylanase activity on xylan from beechwood was performed using thin layer chromatography (TLC). The reaction mixture (0.01 mL) was applied on silica gel plates (G-60, 10×15 cm) and subjected to two sequential ascending chromatography runs using butanol/ethanol/water (5:3:2) as the solvent system. After air drying the plate, spots developed by spraying with a solution of H_2SO_4 and methanol (1:9) containing 0.2% orcinol and heating at 100 °C (Silva et al., 2013). Xylose and xylan (0.1% w/v) were used as standards.

2.8. In vitro digestibility using xylanase as an enzymatic supplement

To evaluate the effect of xylanase addition on the digestibility of ruminants, five different foods were evaluated: *Brachiaria brizantha*, *Panicum maximum* 'Mombaça', Tifton 185 Hay, sugarcane silage, and corn silage. *In vitro* digestibility coefficients were determined using ruminal inoculum and a xylanase buffer solution 6 U.L^{-1} (recommended amount of commercial Fibrozyme™ xylanase). The experiments were carried out six times, and the presented values represent the means of the experiments. Assays without addition of enzymes were performed as controls. The ruminal inoculum was obtained from two adult castrated Jersey cattle, with an average body weight of 380 kg, and provided with a ruminal cannula. The animals were in grass pasture Marandu - *U. brizantha*. The *in vitro* dry matter digestibility (IVDMD) of the diets was determined according to the methodology described by Tilley and Terry (1963), using artificial rumen TE-150 (Tecnal). Samples (0.5 g) were weighed in TNT 100 g/m bags, cut, and sealed to a size of 5×5 cm as described by Casali et al. (2008). Two blank sachets (white) were used in each jar to correct the

data. The sample bags were placed in the jars and evenly distributed. Then, 1280 mL of buffer solution and 320 mL of ruminal inoculum were added, and CO_2 was added to maintain anaerobic conditions in each jar. After this procedure, the jars remained in the artificial rumen at 39 °C for 48 h with continuous agitation, according to the method described by Tilley and Terry (1963). The sachets were washed with distilled water and transferred to the oven for drying at 105 °C for 12 h. The sachets were weighed and analyzed for IVDMD and neutral detergent fiber (NDF) content. The *in vitro* NDF digestibility (NDFD) was obtained by calculating the difference between the fiber concentration in the sample before and after the incubation. The fractions of NDF were determined by the methodology described by Van Soest et al. (1991), using the Tecnal fiber analyzer (TE-149). To compare the samples that underwent *in vitro* digestion with the undigested samples, 500 mg of each of the forages was evaluated for NDF content (%) and acid detergent fiber (ADF), according to the methodology described by Van Soest et al. (1991). The percentage of hemicellulose present in fodder was determined by the difference in NDF and ADF values.

2.9. Statistical analysis

All experiments were carried out in triplicate, and the results were presented as the mean of three independent assays and their standard deviations. Statistical analyses of the data included a one-way ANOVA, followed by Tukey's test with 5% significance.

3. Results and discussion

3.1. Solid-state cultivation for xylanase production

Among the agro-industrial residues used to grow microorganisms for the production of xylanases, wheat bran was more promising, reaching about 270 U g^{-1} of dry substrate (27 U mL^{-1}) by the two fungi (Table 1), and this result was statistically significant at $p < 0.05$.

Kar et al. (2013) obtained xylanase high production by *T. reesei* when this fungus was cultivated in wheat bran. Thomas et al. (2013) also obtained good xylanase production by *Streptomyces* sp. When they used wheat bran as a substrate for xylanase production. Leite et al. (2007) obtained optimal xylanase production by the fungus *Aureobasidium pullulans* using wheat bran as a substrate. They obtained a production of about 5 U mL^{-1}, which is lower than the value obtained in this study for the two *Trichoderma* species.

Wheat bran is a complex substrate, which is rich in nutrients and has great water absorption capacity. Other authors have also reported that wheat bran is as a good substrate for SSC and for xylanase production (Costa et al., 2016; Gowdhaman et al., 2014; Masui et al., 2012; Rodriguez-Zuniga et al., 2011). As it is an agro-industrial residue and thus is less costly, it can reduce the production cost for the enzyme of interest (Delabona et al., 2012; Masui et al., 2012; Mittal et al., 2013).

Considering the results obtained in the present work, different proportions of wheat bran and sugarcane bagasse were evaluated for microbial cultivation. Sugarcane bagasse was used to reduce the compaction of wheat bran, thus aiming to favor mass transfer during microbial growth, which would increase the speed of growth and enzymatic production (Delabona et al., 2012; Penha et al., 2012). However, this was not observed for *T. piluliferum* and *T. viride* (Fig. 1A) because xylanase production reduced with the addition of sugarcane bagasse to the crop. This result can be explained by the composition of wheat bran and sugarcane bagasse. Wheat bran has an adequate balance between carbon and nitrogen sources and is rich in B vitamins (Delabona et al., 2012; Leite et al., 2007), while sugarcane bagasse presents high recalcitrance due to its rich lignin composition, making it difficult for microbes to access cellulose fibers and hemicellulose, resulting in less enzymatic production by the microorganisms (Penha et al., 2012). Therefore, the addition of sugarcane bagasse did not favor the nutritional composition of the substrate used for microbial cultivation. Thus,

subsequent cultures were prepared using only wheat bran as the substrate.

To establish the ideal substrate moisture, the two strains were grown in wheat bran containing different percentages of initial moisture. For *T. piluliferum*, the ideal initial moisture content for xylanase production was 75 and 80%, with no significant difference ($p < 0.05$) between these two humidities, but to use smaller amounts of water, the value of 75% was adopted for subsequent trials. For *T. viride*, the highest production of xylanase was observed in the cultures containing 65, 70, and 75% of moisture, and 65% of humidity was selected for the execution of the following experiments (Fig. 1B).

SSC simulates the natural environment in which filamentous fungi are placed. This type of culture requires no free water between the substrate particles of the solid matrix. However, sufficient moisture is required to ensure the metabolic activity of the fungus, without reducing the porosity of the substrate. High moisture compromises the diffusion of oxygen in the medium, besides providing an environment conducive to bacterial unwanted contamination (Da-silva et al., 2005; Sadaf and Khare, 2014; Knob et al., 2014).

To determine the optimal culture time for xylanase production by fungal strains, samples were taken every 24 h, completing a total of 120 h of culture. The highest production of xylanase by *T. piluliferum* was obtained between 72 and 96 h of culture (approximately 301 U g^{-1} or 30 U mL^{-1} of dry substrate; Fig. 1C). The fungus *T. viride* showed high enzyme production with only 48 h of cultivation (approximately 671 U g^{-1} or 67 U mL^{-1} of dry substrate; Fig. 1C). Pereira et al. (2015) obtained optimal production of xylanase by the fungus *Myceliophthora thermophila* also with 48 h of SSC, obtaining approximately 1138 U g^{-1} using cane bagasse with soybean meal as substrate. The best production of xylanase by the fungus *Penicillium janczewskii* (371 U g^{-1}) obtained by Terrasan and Carmona (2015) was achieved in 168 h of SSC using barley bagasse as substrate. Alves-prado et al. (2010) reported optimal xylanase production, approximately 20 U mL^{-1}, by the fungus *Neosartorya spinosa* in 72 h.

The results obtained in the present work compared to those described in the cited scientific literature allow us to infer that both the microorganisms used in this study have potential for xylanase production in culture media with low added value and with reduced culture time, which favors the final cost of obtaining these biocatalysts making the biocatalyst production process cost-effective.

3.2. Biochemical characterization of produced xylanases

The enzymes produced under optimal culture conditions were characterized biochemically. The enzyme produced by *T. viride* presented optimal activity at pH 6.0. This result differs from that found by Irfan and Syed (2012) when studying xylanase from *T. viride* and found optimal activity at pH 5.0. This divergence can be because these are distinct strains isolated from different environments (Fig. 2A).

The xylanase of the fungus *T. piluliferum* presented activity peaks at

Table 1
Production of xylanase in different lignocellulosic residues by *T. piluliferum* and *T. viride* by solid-state cultivation.

Substrates	Xylanase (U g^{-1})	
	T. piluliferum	*T. viride*
Wheat bran	273.8 ± 8.1 [a]	274.1 ± 10.5 [a]
Soy bran	39.1 ± 3.2 [b]	26.5 ± 0.2 [b]
Rice peel	21.3 ± 1.6 [c]	18.7 ± 0.9 [bc]
Sugarcane bagasse	14.6 ± 2.5 [cd]	10.3 ± 1.2 [c]
Corn straw	7.6 ± 0.2 [d]	26.4 ± 0.1 [b]
Corn cob	7.0 ± 3 [d]	9.8 ± 0.5 [c]

Avarages with different letters in each column indicate significant differences according to the Tukey's test for $p < 0.05$.
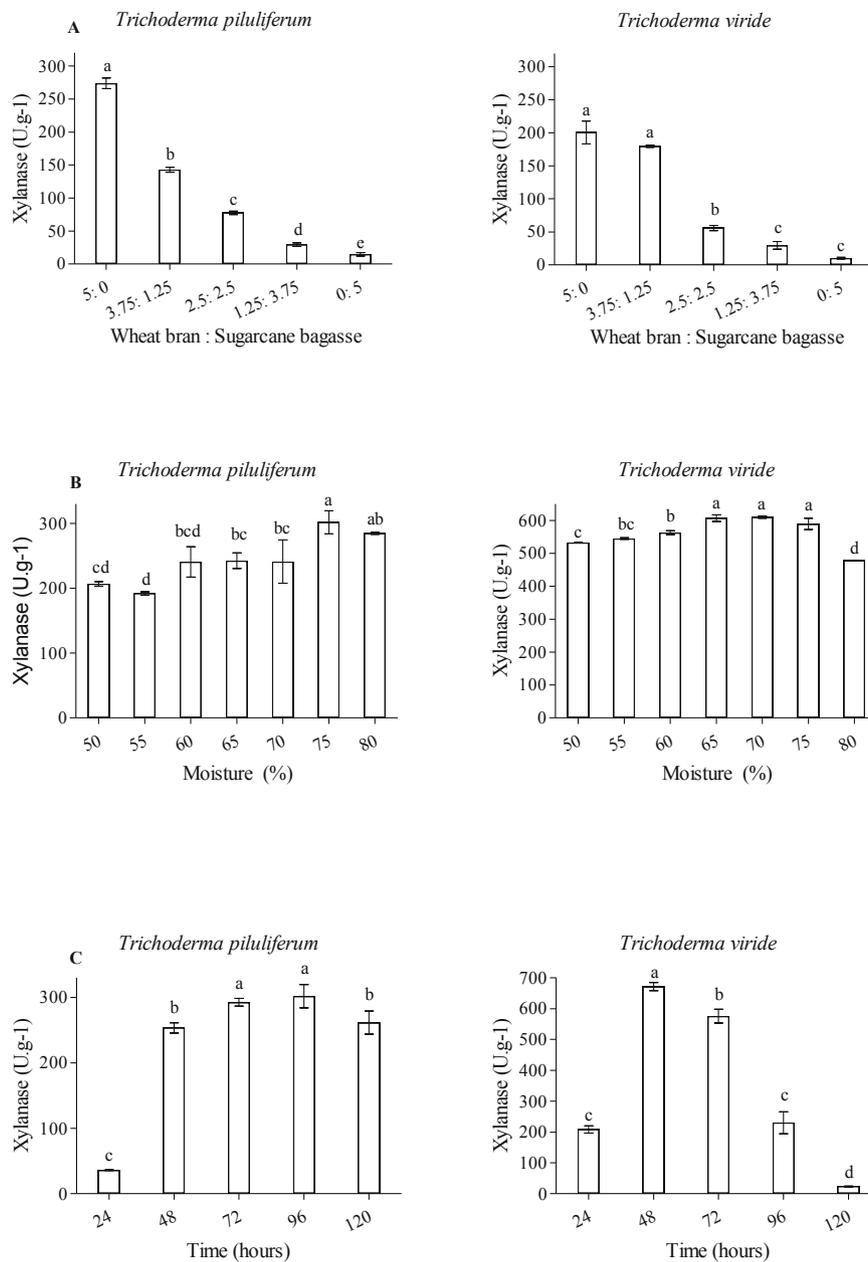
Fig. 1. Production of xylanase by the fungi *Trichoderma piluliferum* and *Trichoderma viride* by solid-state cultivation. A) Influence of different proportions of wheat bran and sugarcane bagasse; B) influence of different moisture content; and C) influence of growing time. The average production with different letters in the same graph indicates significant differences according to Tukey's test (p < 0.05).

pH 4.5 and 6.0, the higher being at pH 4.5. This result suggests the production of isoforms of the xylanase enzyme by the microorganism (Fig. 2A).

Alves-prado et al. (2010) studied the effect of pH on xylanase of *N. spinosa* and found two peaks of optimal activity, suggesting the existence of isoenzymes in the enzymatic extract. Thomas et al. (2013) discussed the presence of four xylanase isoforms produced by *Streptomyces* sp., suggesting that such differences occurred because of nucleotide sequence polymorphisms, small genetic differences between alleles of the same gene, or the presence of more than one xylanase gene copy in the genetic material. Masui et al. (2012) studied the xylanase of *Humicola brevis* and found optimal activity of the enzyme at pH 5.5. Our results are similar to most of the data found in the literature, indicating that the optimal pH of xylanases produced by fungal species is within the range of 4.0–6.0 (Guimarães et al., 2013).

The optimal temperature observed for xylanase produced by *T.*

piluliferum was 50 °C, while that by *T. viride* was 45 °C (Fig. 2B). Guimarães et al. (2013) reported that the optimal temperature for xylanase produced by the fungus *Aspergillus japonicus* is 55 °C, which is close to that found in the present work.

After 24-h incubation at different pH values, the xylanases produced by the two isolates remained stable in the pH range of 3.0–10.0 (Fig. 2C). The xylanases produced by the fungus *A. pullulans* in the study carried out by Leite et al. (2007) were stable for 24 h at pH 3.0 to 8.0. Xylanase produced by *Streptomyces* sp. in the study of Brito-Cunha et al. (2013) remained stable in the range of 5.0–9.0, whose amplitude is lower when compared with than the results obtained in this study.

As for thermostability, the enzymes remained remarkably stable for 1 h at 40 °C (Fig. 2D). This can be explained by the mesophyll characteristics of the two strains. Guimarães et al. (2013) also described the stability of xylanase produced by the fungus *A. japonicus* in the range of 45–50 °C. Silva et al. (2015) described the purified xylanase of the
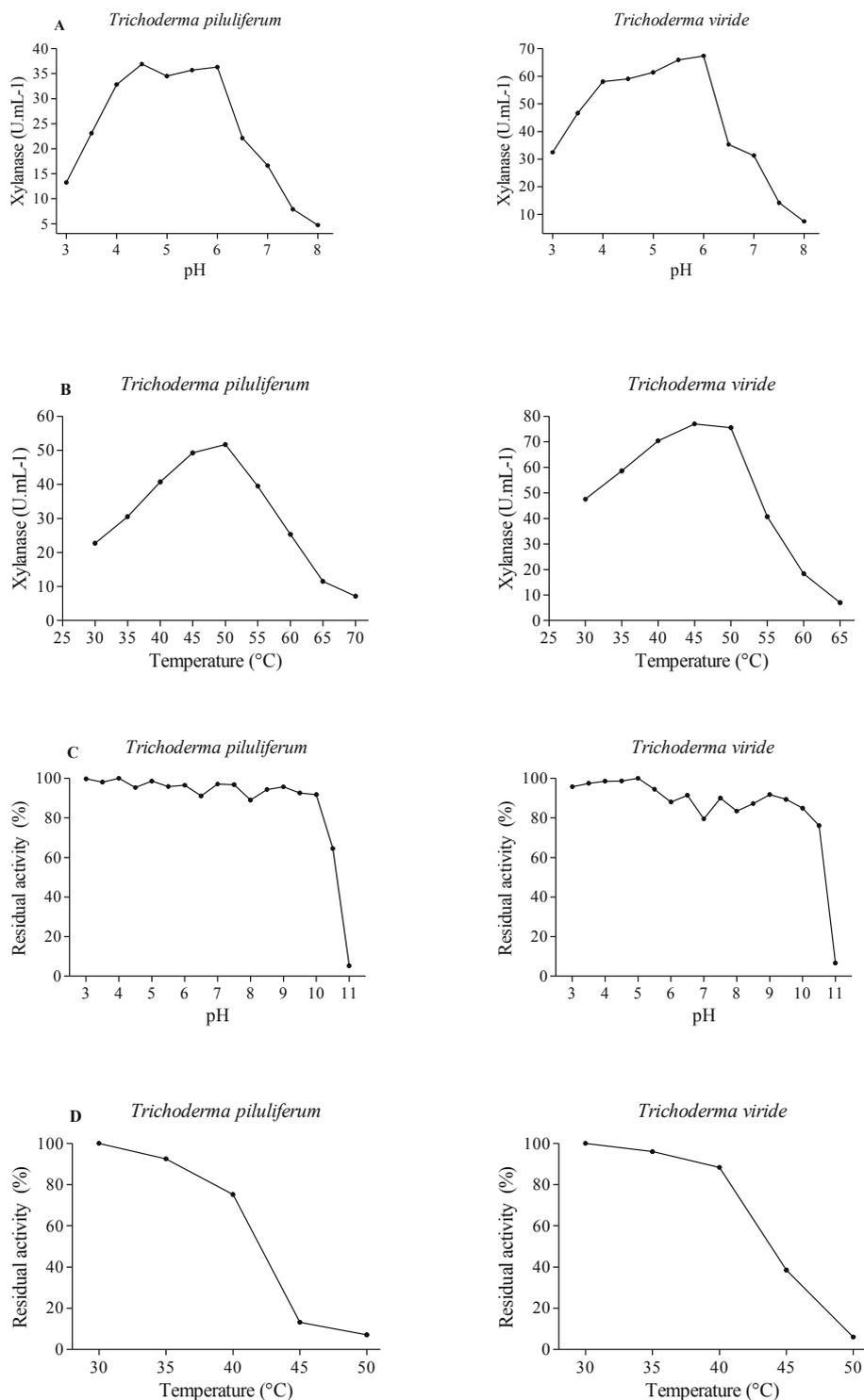
Fig. 2. Biochemical characterization of xylanase produced by the fungi *Trichoderma piluliferum* and *Trichoderma viride*. A) Optimal pH; B) optimal temperature; C) stability at pH; and D) thermostability.

mesophyll fungus *T. inhamatum* as stable at 40 °C, values close to those found for the enzymes of the two isolates.

The half-life ($t_{1/2}$) of the xylanases produced by *T. piluliferum* and *T. viride* when incubated at 45 °C was reached in approximately 25 and 60 min, respectively (Fig. 3). Thus, it is possible that the xylanase produced by *T. viride* presents greater thermal stability compared to the enzyme produced by *T. piluliferum*. Temperature is the factor that most influences the biological function of biocatalysts. The difference between stable and poorly stable enzymes is in the amino acid

composition and sequence, presence of disulfide bonds, ionic interactions, and hydrophobic interactions within the molecule. The increase in temperature results in the disruption of these interactions, consequently causing structural disorganization of the protein (Gomes et al., 2007).

Previous studies reported that xylanases might contribute to the digestibility of fibrous food by ruminant animals, indicating that these enzymes act in ruminal conditions and remain stable during food processing (Fagundes et al., 2008; Nogueira et al., 2013; Cysneiros et al.,
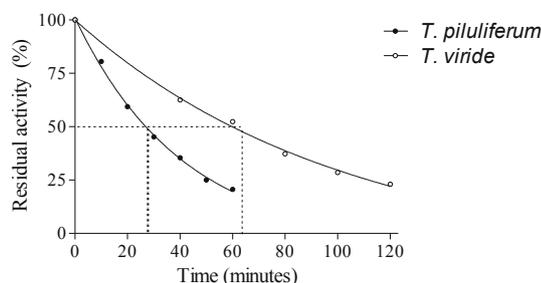
Fig. 3. Half-life (t½) of the xylanase extracts of *Trichoderma piluliferum* and *Trichoderma viride* at 45 °C.
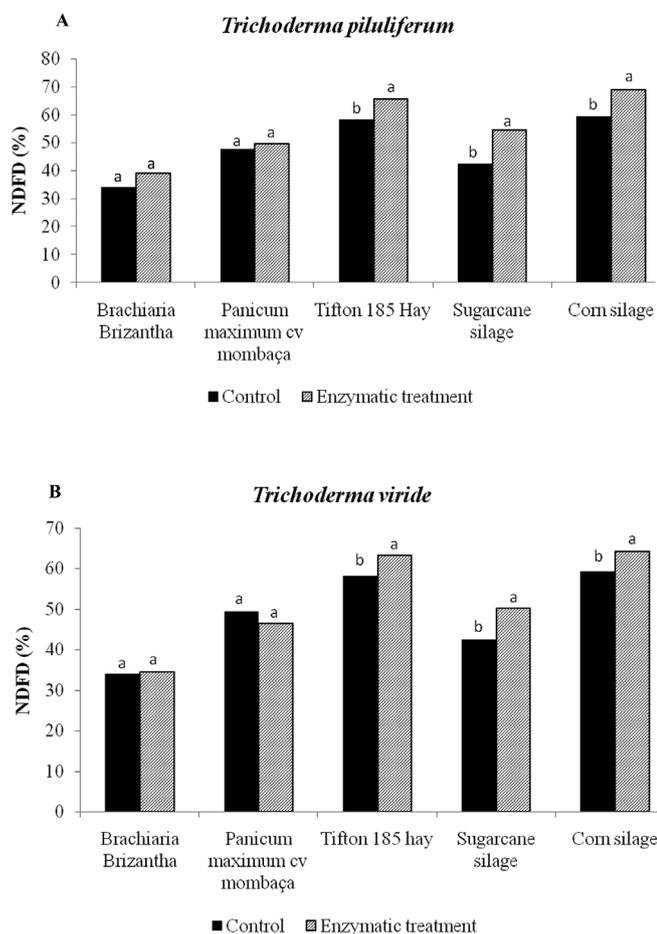

Fig. 4. Percentage of *in vitro* neutral detergent fiber digestibility (NDFD) of different forages not treated (control) and treated with xylanases (6 U L^{-1} of ruminal liquid). A) *Trichoderma piluliferum* and B) *Trichoderma viride*. Averages with distinct letters in the graph are the differences according to Tukey's test (p < 0.05).

Table 2
Average contents of dry matter (DM), neutral detergent fiber (NDF), acid detergent fiber (ADF) and hemicellulose of the analyzed foods, established by the Laboratory of Animal Nutrition of the UFGD.

Food	DM (%)	NDF (%)	ADF (%)	Hemicelulose (%)[a]
Sugarcane silage	27	65	42	23
Corn silage	28	63	35	27
Panicum maximum cv mombaça	25	52	41	11
Brachiaria Brizantha	17	62	45	17
Tifton185 Hay	89	76	43	32

[a] NDF-ADF.

2013).

The temperature of the bovine rumen is around 39 °C and may have minor changes (Cysneiros et al., 2013). The enzymes produced by the two microorganisms evaluated in the present study act in conditions similar to those of the ruminal environment, which can contribute to the digestion process of these animals. In this way, both enzymatic extracts were used *in vitro* for the digestion of possible feed for cattle.

3.3. In vitro digestibility

The enzymatic extracts of fungi *T. piluliferum* and *T. viride* were evaluated for their application in the digestibility of the hemicellulose, cellulose, and lignin complex (NDFs) of different forages used in bovine diets.

The enzymatic extract of *T. piluliferum* increased the *in vitro* digestibility of Tifton 185 hay, sugarcane silage, and corn silage by approximately 7%, 12%, and 9%, respectively, compared to the digestibility of controls (Fig. 4A). In the assays using the enzyme extract of *T. viride*, enzyme addition contributed to the digestibility of Tifton 185 hay, sugarcane silage, and corn silage by approximately 5%, 7%, and 5%, respectively (Fig. 4B). The increase in digestibility was detected predominantly in food with a high percentage of hemicellulose in its composition (Table 2).

The differences in the digestibility observed for the different evaluated foods are probably related to the catalytic properties of the enzymatic extracts used, together with the composition and arrangement of the fodder fibers, which present different conformations and interactions between their polymers.

The best result was observed in the digestibility of sugarcane silage using the enzymatic extract of *T. piluliferum* (12%). In general, this food is considered of poor quality because of its low digestibility, observed in the tests performed without enzyme addition (control, Fig. 4A and B). Thus, it is possible to infer that enzymatic supplementation in the present study increased the efficiency of the sugarcane digestion process. This result is promising for countries such as Brazil that have high availability of this product.

Although the microbiota present in the rumen is capable of digesting cellulose, some factors such as plant structure and composition and interactions between hemicellulose and lignin (Cysneiros et al., 2013) make it difficult to digest food. The addition of xylanases in the digestion process causes hemicellulose to break down, thus exposing the cellulose fibers and nutrients present in the food. Therefore, the ruminal microbiota and the endogenous enzymes can have greater access to the food (Nogueira et al., 2013).

Nogueira et al. (2013) used xylanase from *A. niveus* in ruminal conditions of goats and obtained a gain in digestibility of approximately 6% in *in vitro* for corn silage this value is lower than that found in this work for the addition of the enzymatic extract of *T. piluliferum*. The addition of enzymes in the diets of ruminant animals can positively influence milk and meat production, factors not evaluated in the present study. Schingoethe et al. (1999) observed an increase of approximately 11 and 13% in the production of milk and milk protein, respectively, with the addition of cellulase and xylanase in diets containing corn silage. The increase in digestibility by xylanase addition, besides being beneficial in nutritional terms, reduces the amount of nutrients in the feces, reducing the emissions of methane, which contributes to the reduction of environmental pollution caused by livestock. These enzymes may also aid in weaning and decreasing the viscosity of food in the digestive process in the rumen, considering that in a viscous environment the digestibility is compromised (Campestrini et al., 2005; Fagundes et al., 2008). In this context, it is possible to infer that the results obtained in the present study can be even more significant if other parameters related to animal nutrition are evaluated.

Although the promising results obtained in the present study, some difficulties must be overcome in order to enable the use of these enzymes in animal nutrition. The heterogeneity of the solid state

Table 3

Catalytic profile of the enzymatic extracts produced by *Trichoderma piluliferum* and *Trichoderma viride*.

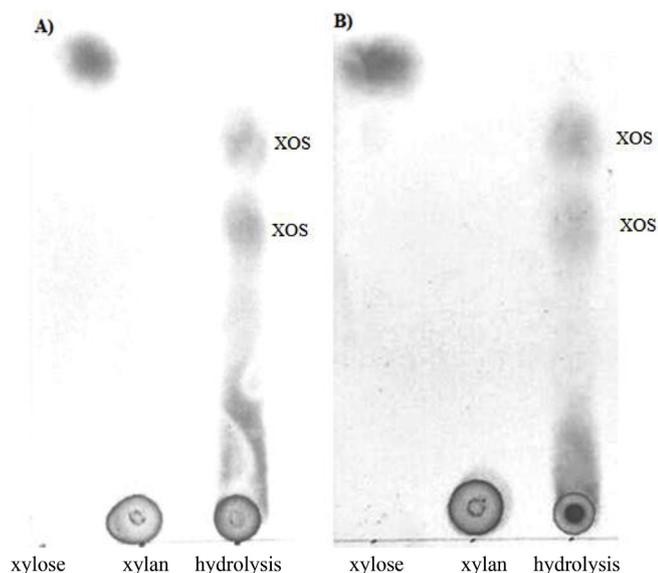| Enzyme	Substrate	*T. piluliferum* (U.mL^{-1})	*T. viride* (U.mL^{-1})
Avicelase	Avicel	0	0.75
CMCase	Carboximetilcellulose	3.64	5.30
β-glucosidase	Nitrofenil β-D glucopyranoside	2.46	2.05
FPase	Filter paper	0.10	0.10
Xylanase	Beechwood xylan	30.18	67.18
β-D-xylosidase	Nitrofenil β-D xylopyranoside	0.31	0.26

Fig. 5. Thin layer chromatography (TLC) of the hydrolysis product (10 min) of xylan beechwood (0.5%) by the enzymes produced by *Trichoderma piluliferum* (A) and *Trichoderma viride* (B). Xylose and xylan (0.1%) were used as standards for the hydrolysate analysis.

cultivation impairs to increase the enzymatic production for industrial scale (Singhania et al., 2009), which justifies the high number of studies that try incessantly to improve this type of process (Costa et al., 2016; Delabona et al., 2012; Garcia et al., 2018; Gonçalves et al., 2013; Kar et al., 2013; Sadaf and Khare, 2014). Alternatively, some authors perform the micelliation of the microorganism directly on the animal feed, contributing to the increase of the digestibility and protein yield (Nayan et al., 2018).

3.4. Catalytic profile of enzymatic extracts

Among the enzymatic activities evaluated, extracts produced by fungi *T. piluliferum* and *T. viride* presented non-significant activity of Avicelase, CMCase, and β-glucosidase. The reduced potential for degrading cellulose was confirmed by the low FPase activity (Table 3). However, in the study published by Nathan et al. (2014), the fungus *T. viride* was able to produce cellulases in submerged culture using CMC as the carbon source. According to Okeke (2014), species of *Trichoderma* spp. produced high concentrations of cellulases and xylanases but low levels of β-glucosidase. This differs from that found in the present work to produce cellulases by the two isolates belonging to the genus *Trichoderma*. This fact indicates microbial metabolic diversity enables the identification of different catalytic potentials within the same species.

Generally, the cultivation of fungal decomposers of vegetal biomass in agro-industrial residues results in the production of extracts rich in several enzymes for degradation of the vegetal cell wall (Garcia et al.,

2018). The presence of other enzymes in the fermented medium can increase the cost of obtaining the enzyme of interest, often requiring the addition of protein purification steps, especially when the application of this biocatalyst undergoes interference from other enzymes, resulting in poor quality (Costa et al., 2016).

A typical example of this type of problem is the production of xylanase-rich and cellulase-free enzymatic extracts from the microbial culture in agro-industrial waste for application in the paper and cellulose industries. Xylanases can be used in wood processing to remove lignin by disorganization of the vegetal cell wall caused by hemicellulose degradation. This reduces the amount of chemical compounds necessary for bleaching of the cellulose pulp. The presence of cellulases in the bleaching stage of the cellulose pulp can affect the quality of the cellulose, and hence an enzymatic extract rich in xylanases and free of cellulases is necessary in this step (Carmona et al., 2005; Da-silva et al., 2005). Thus, it is possible to conclude that the enzymatic extracts produced by both microorganisms show potential for the treatment of paper pulp and cellulose, considering the reduced hydrolysis cellulose potential observed (Table 3). The activity of β-D-xylosidase was negligible in both enzymatic extracts (Table 3), indicating the predominance of endoxylanolytic activity (endo-1,4-β-xylanase). This was confirmed by TLC, and the predominance of XOS as a hydrolysis product of xylan was observed (Fig. 5). This characteristic indicates that the xylanases produced in the present study internally cleave the xylan molecule, releasing oligosaccharides of xylose as the main product. Previous studies described the prebiotic function of XOS in the human intestine; they increase and control the proliferation of bifidobacteria and lactobacilli, which is beneficial for the intestine (Lecerf et al., 2012; Menezes and Durrant, 2008).

The proliferation of bifidobacteria and lactobacilli also benefits the health of ruminant animals. The control of the microbiota present in the rumen favors digestion, prevents infectious diseases, and reduces the occurrence of gastrointestinal disorders (Campestrini et al., 2005; Nogueira et al., 2013). This reinforces the potential for application of the enzymatic extracts obtained in the present study as additives in cattle feed.

4. Conclusion

From the results obtained in the present study, we can infer that both microorganisms presented considerable potential for xylanase production at low cost and at low culture times, and the optimization of the culture parameters increased xylanase production by *T. piluliferum* and *T. viride* by approximately 12 and 150%, respectively.

The biochemical characteristics of the xylanases obtained favor the application of these enzymes in ruminant animal diet because they are active in ruminal conditions, as supported by the preliminary *in vitro* digestibility tests that showed a significant increase in the digestibility of foods mainly rich in hemicellulose.

The enzymatic extracts produced in optimized conditions showed potential for application in different industrial segments such as paper and cellulose industries because of the reduced cellulolytic activity, besides the predominant endoxylanolytic activity, which enables their use to obtain XOS.

Conflict of interest

The authors declare no conflicts of interest.

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