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Enzymatic extract *Lentinus tigrinus* and *Trametes villosa* fungi obtained by semi solid fermentation by processing agroindustrial residues for animal feeding

Uso do extrato enzimático dos fungos *Lentinus tigrinus* e *Trametes villosa* obtidos por fermentação semissólida no processamento de resíduos agroindustriais para alimentação animal

Uso del extracto enzimático de los hongos *Lentinus tigrinus* y *Trametes villosa* obtenidos por fermentación semisólida en el procesamiento de productos agroindustriales para la alimentación animal

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Abstract
Ligninolytic enzymes from differents Basidiomycete fungi on different substrates demonstrate microorganisms biotechnological potential on agro-industrial byproducts. The aim of this study was to evaluate the use of enzymatic extracts of *Lentinus tigrinus* and *Trametes villosa* produced by semi-solid fermentation (SSF) for processing sisal coproduct and sugarcane bagasse. The strains were evaluated for RBBR test to confirm enzymes production and subsequently subcultured for 7 days until visible growth of the mycelium. For the SSF, 48 Erlenmeyers distributed in different treatments were used. The enzymatic extract was obtained and added in BC and CDS for further Bromatological analysis. The cultivation methods used were satisfactory in terms growth fungal and ligninase production from RBBR test. The fungi showed good potential in agro-industrial by-products lignocellulosic reduction. The enzyme synthesis behavior of the fungi was different, demonstrating to be in the initial days for *Lentinus tigrinus* and at 14 days for *Trametes villosa.*
Keywords: Basidiomicetos; Ruminant; Lignin; Forage; Cellulose.

Resumo
As enzimas ligninolíticas de diferentes fungos basidiomicetos, em diferentes substratos, demonstram o potencial biotecnológico de microrganismos em subprodutos agroindustriais. O objetivo deste trabalho foi avaliar o uso de extratos enzimáticos de *Lentinus tigrinus* e *Trametes villosa* produzidos por fermentação semissólida (SSF) para o processamento de coprodutos de sisal e bagaço de cana-de-açúcar. As cepas foram avaliadas pelo teste RBBR para confirmar a produção da enzima e subsequentemente subcultivadas por sete dias até o crescimento micelial visível. Para o SSF, foram utilizados 48 frascos *Erlenmeyer*, distribuídos em diferentes tratamentos. O extrato enzimático foi obtido e adicionado ao BC e CDS para posterior análise bromatológica. Os métodos de cultivo utilizados foram satisfatórios em termos de crescimento fúngico e de produção de ligninase, a partir do teste RBBR. Os fungos apresentaram bom potencial na redução lignocelulósica de subprodutos agroindustriais, desenvolveram uma boa síntese de enzimas e seu comportamento foi diferente, sendo demonstrado nos dias iniciais para *Lentinus tigrinus* e aos 14 dias para *Trametes villosa.*
Palavras-chave: Basidiomicetos; Ruminante; Lignina; Forragem; Celulose.

Resumen
Las enzimas ligninolíticas de diferentes hongos basidiomicetos en diferentes sustratos demuestran el potencial biotecnológico de los microorganismos en subproductos agroindustriales. El objetivo de este estudio fue evaluar el uso de extractos enzimáticos de *Lentinus tigrinus* y *Trametes villosa* producidos por fermentación semisólida (SSF)

para el procesamiento de coproductos de sisal y bagazo de caña de azúcar. Las cepas se evaluaron para la prueba RBBR para confirmar la producción de enzimas y posteriormente se subcultivaron durante 7 días hasta el crecimiento visible del micelio. Para el SSF se utilizaron 48 Erlenmeyers distribuidos en diferentes tratamientos. El extracto enzimático se obtuvo y se agregó en BC y CDS para su posterior análisis bromatológico. Los métodos de cultivo utilizados fueron satisfactorios en términos de crecimiento fúngico y producción de ligninasa a partir de la prueba RBBR. Los hongos mostraron buen potencial en la reducción lignocelulósica de subproductos agroindustriales. La síntesis de enzimas el comportamiento de los hongos fue diferente, demostrándose en los días iniciales para *Lentinus. tigrinus* ya los 14 días para *Trametes villosa.*
Palabras clave: Basidiomicetos; Rumiante; Lignina; Forraje; Celulosa.

1. Introduction

Agribusiness has been recognized as a crucial vector of Brazilian economic growth. According to Brasil (2020), agribusiness reached BRL1.55 trillion or 21,4% of Brazil's gross domestic product (GDP). Parallel to this food production scenario, there is the generation of residues and/or agro-industrial co-products. Thus, materials from agroindustry, when treated as waste, can represent a loss of biomass and nutrient, along with increasing the potential for environmental pollutants (Rosa, et al., 2011).

Several authors (Santos et al., 2013; Santos & Silva, 2017) describe that the use of agro-industrial co-products has great potential for animal feeding, and it is possible to incorporate them into the diet of ruminants. However, it is important that these co-products do not present toxicity, harmful compounds and have good nutritional value.

Nationally, the culture of sisal (*Agave sisalana*) in Bahia is a reference (Santos & Silva, 2017). Sisal production process provides important social and economic development, whether in fiber production or its co-products for ruminant feed (Andrade et al., 2009). However, studies evaluating sisal defibration co-products used for animal feeding confirm its high fibrous portion composition, low digestibility and use of its nutrients (Brandão et al., 2011; Santos, 2013; Silva & Beltrão, 1999).

With a similar fiber composition, sugarcane bagasse (*Saccharum sp*) also stands out as a co-product of high economic importance. Sugarcane is the second largest bioenergy crop in the world and represents nearly 80% of the world's sugar production (Yang, 2021). Sugarcane bagasse production is approximately 2.5 thousand tons per day in Brazil (Conab, 2018) and it means a high availability of low-cost cellulosic biomass (Basso et al., 2010).

Cellulose is the main constituent of the cell wall of the plant, having its glucose units joined through a β -1,4-glycosidic bond. To break this bond and release glucose molecules, a group of enzymes is needed (Ghazanfar et al., 2019) that are synthesized by microorganisms during their development. Thus, the Basidiomycete fungi with white-rod decomposition, mainly *Trametes villosa* and *Lentinus tigrinus* stand out as being able to synthesize the enzymes Manganese Peroxidase, Lignin Peroxidase and laccase responsible for lignin degradation (Silva, 2014; Carneiro et al., 2017).

The semisolid fermentation process (SSF) by Basidiomycete fungi is an excellent alternative for the production of ligninolytic enzymes, mainly due to its low production cost. According to Basso et al. (2010), substrate selection is important as it depends on factors mainly related to cost and availability. Likewise, several authors have studied the production of lignases from different Basidiomycetes fungi in different substrates, such as: rice straw (Kang et al., 2004), sugarcane bagasse, coconut husk, sisal fiber (Silva et al., 2014), wheat bran (Camassola & Dillon, 2007), cottonseed cedar (Zahra et al., 2020), jackfruit (Marques et al., 2019) and demonstrated the biotechnological potential of these microorganisms on agro-industrial by-products.

According to Oliveira et al. (2013), the use of co-products in animal feeding, especially in ruminant nutrition, has several advantages over traditional ingredients. Among them, it is possible to mention the lower cost of co-products, potential usage during dry season and lack of pasture, as well as the minimization of environmental impacts with the improper waste

disposal. Thus, enzymatic extracts in *in natura* co-products for animal feed data deserves further studies.

The aim of this study was to evaluate the enzymatic extracts *Lentinus tigrinus* and *Trametes villosa* produced by SSF in the processing sisal defibration co-product (SDC) and sugarcane bagasse (SB).

2. Methodology

The fungi *Lentinus tigrinus* CCMB553 and *Trametes villosa* CCMB561 used belong to the culture collection of the Microbiology Research Laboratory of Feira de Santana State University. The strains were subcultured on agar containing 8g of agar I (HiMedia); 8g of sugarcane bagasse obtained after extracting the juice at a cafeteria nearby; 2g of ammonium sulphate and 400 mL of distilled water, and then incubated at 27º (± 2°C) for 7 days until visible growth of the mycelium to proceed with SSF on SDC and SB substrates.

For the qualitative evaluation of the production of ligninolytic enzymes, a RBBR (Remazol Brilliant Blue R) test was performed. For this, a plug of each reactivated fungus was placed in a Petri dish containing 10g of agar, together with 2.5g of malt extract, 8g of sugarcane bagasse and 1g RBBR dye, mixed in 500 mL of distilled water. The medium solidified with the fungus was submitted to a 27ºC temperature.

For the SSF, 48 erlenmeyers (250 mL) were used as bioreactors (24 for each substrate) distributed in different treatments in triplicate, as shown in Table 1.

Table 1 - Treatments used in triplicate to obtain enzymatic extracts of the fungi *Lentinus tigrinus* and *Trametes villosa* through Semi-Solid-State Fermentation (SSSF).

TREATMENT	FUNGUS	COMPOSITION (in 400 mL)	FERMENTATION TIME
Sugarcane bagasse (SB)	*Lentinus tigrinus*	20 g SB; NH_4SO_4 and 0.01% $MgSO_4$ (w/v) and water	1, 7, 10 and 14 days
	Trametes villosa	2 0 g SB; NH_4SO_4 and 0.01% $MgSO_4$ (w/v) and water	1, 7, 10 and 14 days
Sisal defibration co-product (SDC)	*Lentinus tigrinus*	20 g SDCP; NH_4SO_4 and $MgSO_4$ 0,01% (p/v) and water	1, 7, 10 and 14 days
	Trametes villosa	20 g SDCP; NH_4SO_4 and $MgSO_4$ 0,01% (p/v) and water	1, 7, 10 and 14 days

Source: Self elaboration (2023).

The 48 bioreactors containing the substrates previously ground and enriched with a 1% NH_4SO_4 solution (w/v) and 0.01% $MgSO_4$x7H2O (w/v) and sterile water (50% moisture) were sterilized in a vertical autoclave at 121°C during 15 minutes. As cooling procedure, the material was placed in a biological safety cabinet under the incidence of ultraviolet lights (Vertical Laminar Flow, Filterflux).

The inoculation of five mycelium discs (5 mm in diameter) was carried out on each bioreactor in the conditions: 80% humidity, after correction, pH 9.4 (with the addition of buffer) and incubated at 20˚C according to Silva et al. (2014) recommendations for greater enzyme production. The enzymatic extracts of each treatment were collected after 1, 7, 10 and 14 days (named T1, T2, T3 and T4, respectively).

The enzymatic extract was obtained by maceration of the mycelia at the end of the respective time in cold sterile distilled water, followed by vacuum filtration in a Buchner funnel and centrifugation at 3,000x. The supernatant (enzymatic extract) was preserved in an ice bath for further evaluation of lignocellulolytic activity through the chemical composition of the co-products, carried out before and after treatment with the extracts.

To evaluate the lignocellulolytic activity, enzymatic extracts were added to flasks containing 2g of SB and SDC residues, and homogenized at 150 rpm and 39˚C during eight hours for further Bromatological analysis. The co-products

treated, as well as *in natura* (without the use of extract), were analyzed for neutral detergent fiber (NDF), acid detergent fiber (ADF), cellulose (CEL) and lignin (LIG) contents in accordance with Silva & Queiroz (2002) as well as Hemicellulose (HEM) which was obtained by difference (NDF - ADF).

The two co-products were evaluated separately for fungus strain and fermentation time using a completely randomized experimental design in a 2x4x3 factorial scheme, with two fungi, four fermentation times and three replications for each substrate. Means were compared by the Tukey 5% test using Statistica software (6.0).

3. Results and Discussion

The analysis of the fungi in Remazol Brilliant Blue R (RBBR) medium was positive for both fungi under study with the expected discoloration as shown in Figure 1.

Figure 1 - Discoloration of Remazol Brilliant Blue R (RBBR) medium under the action of the fungi *Trametes villosa* (above) and *Lentinus tigrinus.*

Source: Self elaboration (2023).

The discoloration capacity of the RBBR dye by fungi occurs due to the production of lignolytic enzymes, such as laccase and peroxidases (Conceição, 2010). The RBBR cultivation methodology has revealed to be a simple and quick alternative for the identification of fungi with ligninolitic activity of polymeric dyes, similar to the lignin polymer. These dyes are used as a substrate for the lignin degrading system and they can also determine the start of secondary metabolism in ligninolitic fungi (Pasti & Crowford, 1991).

Teixeira et al. (2016), after analyzing the production of ligninolytic enzymes by fungi in agro-industrial residues, verified that fungi of the genus *Lentinus* were able to discolor RBBR. Costa (2011) verified the total degradation of the RBBR medium by the fungus *Trametes villosa*, confirming the ligninolytic potential of this fungus, also described by Yamanaka et al. (2010) and Oliveira et al. (2010). The data obtained in this study corroborate the results already reported by several authors (Zhou et al., 2013; Varela et al., 2000; Conceição, 2010), which confirms the production and action of ligninolytic enzymes in the studied fungi specimens.

According to Karp et al. (2013) sugarcane bagasse (SB) contains 32-44% cellulose (CEL), 27-32% hemicellulose (HEM) and 19-24% lignin (LIG). Missio (2016) confirmed this variation after compiling several data from this co-product. Brandão et al. (2013) and Santos et al. (2013) evaluating sisal defibration co-product (SDC) reported mean levels of 35% NDF,

26% ADF, 9% HEM and 11% LIG. Thus, the data found in this paper related to fibrous fractions on SB and SDCP in natura corroborates the literature citations, as shown in Table 2.

Table 2 - Valores de fibra em detergente neutro (FDN), fibra em detergente ácido (FDA), celulose (CEL), hemicelulose (HEM) e lignina (LIG) em % da Matéria Seca (MS) do Bagaço de cana (BC) e do Coproduto do desfibramento do Sisal (CDS) *in natura*.

CO-PRODUCT	NDF	ADF	CEL	HEM	LIG
SB	68,8	54,7	32,1	14,2	21,4
SDCP	32,1	23,3	13,4	8,8	9,6

Source: Self elaboration (2023).

From a statistical point of view, there was a significant difference ($P<0.05$) for the NDF values of the treatments compared to in natura co-products (Table 3). This demonstrates that the use of the enzymatic extract allowed the degradation of cell wall components since, according to Silva and Neumann (2012), NDF is basically constituted by cellulose, a portion of nitrogen bound to the fiber, hemicellulose and lignin.

In both co-products, the lowest levels of NDF were found on T1 and T4 times for *Lentinus tigrinus* and *Trametes villosa*, respectively. The greatest fiber degradation in SB observed with *Trametes villosa* extract after 14 days of fermentation corroborates what was found by Silva (2014): after evaluating the action of Manganese Peroxidase (MnP) of this fungus on agro-industrial residues, Silva (2014) observed excellent enzymatic conditions after 15 days of fermentation at 20º C and pH 9.38, conditions established in this study for the evaluated co-products.

Kundu et al. (2005) reported reduction in fiber contents and describe that brown and sweet rot fungi are also able to reduce NDF; however, they use more cellulose and hemicellulose for their growth, leaving behind low digestibility compounds such as lignin. Jung et al. (1992) also noted similar behaviors evaluating five white decaying basidiomycetes grown on oat straw and alfalfa stalks and observed that cell wall polysaccharides were removed from both substrates. Ramirez-Bribiesca et al. (2010) reported that treatment with Pleurotus ostreatus for 15 days on corn husks reduced NDF by 14.5%.

In this study, SDC NDF values were significantly reduced ($P<0.05$) with the *Trametes villosa* extract after 14 days and *Lentinus tigrinus* at the 1st day, suggesting different behaviors between the fermentation processes. It was observed that the results of the extract of *Lentinus tigrinus* after 14 days (T4) were not satisfactory. Silva (2014) mentions that there is a consensus among researchers that the type and composition of the substrate, as well as the established conditions, interfere with the fungus' growth and lignolytic activity. However, the lower NDF values with *Lentinus tigrinus* in T1, 57.5% and 26.0% in SB and SDC respectively, suggests that the behavior of the fungus itself is a greater enzyme production in the first days, demanding more research in this regard.

The feasibility of fibrous co-products in animal feed predicts an increase in NDF digestibility and, consequently, greater availability of plant nutrients (Kotsampasi et al., 2017). However, the degradation of potentially digestible fiber may be related in part to the reduction in the amount of lignin. Thus, lignin incrustations to wall carbohydrates must be considered (Grabber, 2005) and the reduction in NDF values cannot be observed in isolation considering different degradability rates of its constituents in the rumen (Silva et al., 2013). Thus, it is relevant to analyze the enzymatic activities of the extracts as well as to measure the fiber fractions (cellulose, hemicellulose and lignin) in the co-products.

Table 3 - Values on neutral detergent fiber (NDF), acid detergent fiber (ADF), cellulose (CEL), hemicellulose (HEM) and lignin (LIG) in %DM of sugarcane bagasse (SB) and co-product of sugarcane defibration sisal (SDCP) *in natura* and after using the enzymatic extract of *Lentinus tigrinus* and *Trametes villosa* obtained by SSSF after 1, 7, 10 and 14 days.

		Lentinus tigrinus				***Trametes villosa***				
		T1	**T2**	**T3**	**T4**	**T1**	**T2**	**T3**	**T4**	
	SB - *in natura*	**1**	**7**	**10**	**14**	**1**	**7**	**10**	**14**	**CV%**
NDF	68,8a	57,5c	61,8b	61,6b	65,0ab	60,8b	61,3b	61,3b	58,5c	3,6
ADF	54,7a	45,2d	49,9b	49,2b	52,6ab	48,4c	49,1b	49,7b	43,7d	3,8
LIG	21,4a	16,2b	17,2b	17,7b	19,2a	16,5b	16,9b	17,1b	15,2c	7,5
HEM	14,2a	12,3a	11,9a	12,3a	12,3a	12,4a	12,2a	11,6a	14,8a	10,2
	SDCP - *in natura*	**1**	**7**	**10**	**14**	**1**	**7**	**10**	**14**	**CV%**
NDF	32,1a	26,0b	27,9ab	28,1ab	27,8ab	28,2ab	27,9ab	28,0ab	25,2b	5,9
ADF	23,3a	18,3b	20,3ab	20,5ab	20,5ab	20,6ab	20,8ab	20,2ab	17,9b	7,2
LIG	9,6a	6,2b	8,2ab	8,2ab	8,5ab	8,5ab	8,5ab	8,1ab	6,1b	13,9
HEM	8,8a	7,6a	7,1a	7,7a	7,2a	7,7a	7,4a	7,7a	7,4a	12,8

Means followed by different letters on the same line differ from each other (P<0.05) by the Tukey test.
Source: Self elaboration (2023).

Similar behavior to NDF was observed in ADF levels (P<0.05). The first and last days of fermentation for *Lentinus tigrinus* and *Trametes villosa*, respectively, proved to be more interesting with regard to the synthesis of ligninolytic enzymes and, consequently, reduction in ADF. It should be noted that, in accordance with Silva ?& Neumann (2012), ADF contains basically cellulose and lignin portions of the plant cell. That is, the degradation of hemicellulose is responsible for the difference between the NDF and ADF. It is possible to point out that the degradation of HEM occurred in a similar way in both co-products, not differing significantly (P>0.05) from in natura material.

In an extensive review of lignocellulolytic fungi, Andlar et al. (2018), reports that the hydrolysis of hemicellulose requires cooperative action of several types of enzymes working at different levels of the hemicellulite matrix and justifies that this synergistic activity is necessary because of hemicellulose complexity and its connection with the other components of the plant cell wall. In addition, Jaramillo et al. (2015) report that the combination of hemicellulose and lignin forms a protective barrier around the cellulose, which must be modified (or removed) before hydrolysis of the cellulose. However, lignin removal is a fundamental challenge to increase enzyme access to hemicellulose and cellulose. These observations explain the maintenance (P>0.05) of HEM levels after using the enzyme extract in SDC and SB.

Considering co-products usage in animal feeding, as lignin is a compound that has the function of protecting the plant structure against chemical and biological attacks (Gonzalo et al., 2016) and cellulose is more susceptible to encrustation, so it is hemicellulose as the most available polymer for degradation of ruminal bacteria. The cross-disposition of the bonds of the polymers that form hemicellulose gives less resistance to the degradation of this carbohydrate at the ruminal level when compared to cellulose (Van Soest, 1994). This confirms that the use of enzyme extracts from fungi analyzed in co-products allows ADF reduction, an indigestible portion for ruminants, keeping hemicellulose and non-fibrous carbohydrates in food. Thus, it is possible to predict that the extracts used would allow an increase in the ruminal degradability of the treated co-products, suggesting in vitro digestibility techniques for confirmation.

According to Kamra and Zadražil (1988), an improvement in fiber digestibility should be the objective of SSSF when the product is intended for ruminant nutrition. The authors also state that this improvement must be characterized by marked decomposition of lignin and release of nutrients from the lignocellulosic matrix with an accumulation of digestible fractions

and a reduction in saccharide consumption. These reports were confirmed by Mahesh & Mohini (2013) in a long review of biological residue treatments for ruminant feeding. It is noteworthy that, in parallel with the reduction of fibrous fractions, the use of enzymatic extracts can collaborate with the enrichment of the final product with regard to crude protein (Villas-Bôas et al., 2002) due to the addition of enzymes in co-products.

There was a significant difference ($P<0.05$) for LIG values between treatments. As already observed in NDF and ADF contents, the fibrous constituents of the co-products were degraded mainly in T1 and T4, from *Lentinus tigrinus* and *Trametes villosa* extracts, respectively. The lowest LIG levels in SDC were 6.1% (*Trametes villosa* extract in T4) and 6.2% (*Lentinus tigrinus* extract in T1), which means a 32% reduction compared to in natura SDC. These results suggest that a greater enzymatic synthesis (MnP, LiP and Lacase) by *Lentinus tigrinus* may occur at the beginning of its fermentation process and a little later for *Trametes villosa.*

After evaluating ligninolytic enzymes in *Lentinus tigrinus* strains, Zavarzina et al. (2018) identified laccases and MnP at the beginning of the microorganism's development. Isikhuemhen et al. (2012) found the maximum enzyme activity after six days of culture when evaluating the potential of ligninolytic enzymes from the fungus *Lentinus* squarrosulus using SSSF. Singh & Chen (2008) report that MnP and LiP activities in many white decaying fungi appear when an available nutrient source is limited or depleted. Therefore, studies of the enzymatic activity of *Lentinus tigrinus* in SSSF in the first seven days are important and they will be described later.

Concerning LIG values in SB for *Lentinus tigrinus* and *Trametes villosa* at T1 and T4 times, respectively, the results were 16.4% and 14.9%; reaching a reduction of 26.0 (±3) % of this component in waste. Thus, LIG data prove that the greatest reductions in cell wall components are due to degradations of this component in two co-products, especially the *Trametes villosa* extract in T4 of SB. Considering a fermentation time of 14 days, *Lentinus tigrinus* extract allowed the lowest delignification rates in the co-products (below 2%).

When analyzing cottonseed fiber degradation by Pleurotus ostreatus, Li et al. (2001) observed that, after 45 days of incubation, the lignin content decreased from 17% to 11% - a 35% reduction. Already using wheat straw as substrate for Fusarium concolor, Li et al. (2008) noticed a 13.07% removal of lignin after 5 days of incubation. Deswal et al. (2013) detected 7.9% of lignin degradation in sugarcane bagasse and Silva et al (2014) showed that the crude enzyme extract of *Trametes villosa* was able to reduce by 35.0% the lignin content for sugarcane bagasse and 63.1% for sisal fibre. Results obtained on this study are aligned with the average observed in the literature. Although the delignification of the co-products has not been achieved, as reported by other authors, a valuable reduction in the fibrous components and feasibility of using the studied fungi were noticed.

4. Conclusion

The cultivation methods used for *Lentinus tigrinus* and *Trametes villosa* were satisfactory in terms of fungi development and ligninase production confirmed in RBBR test. *Lentinus tigrinus* and *Trametes villosa* proved to be important microorganisms for use in semi-solid state fermentation and lignocellulosic material reduction in agro-industrial by-products. The enzymatic synthesis behavior was different for each fungus, showing to occur on the initial days for *Lentinus tigrinus* and on the 14th day for *Trametes villosa*. Additional in vitro digestibility tests of materials delignified with enzymatic extracts of *Lentinus tigrinus* are suggested as well as the enzymatic quantification of these extract, since the enzymatic synthesis time has shown to be faster with *Lentinus tigrinus.*

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