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Cellulase activity of filamentous fungi induced by rice husk

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The objective of this study was to determine the potential of different filamentous fungi to degrade cellulose in rice husk pre-treated with steam explosion or alkaline hydrolysis. A preliminary test performed with carboxymethyl cellulose and nine fungi (*Trichoderma* 1, 2, 3, 4, 5; *Trichoderma reesei*; *Aspergillus niger*; *Rhizopus oryzae* and an isolated fungus from rice husk) allowed the selection of the fungi that can degrade cellulose the most. Subsequently, the fastest growing fungi on the substrate (carboxymethyl cellulose) were subjected to a fermentation bioreactor (18 mL of the fungus with 2 mL of conidial suspension at a concentration of 5 x 10^6 conidia mL^{-1}). Their potential to degrade cellulose was determined. This was done by measuring the amount of total carbohydrate and reducing sugars using the anthrone method and 3,5 dinitrosalicylic acid respectively. On the other hand, the endoglucanase, exoglucanase and β-glucosidase activity of the two most promising fungus (*Trichoderma* sp. 1 and *Aspergillus* sp.) was evaluated. Statistical analysis revealed no significant differences; however, the rice husk pre-treated with steam explosion before the fungal strains had the greatest amount of total carbohydrates; it produces 2.9 and 1.4 times more than those not treated with alkaline hydrolysis. Moreover, fungi *Trichoderma* sp. 1 and *Aspergillus* sp. had higher number of total released carbohydrate and reducing respectively, which demonstrated the difference in the enzyme system of the two microorganisms. Endoglucanase and exoglucanase activities had similar performance for *Aspergillus* sp., and *Trichoderma* sp. 1, during the 288 h of the test. Likewise, β-glucosidase activity was similar. After 192 h, values of 0.150 and 0.140 IU mL^{-1} were obtained for *Aspergillus* sp. and *Trichoderma* sp. 1, respectively. Finally, the applicability of rice husk in agribusiness as a raw material for subsequent fermentation and for obtaining added-value compounds is shown.

Key words: Enzymatic activity, rice husk, fermentable sugar, agroindustrial wastes, filamentous fungi.

INTRODUCTION

The use of agro-industrial byproducts as raw materials for the production of high added-value products such as bio-fuels, compost, xylitol, enzymes and compounds for human and animal consumption, among others, has become increasingly important (Sánchez, 2009). In Colombia, rice farming is a major component of the agricultural sector, with a semiannual output of 1,376,385 t (Dane-Fedearroz, 2013), from which about 50% of rice husk can be obtained (Ahumada and Rodríguez-Páez, 2006). Due to its recalci-trant structure (Yu et al., 2009), abrasive nature, low nutri-

tional value and high ash content (Jurado et al., 2003), this residue has limited use. Additionally, its incineration is questioned, given the high environmental costs of its combustion (Camassola and Dillon, 2009).

Different physical and chemical treatments are used to transform cellulosic wastes (Sun and Cheng, 2002). As a clean alternative, the industry uses enzymes that convert the constituent polymers of the plant cell wall (lignin, cellulose and hemicellulose) into simple sugars (Pérez et al., 2002), but the high cost of these processes is an obstacle for their usage (Biswas et al., 2006). Consequently, the use of microorganisms is gaining relevance because of their ability to degrade polymers such as cellulose and starch which are the major constituents of plant biomass (Ramírez and Coha, 2003). Moreover, it is important to highlight the role of microorganisms in the degradation of agro-products, for two main reasons: 1) the cost of producing the enzymes for the process is 50% (Galbe and Zacchi, 2002), and 2) the decrease in the inhibitory effect on fermentation processes caused by the preservatives and stabilizers that accompany the use of commercial enzymes (Fujita et al., 2004; Golias et al., 2000).

Different strains of fungi are used in agro-industrial waste degradation, especially those that have exhibited activity on cellulosic substrates. The *Trichoderma* genus was analyzed because of its ability to produce high cellulolytic enzymes activity (Miettinen-Oinonen and Suominen, 2002), that allows the transformation of plant cell-wall constituents or wastes, such as husk, into simple sugars that may become alcohols after the fermentation process. This leads to the conservation of non-renewable resources (Valverde et al., 2007). Therefore, ethanol production becomes relevant, given the possibility of producing 0.25 L of 96°GL alcohol per Kg of husk, which, according to the per liter price Colombia (USD 0.91), could represent an additional income source for producers (Rojas and Cabanillas, 2008). The use of *Penicillium echinulatum* on sugarcane bagasse yields 1.60, 0.21 and 1.49 U mL^{-1} for endoglucanase, β-glucosidase and xylanase, respectively; for control cellulose, values of 1.20, 0.20 and 1.46 U mL^{-1}were obtained (Camassola and Dillon, 2009). Also, *Aspergillus niger* cellulases, cross linked by glutaraldehyde, maintain their degrading activity during a longer period of time, and hence, further degradation of rice husk at lower cost can be obtained (Sohail et al., 2009).

Therefore, the search for native microorganisms from substrates could be an alternative for obtaining fungal strains with high potential for a cleaner conversion of lignocellulosic materials, and the use of physical and chemical pretreatments will generate cleaner, cheaper processes and without demanding specialized infrastructure (Llacza and Castellanos, 2012; Martínez-Anaya et al., 2008). In this regard, the objective of this study is to compare the cellulolytic activity of fungal reference strains against those isolated from rice husk, identifying the potential of converting this residue into fermentable sugars.

MATERIALS AND METHODS

Plant material

Rice husk was obtained in rice mills located in El Espinal - Tolima Department, Colombia, during the second half of 2011 and was subsequently treated in an electric mill to obtain a size of 1-2 mm. Then, a bromatologica was performed to determine humidity, crude fiber, ether extract, cinder, protein, nitrogen, potassium, phosphorus, cooper, zinc, iron, manganese, bore, sulfur, sodium, calcium, and magnesium was done using the methods of AOAC (2012). Analysis was performed in order to determine the percentages of cellulose, hemicellulose, lignin and some oligoelements that could influence fungal growth and cellulase activity.

Biological material

Fungi isolation and identification

Untreated samples (rice husk) were introduced into sterile Petri dishes with potato dextrose agar (PDA, Oxoid) and incubated 8 days at 25°C to allow the growth of microorganisms. Later, subcultures were made in order to separate and individualize each fungus. Preliminary identification was performed on a microscope (Advanced Optical, Model XS-402) after staining the fungi with blue-lactophenol; and through taxonomic keys, genera identification was possible.

Preliminary evaluation of cellulolytic capacity

With some modifications, the methodology proposed by Mikán and Castellanos-Suárez (2004) was used. Strains of *Rhizopus oryzae*, *Aspergillus niger*, *Trichoderma reesei* and *Trichoderma* sp. (five strains) were obtained from the microbiology laboratory of the Research Group of Natural Products of University of Tolima – Colombia. They were identified as follows: T.1, T.2, T.3, T.4, and T.5 and determined for their cellulolytic potential. Also, a strain isolated from rice husk was used. These fungi were placed into a solid culture medium that contained agar-agar and CMC (1 and 2% w/v). Inoculation was performed by placing in the CMC agar center a 5 mm diameter disk of potato dextrose agar (PDA, Oxoid) that was previously inoculated with fungal mycelium. Growth kinetics measurement was performed by triplicate, incubating the microorganisms at 25°C, until the growth of the control samples was observed in the entire 9 mm Petri dish. The degradative activity was manifested through the presence of yellow or unstained areas after the application of Congo red solution (Merck).

Pre-treatment

Steam explosion (SE)

The methodology proposed by Sun and Cheng (2002) was used,

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Table 1. Experimental design and treatments.

Pre-treatment	Fungi		
	***Trichoderma* sp.**	***Aspergillus* sp.**	***Rhizopus oryzae***
SE	T.SE	A.SE	R.SE
LIME	T.LIME	A.LIME	R.LIME
BLANCK	T.WT	A.WT	R.WT

SE: Steam explosion; LIME: Alkaline hydrolysis; Blank: rise husk without pre-treatment.

with some modifications. The lignocellulosic material (rice husk) was treated with high-pressure saturated steam and then the pressure was swiftly reduced. The process was performed under autoclave conditions (120°C, 15 psi) during an interval of 45 and 60 min.

Alkaline hydrolysis (LIME)

For this assay, the methodology described by Sun and Cheng (2002) was used. 100 g of the rice husk was treated with saturated solution of calcium hydroxide diluted (2 L) in 1:20 ratio, at 60°C for 24 h. Finally, rice husk was washed with distilled water three times.

Fermentation

The material was exposed to a fermentation that included a pre-treatment (SE or LIME) coupled with the subsequent degradation of one of the fungal strains used. The total number of treatments was 6, with 3 replicates for each one, wherein blank was included (rise husk without pre-treatment).

The fermentation process of 3 fungi [*Trichoderma* sp., *Aspergillus* sp. (isolated from rice husk) and *Rhizopus oryzae*] with the best performance from the CMC assay was developed in bioreactors of 500 mL, containing 10 g of husk, 18 mL of sterile water and 2 mL of spore suspension ($5x10^6$ conidia mL^{-1}). Finally, the pH was adjusted to 6.5 with 0.1 N HCl and 0.1 N NaOH, and the solution was incubated at room temperature (25°C) with constant stirring (150 rpm) for 30 days. Finally, the leachate samples from the bioreactors were taken every 7 days for a month, whereupon they were vacuum filtered in order to quantify total and reducing sugars.

Quantification of carbohydrates

Total carbohydrates were quantified by a spectrophotometer (UV-V Thermo Scientific Helios Gamma UVG154501 model), using the anthrone method described by Witham et al. (1971). Moreover, reducing sugars were quantified by the 3.5-dinitrosalicylic method, described by Miller (1959). Calibration curves were made from 10 to 100 µg mL^{-1} for DNS method and 120-2000 µg mL^{-1} for anthrone method, and validated according to Quattrocchi et al. (1992).

Cellulose activity

Endoglucanase activity

The methodology used for this purpose was the one proposed by Gunjikar et al. (2001) and Berghem and Pettersson (1973) . A CMC solution (1%) was prepared in sodium acetate buffer (0.05 M, pH 5) and one (1) mL of this solution was incubated with 0.28 mL of the enzyme solution (leachate filter) and assayed at 50°C for 30 min. After reaction completion, DNS reagent (1%) was added. The reducing sugars concentration produced by the enzyme reaction was measured according to the equation proposed by Eveleigh et al. (2009) and Gunjikar et al. (2001): Endoglucanase activity (U mL^{-1}) = reducing sugars released (mg) x 0.66.

Exoglucanase activity

In this assay, the methodology used was the one proposed by Gunjikar et al. (2001) and Berghem and Pettersson (1973) . One (1) mL of tested enzyme solution (leachate filter) was added to 50 mg of filter paper previously dipped in Buffer sodium acetate (0.05 M, pH 5). After 30 min of incubation at 40°C, DNS reagent (1%) was added and the reducing sugar concentration was measured. Exoglucanase activity was calculated according to the equation proposed by Afolabi (1997): Exoglucanase activity (U ml^{-1}) = reducing sugars released (mg) x 0.185.

β-Glucosidase activity (cellobiose)

The methodology used was the one proposed by Klesov (1981). Three test tubes were used: the first blank tube contained 1 mL of each solution (cellobiose 15 mM, citrate buffer at pH 4.8 and water), the second blank tube contained 1 mL of the sample (filter leachate) and 2 mL of water, and the third tube contained 1 mL of cellobiose solution, buffer and test sample. All tubes were mixed and incubated at 50°C for 30 min. DNS reagent (1%) was added and the reducing sugars concentration (glucose) was measured by the DNS method. The concentration measurement was obtained by subtracting the absorbance sample from that of the sample blank and cellobiose blank. The β-glucosidase activity was determined according to the equation of Afolabi (1997): β -glucosidase activity (U mL^{-1}) = Glucose liberation (mg) x 0.0926.

All tests were made with leachates extracted from a submerged culture assay as described above. But in this case only *Trichoderma* sp.1 and Aspergillus sp. were used; moreover, a kinetics analysis was performed every 48 h reaching 196 h.

Statistical analysis

All variables were subjected to a Kolmogorov-Smirnov test, in order to obtain a normal data distribution. Then a one-way variance analysis (ANOVA) and a LSD test ($p \leq 0.05$) were made using the Info Stat program (free version) (Di Rienzo et al., 2011). Treatments abbreviations are described in Table 1, which were employed in subsequent graphs.

RESULTS AND DISCUSSION

As a result of the bromatological test applied, percentages of cellulose, hemicellulose and lignin were determined

Table 2. Bromatological test results from rice husk.

Parameter	Value
Cellulose (%)	37.63
Hemicellulose (%)	10.23
Lignin (%)	12.5
Humidity (%)	11
Cinder (%)	19
Crude Protein (%)	1.7
Ether extract (%)	2.6
Brute protein (%)	34
Nitrogen (%)	0.27
Potassium (%K)	0.31
Phosphorus (%P)	0.56
Cooper (mg Kg^{-1} Cu)	1.2
Zinc (mg Kg^{-1} Zn)	18
Iron (mg Kg^{-1} Fe)	12
Manganese (mg Kg^{-1} Mn)	39
Bore	ND
Sulfur (%S)	0.20
Sodium (mg Kg^{-1} NA)	46

(Table 2). These results were used in the calculation of the material conversion into total carbohydrates and reducing sugars. These findings were compared with reports from other authors regarding the same waste (rice husk), and similar results to those reported were obtained by Sánchez (2009) and Valverde et al. (2007).

The ash (19%) indicated the presence of minerals, such as manganese (39 mg kg^{-1}), iron (12 mg kg^{-1}) and zinc (18 mg kg^{-1}). Likewise, other minerals were found, but in smaller proportions. It is noteworthy that some of the minerals (manganese, iron and zinc) are part of the most widely culture media used in cellulose degradation studies.

Growth kinetics

Some of the fungal strains (*R. oryzae, T. reesei* and *Trichoderma* 1, 2, 3, 4, 5) were present in the microbiology laboratory and *Aspergillus* sp. was recovered from waste. Growth assay on one material cellulosic like CMC allowed the identification of the cellulolytic activity from the strains used as shown in Figure 1. This allowed the identification of *R. oryzae*, *Aspergillus* sp. and *Trichoderma* sp.1 as the ones with the highest speed growth. Husk degradation tests were done with those strains. The fungus *R. oryzae* filled Petri dish in just 48 h, probably for its capacity to grow in different substrates.

Quantification of carbohydrates

The statistical analysis showed that there is no significant difference between the applied pretreatments; however, the best performance was the one showed by steam explosion. This treatment released 878.26 µg of total carbohydrates, generating 2.9 (304.44 µg) and 1.4 (643.44 µg) more than those released from the treated (LIME) and untreated husk, respectively. Regarding reducing sugars, the untreated material was the top performer: it released 509.56 µg, generating 1.5 (343.15 µg) and 1.3 (387.49 µg) more than those released with the LIME and steam explosion pre-treatments respectively (Figure 2).

As shown in Figures 2 and 3, the steam explosion pretreatment favored carbohydrate release. Probably this effect is due to the physical and chemical changes that may occur in this process, such as depolymerisation and breakage of fiber and links with the subsequent release of oligosaccharides; processes that have been previously described by Sun and Cheng (2002). Nonetheless, the performance of reducing sugar release was significantly lower, probably due to other factors such as substrate fungal colonization and their enzymatic efficiency.

Likewise, between the two most efficient fungi (*Aspergillus* sp., and *Trichoderma* sp.1) statistically significant differences were observed. *Aspergillus* sp. released more reducing sugars and *Trichoderma* sp. 1 produced the largest amount of total carbohydrates (probably related to the β-glucosidases production, responsible for monomeric sugars release). This performance was also observed in *Trichoderma reesei* strains as previously reported by Saloheimo et al. (2007) and Lynd et al. (2002). This will be clarified later in the enzymatic activity discussion.

Figures 4 and 5 show the system performance during each week. In Figure 4, high total carbohydrates release can be observed (1489.41 µg mL^{-1} of total carbohydrates from which 610.83 µg mL-1 correspond to reducing sugars). However, that release decreased with time. This phenomenon has also been observed by other authors, who have highlighted that it is due to several factors, such as fungal demand for taking some of the produced sugars to continue their metabolism (Taniguchi et al., 2005), the absorption of enzymes by cellulose and lignin (Garibello and Melissa, 2013), or the enzymatic activity inhibition due to glucose and cellobiose presence (produced by cellulases) in the medium (Qing et al., 2010).

Finally, at week 4 of the treatment, the best conversion ratio, starting with 10 g of husk, was that *Aspergillus* sp. had a transformation percentage of 21.06%. There was a sharp difference in the production of total carbohydrates and reducing sugars, which allowed the choosing of *Trichoderma* sp. 1, and *Aspergillus* sp., as the two microorganisms with the best performance. The cellulase activity was evaluated in order to differentiate their ability to degrade the material.

Cellulase activity

Endoglucanase activity Strains of Aspergillus sp. and Trichoderma sp. 1 showed similar performance during

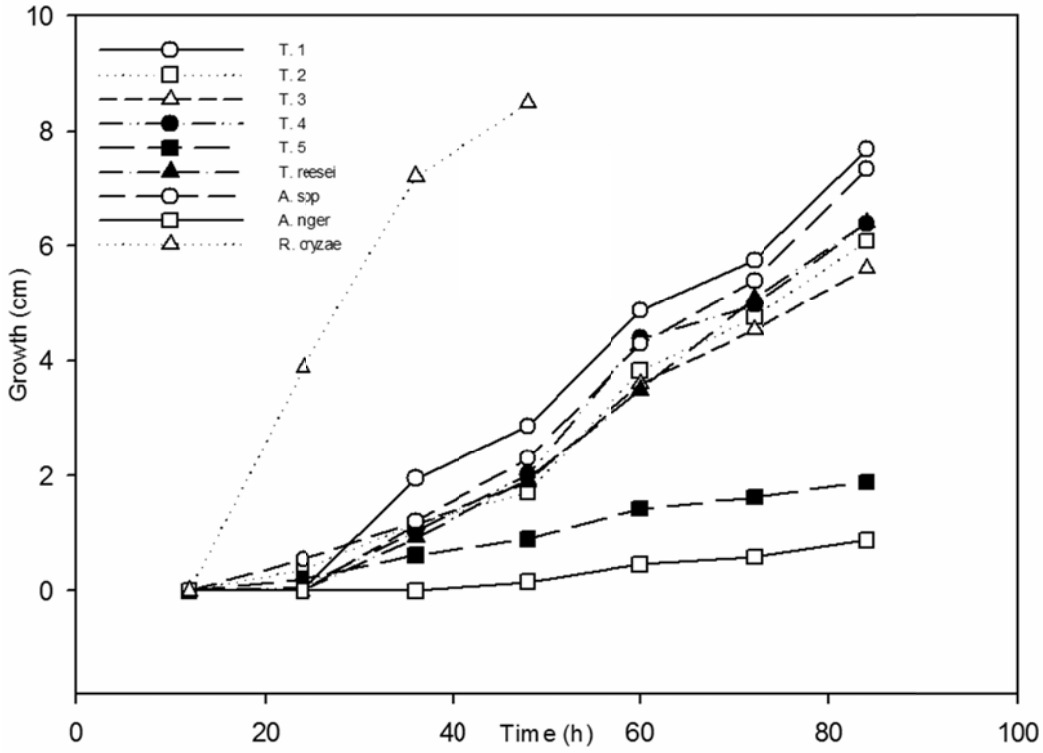

Figure 1. Growth kinetics from strains used in agar CMC (2%). T. 1, *Trichoderma* sp 1; T. 2, *Trichoderma* sp 2; T. 3, *Trichoderma* sp 3; T. 4, *Trichoderma* sp 4; T. 5, *Trichoderma* sp 5; T. reseei, *Trichoderma reesei*; A. sp (Asl), *Aspergillus* sp; A. niger, *Aspergillus niger*; *R. oryzae*, *Rhizopus oryzae*.

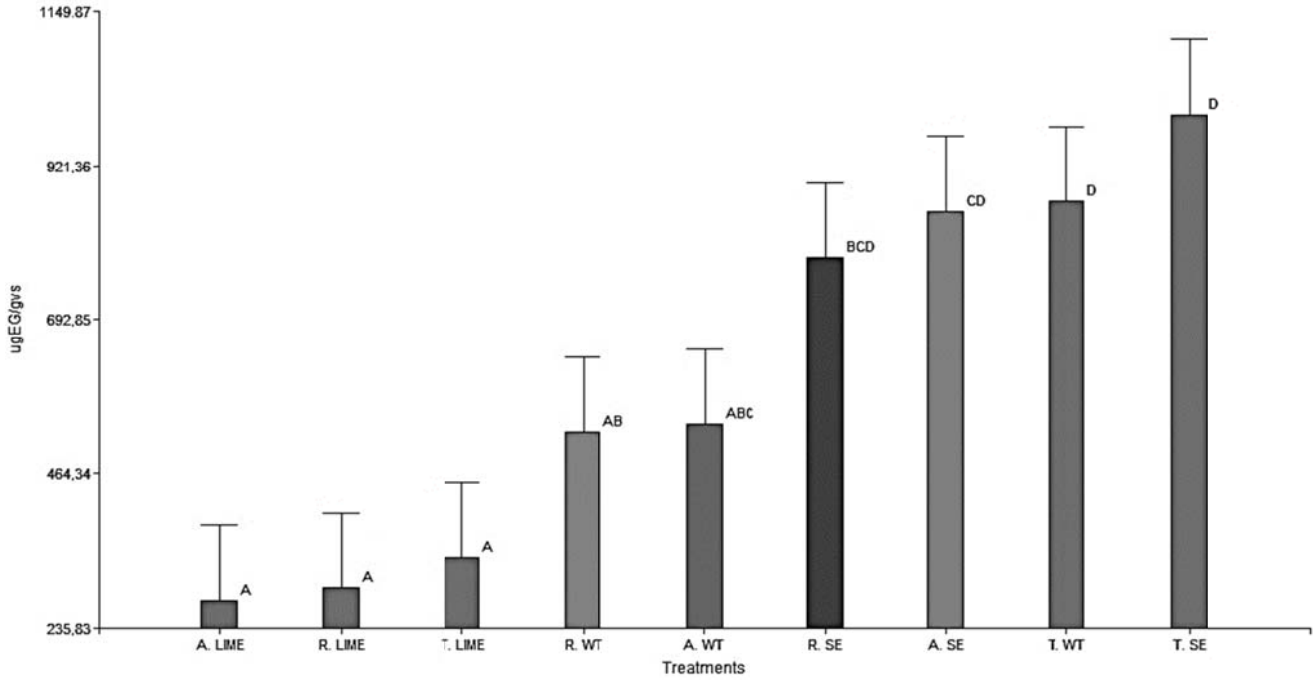

Figure 2. Least significant difference (LSD) of treatments. .LIME, Rhizopus-LIME; A.LIME, Aspergillus-LIME; T.LIME, Trichoderma-LIME; R.WT, Rhizopus-Without treatment; A.WT, Aspergillus-Without treatment; T.WT, Trichoderma-Without treatment; R.SE, Rhizopus-steam explosion; A.SE, Aspergillus steam-explosion; T.SE, Trichoderma steam-explosion.

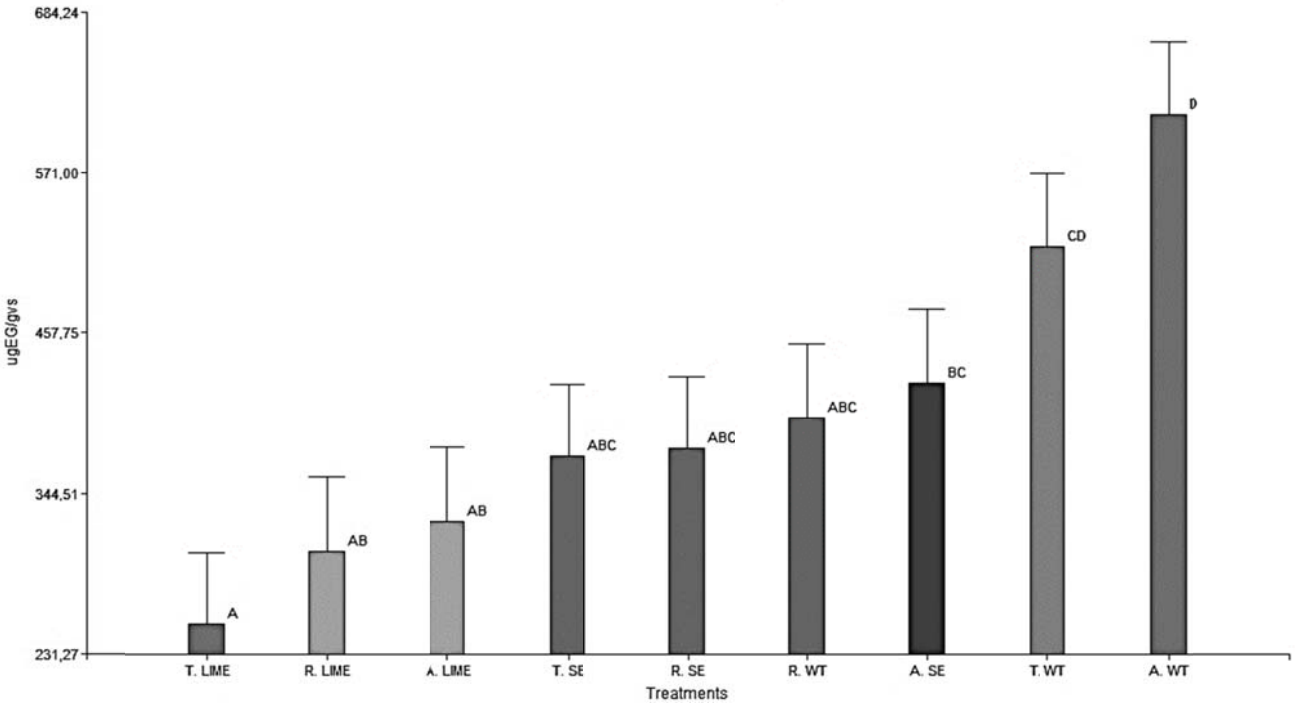

Figure 3. Least significant difference (LSD) treatments. T.LIME (Trichoderma-LIME), R.LIME (Rhizopus-LIME), A.LIME (Aspergillus-LIME), T.SE (Trichoderma steam-explosion), R.SE (Rhizopus-steam explosion), R.WT (Rhizopus-Without treatment), A.SE (Aspergillus steam-explosion), T.WT (Trichoderma-Without treatment), A.WT (Aspergillus-Without treatment) in the release of reducing sugars.

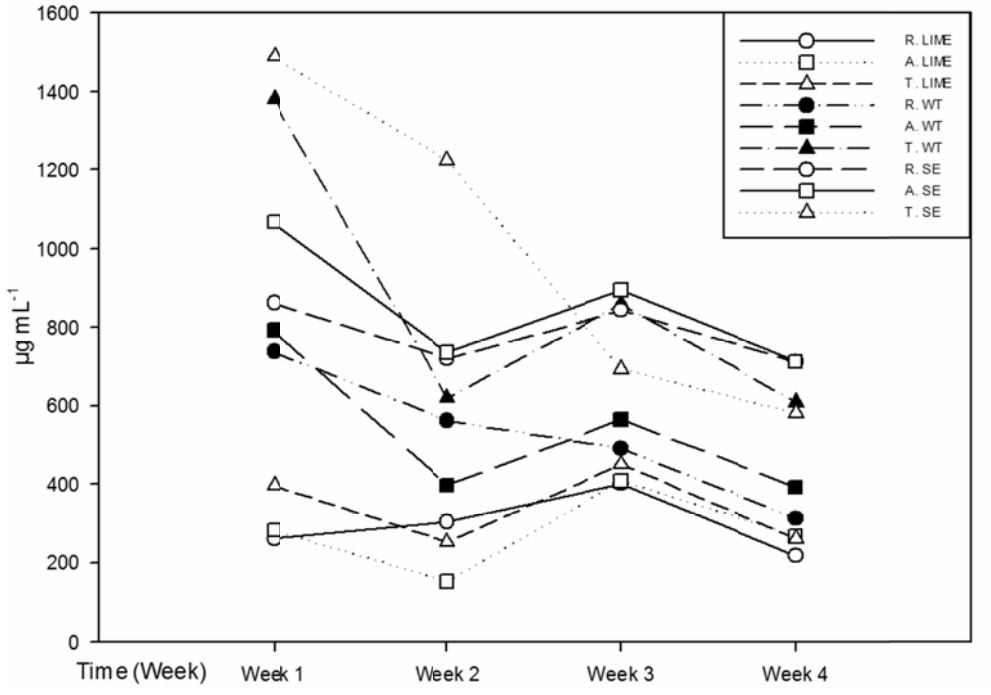

Figure 4. Kinetics of total carbohydrates released by the treatment used. R.LIME, Rhizopus-LIME; A.LIME, Aspergillus-LIME; T.LIME, Trichoderma-LIME; R.WT, Rhizopus-Without treatment; A.WT, Aspergillus-Without treatment; T.WT, Trichoderma-Without treatment; R.SE, Rhizopus-steam explosion; A.SE, Aspergillus steam-explosion; T.SE, Trichoderma steam-explosion.

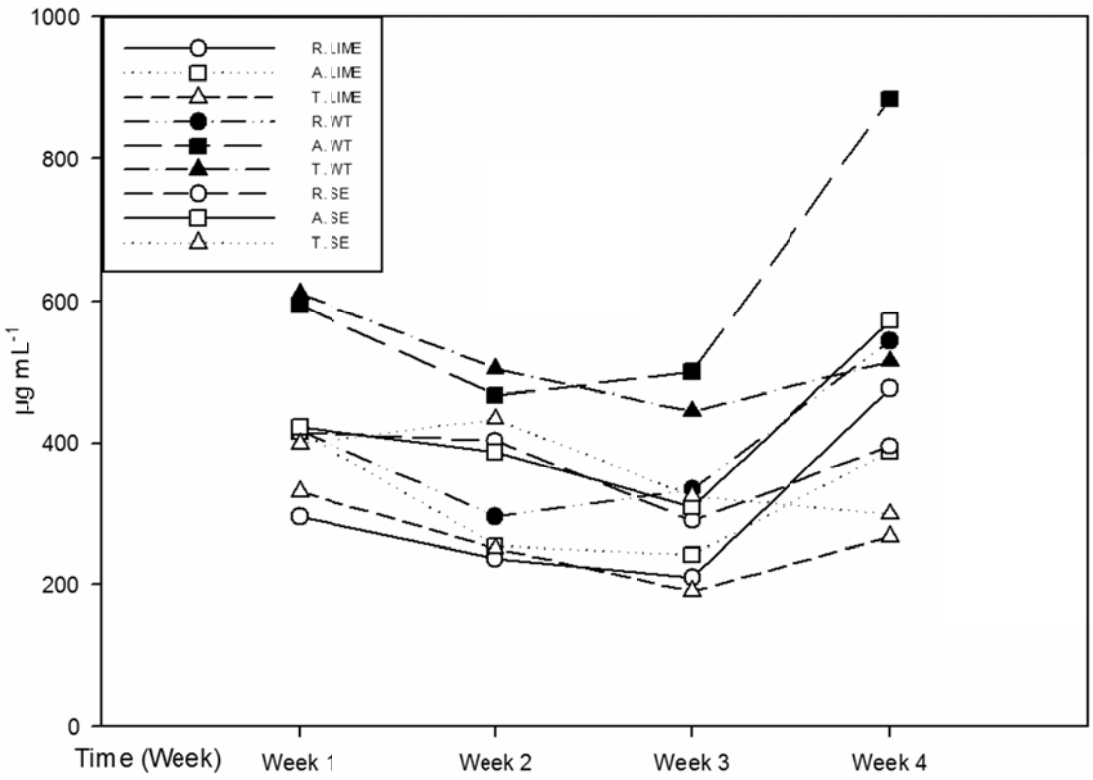

Figure 5. Kinetics of reducing sugars released by the treatment used. R.LIME, Rhizopus-LIME; A.LIME, Aspergillus-LIME; T.LIME, Trichoderma-LIME; R.WT, Rhizopus-Without treatment; A.WT, Aspergillus-Without treatment; T.WT, Trichoderma-Without treatment; R.SE, Rhizopus-steam explosion; A.SE, Aspergillus steam-explosion; T.SE, Trichoderma steam-explosion.

monitoring, with a unique difference at 240 h wherein *Trichoderma* sp.1 showed higher endoglucanase activity. This result contrasts with previous reports, which indicate that the activity of *Aspergillus* genus has been greater than that of *Trichoderma*. In the present study, at day 10, Trichoderma sp.1 showed the highest activity, with 0.350 IU mL^{-1} followed by *Aspergillus* sp. with a production of 0.225 IU mL^{-1}, as shown in Figure 6. Furthermore, the fungal endoglucanases from the strains assessed, proved to have a production comparable with others enzymes from different investigations performed on different substrates (Ahamed and Vermette, 2010).

Exoglucanase activity

The exoglucanase activity showed no significant differences among the used fungi (Figure 7), emphasizing that the activity is the same on this substrate. However, different performances are observed in the literature when compared strains from the same genus are placed on filter paper substrates (Fang et al., 2010). This indicates the importance of studying the performance of several strains on different substrates and under different culture conditions.

β-Glucosidase activity

Regarding this activity, there was a similarity between *Aspergillus* sp. and *Trichoderma* sp.1 with maximum values of 0.150 and 0.140 IU mL^{-1} at 192 and 288 h, respectively (Figure 8). The β-glucosidase activity in *Trichoderma* sp.1 was lower than the one of *Aspergillus* sp.. This is contrary to the reports of Manjarrés et al. (2011), Fang et al. (2010) and Flachner et al. (1999), wherein an inverse performance is pointed, compared with the one found in the present study. Finally, the enzymatic assay allowed the relating of the endoglucanases production, the greater release of total carbohydrates in *Trichoderma* sp. 1 as well as the greater production of reducing sugars and β-glucosidase in *Aspergillus* sp.

Conclusions

The study presented here showed the efficiency of using filamentous fungi for splitting rice husk. It allows the production of significant amounts of fermentable sugars, which can be subsequently used to produce various added-value compounds, including ethanol. Native fungal

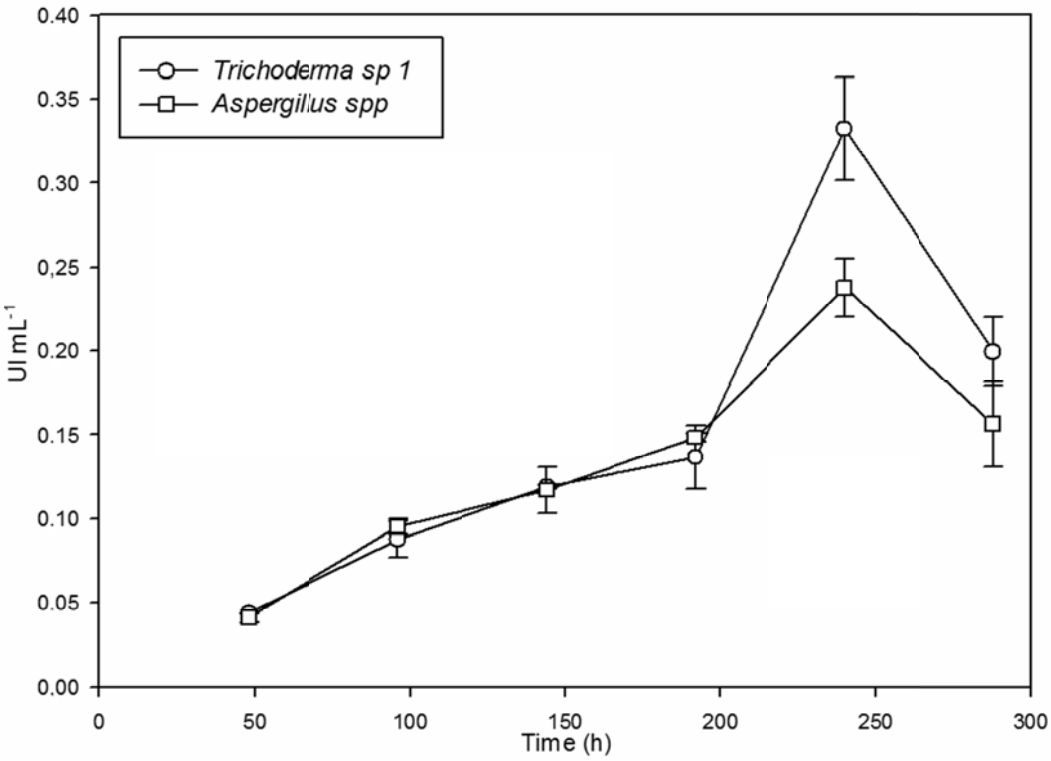

Figure 6. Enzyme kinetics of two fungal endoglucanase activity with increased release of total carbohydrate and reducing sugars (*Trichoderma* sp 1 and *Aspergillus* spp).

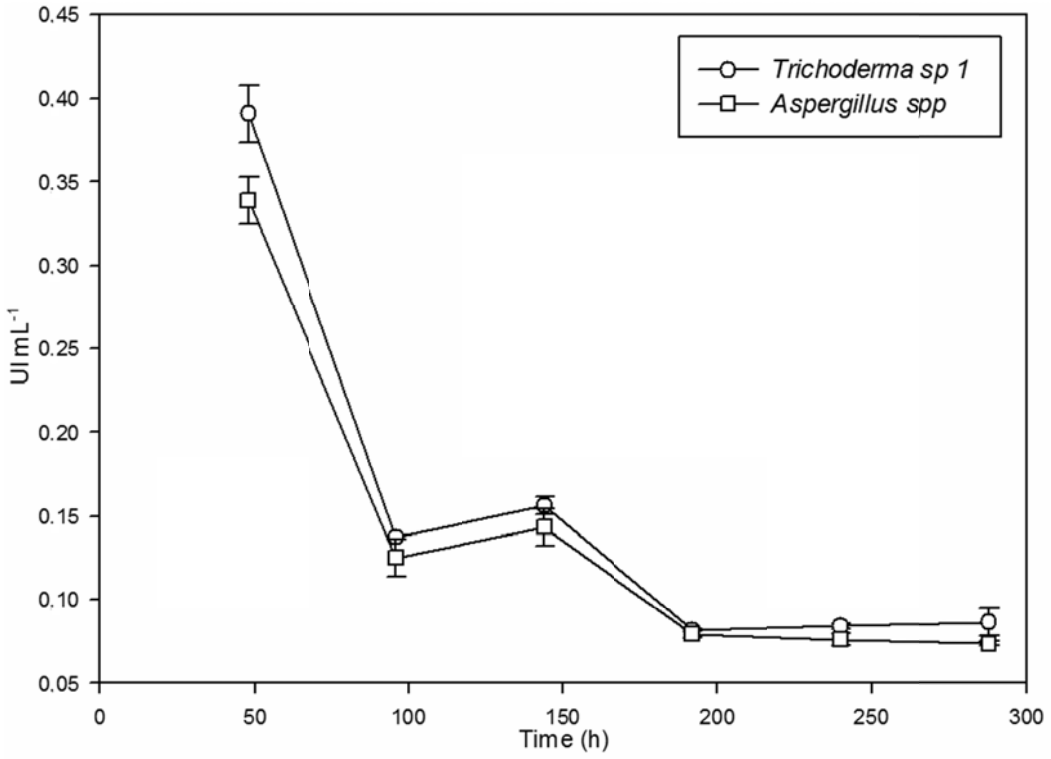

Figure 7. Enzyme kinetics of two fungal exoglucanase activity with increased release of total carbohydrate and reducing sugars (*Trichoderma* sp 1 and *Aspergillus* spp).

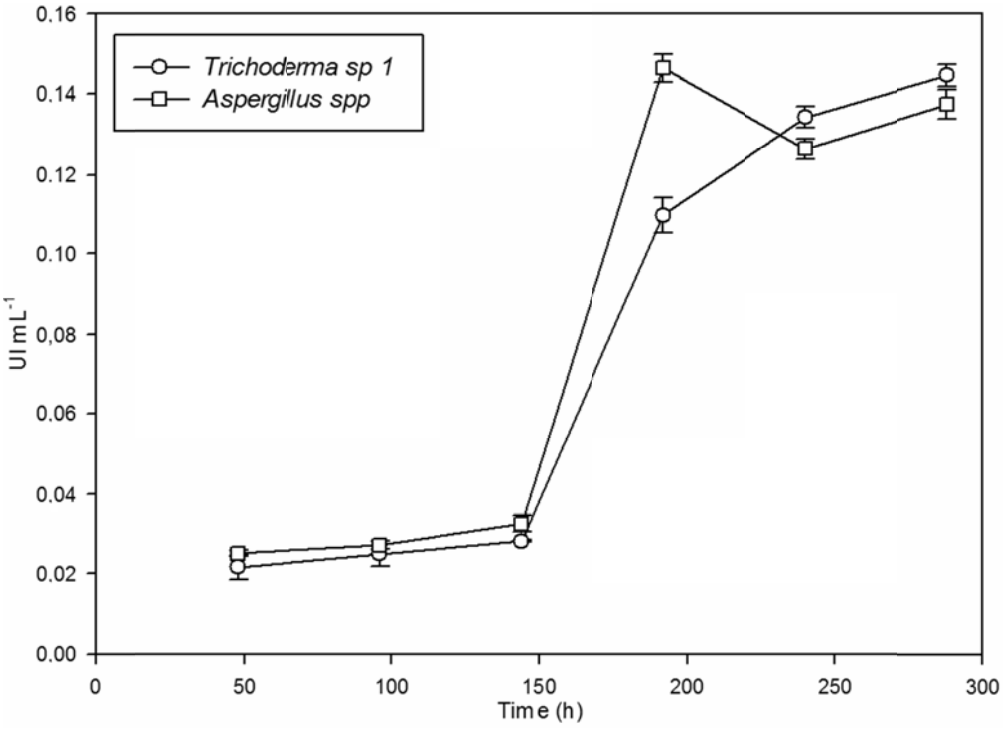

Figure 8. Enzyme kinetics of two fungal β-glucosidase activity with increased release of total carbohydrate and reducing sugars (*Trichoderma* sp 1 and *Aspergillus* spp).

strains from husk, such as *Aspergillus* sp., offer a potential comparable with that of fungi widely used for similar purposes, and hence, may be used in the cellulosic materials degradation processes.

Conflict of Interests

The author(s) have not declared any conflict of interest.

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