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ABSTRACT

Xylanases hydrolyse efficiently the xylan component of hemicelluloses into single xylose monomers. Hence, these enzymes are suitable to be applied in the bioconversion of ligno-cellulosic wastes such as sugarcane bagasse to improve the bioethanol process. Misiones rainforest in the northeast of Argentina has a huge biodiversity which becomes the most likely site to screen new fungal isolates for powerful xylanolytic enzymes. The aim of this work was to find efficient xylanolytic *Aspergillus* with low levels of proteolytic enzymes through the isolation and exploration of native fungi from Misiones. Fungi were isolated from different locations of this subtropical region and 25 *Aspergillus* isolates were screened based on their ability to produce xylanolytic and proteolytic enzymes. From the principal component and conglomerate analysis, six groups were discriminated. The most promising fungi presented the highest endoxylanase and β-xylosidase activities and the lowest proteo-lytic activities. These isolates were *A. niger* LBM 055 and *A. niger* LBM 134 which reached 29 and 27 UmL^{-1} of endoxylanases, respectively; 0.6 UmL^{-1} of β-xylosidases, no casein and very little gelatin proteases. This study proves that the search of new fungi from nature is an important strategy to find novel isolates with the ability to secrete high-performance xylanases and exploit these enzymes in the bioconversion of lignocellulosic wastes.

1. Introduction

Xylan is the major component of hemicelluloses and like cellulose is the most abundant polysaccharide in nature (Menon & Datta, 2017). Xylan and cellulose are the principal wastes from the agroforestry indus-try, providing an abundant source of carbohydrates (Bhattacharya, Bhattacharya, & Pletschke, 2015). Among residues, sugarcane bagasse (SCB) is one of the major agricultural wastes found in great quanti-ties, especially in tropical countries. The principal advantages of SCB are the low ash content (Cardona, Quintero, & Paz, 2010) and the high hemi-cellulose content, 30–35%, mostly xylan (Brienzo, Carvalho, de Figueiredo, & Oliva Neto, 2016).

A promising strategy for the use of SCB is its enzymatic hydrolysis to obtain reducing sugars that can be fermented to second-generation bioethanol (Menon & Datta, 2017). Two principal difficulties in the bioethanol process are the pretreatment and the enzymatic hydrolysis of the lignocellulosic material. The challenge of the pretreatment is to efficiently remove lignin and hemicelluloses to polysaccharides

accessible for the second step, the enzymatic hydrolysis. The challenge of the enzymatic hydrolysis is to find new enzymes able to compete with the commercial enzymes in a cost-efficient manner (Hu, Arantes, & Saddler, 2011; Verardi, De Bari, Ricca, & Calabrò, 2012).

In this sense, microbial enzymes can hydrolyze the xylan, making the lignin removal easier. These enzymes have different modes of action that syner-gistically act to degrade the xylan to xylose mono-mers. This group of enzymes includes endo-1,4-β-xylanase or endoxylanase (EC 3.2.1.8), xylan-1,4-β-xylosidase or β-xylosidase (EC 3.2.1.37), α-glucuro-nidase (EC 3.2.1.139), α-arabinofuranosidase (EC 3.2.1.55) and acetylxylan esterase (EC 3.1.1.72). Among them, endoxylanases and β-xylosidases, col-lectively named as xylanases, are the key enzymes responsible for the total hydrolysis of xylan (Burlacu, Cornea, & Israel-Roming, 2016; Menon & Datta, 2017). Endoxylanases hydrolyze the 1,4-β-linkages on the xylan backbone producing xylo-oligomers which are then degraded to xylose units by the β-xylosi-dases (Juturu & Wu, 2012).

Table 1. *Aspergillus* isolates from Misiones rainforest used in this study.

Isolate collection	Sample	Location	Coordinate position	References
Aspergillus sp. LBM 041	*Carica papaya* fruit	Posadas	Latitude 27°26′04″ S/ longitude 55°53′14″ W	Zini (2013)
Aspergillus sp. LBM 043	*Passiflora edulis* fruit	Posadas	Latitude 27°26′04″ S/ longitude 55°53′14″ W	Zini (2013)
Aspergillus sp. LBM 046	*Psidium guajava* fruit	Posadas	Latitude 27°26′04″ S/ longitude 55°53′14″ W	Zini (2013)
Aspergillus sp. LBM 048	*Citrus sinensis* peal	Posadas	Latitude 27°26′04″ S/ longitude 55°53′14″ W	Zini (2013)
Aspergillus sp. LBM 051	*Passiflora edulis* fruit	Posadas	Latitude 27°26′04″ S/ longitude 55°53′14″ W	Zini (2013)
Aspergillus sp. LBM 053	*Ci. sinensis* peal	Posadas	Latitude 27°26′04″ S/ longitude 55°53′14″ W	Zini (2013)
Aspergillus sp. LBM 054	*Ci. limon* fruit	Posadas	Latitude 27°26′04″ S/ longitude 55°53′14″ W	Zini (2013)
A. niger LBM 055	*Camellia sinensis* leaves	Posadas	Latitude 27°26′04″ S/ longitude 55°53′14″ W	Zini (2013)
Aspergillus sp. LBM 056	*Ilex paraguariensis* leaves	Posadas	Latitude 27°26′04″ S/ longitude 55°53′14″ W	Zini (2013)
Aspergillus sp. LBM 057	*Ca. sinensis* leaves	Posadas	Latitude 27°26′04″ S/ longitude 55°53′14″ W	Zini (2013)
Aspergillus sp. LBM 058	*I. paraguariensis* leaves	Posadas	Latitude 27°26′04″ S/ longitude 55°53′14″ W	Zini (2013)
Aspergillus sp. LBM 059	*Persea americana* fruit	Garupá	Latitude 27°27′28″ S/ longitude 55°51′25″ W	Zini (2013)
Aspergillus sp. LBM 063	*Ca. sinensis* leaves	Posadas	Latitude 27°26′04″ S/ longitude 55°53′14″ W	Zini (2013)
Aspergillus sp. LBM 126	*I. paraguariensis* leaves	Santo Pipó	Latitude 27°13′70″ S/ longitude 55°41′07″ W	This work
Aspergillus sp. LBM 127	*I. paraguariensis* leaves	Santo Pipó	Latitude 27°13′70″ S/ longitude 55°41′07″ W	This work
Aspergillus sp. LBM 128	*I. paraguariensis* leaves	Santo Pipó	Latitude 27°13′70″ S/ longitude 55°41′07″ W	This work
Aspergillus sp. LBM 129	Rotten wood	Alem	Latitude 27°24′39″S/ Longitude 55°54′7″ W	This work
Aspergillus sp. LBM 130	Rotten wood	Posadas	Latitude 27°43′62″ S/ longitude 55°88′77″ W	This work
Aspergillus sp. LBM 131	Rotten wood	Posadas	Latitude 27°43′62″ S/ longitude 55°88′77″ W	This work
Aspergillus sp. LBM 132	Rotten fruit	Posadas	Latitude 27°43′62″ S/ longitude 55°88′77″ W	This work
Aspergillus sp. LBM 133	Rotten fruit	Posadas	Latitude 27°43′62″ S/ longitude 55°88′77″ W	This work
A. niger LBM 134	Rotten wood	Aristóbulo del Valle	Latitude 27°09′05″ S/ longitude 54°86′77″ W	This work
Aspergillus sp. LBM 135	Rotten wood	Aristóbulo del Valle	Latitude 27°09′05″ S/ longitude 54°86′77″ W	This work
Aspergillus sp. LBM 136	Soil	Posadas	Latitude 27°43′62″ S/ longitude 55°88′77″ W	This work
Apergillus sp. LBM 210	Soil	Posadas	Latitude 27°43′62″ S/ longitude 55°88′77″ W	This work

Xylanases have been employed in biological pre-treatments which are specific and non-toxic treatments. The use of xylanases also reduces the cost of the process and increases the xylan hydrolysis efficiency (Kubicek & Kubicek, 2016). Xylanases are produced in diverse groups of organisms including bacteria, algae, fungi, protozoa, gastropods, and arthropods (Collins, Gerday, & Feller, 2005; Menon & Datta, 2017). Among these, xylanases from *Aspergillus* fungi are considered powerful to carry out biomass conversion into sugars. Currently, *Aspergillus* xylanases are commercialized; however, they are expensive and therefore their use for commercial application is still complex (Florencio et al., 2016). To overcome this challenge, different agricultural wastes such as SCB have been used for fungal growth and xylanases production (Barchuk et al., 2016; Pandey, Soccol, Nigam, & Soccol, 2000).

The search for fungal isolates is particularly promising in the genus *Aspergillus,* which has the xylanolytic potential essential for xylanase production. Furthermore, it is desired that these new fungal isolates do not produce proteases, or at least production in low quantities since proteases catalyse the cleavage of peptide bonds of proteins like xylanases, and this is a serious problem for the xylanase stability (Oliveira, Porto, & Tambourgi, 2006).

The search of fungal isolates is essential in biodiverse regions. Misiones rainforest is one of the most biodiverse systems of subtropical regions on earth (Brown, Grau, Lomáscolo, & Gasparri, 2002; Coniglio, Fonseca, Villalba, & Zapata, 2017). This ecoregion is in the northeast of Argentina and is biogeographically linked with the Atlantic Forest of Brazil (Rubio, 2014). Misiones rainforest has a subtropical climate, medium temperature of 19–24 °C and annual precipitations that vary between 1000 and 2200 mm (Grassi, Romano, & Schenone, 2016). Misiones rainforest has a surface area of 29,800 km² and together with Yungas forest (northwest of Argentina) represent less than two per cent of continental Argentina and contain more than 50% of the country's biodiversity (Rubio, 2014).

Our goal was to search for fungal isolates from Misiones rainforest that produce high xylanase levels and no proteases for future biotechnological applications on the bioconversion of agricultural wastes. For that, the aim of this study was to screen new *Aspergillus* isolates using SCB as carbon source and to categorize the most promising isolates by a multivariate analysis.

2. Material and methods

2.1. Isolation of native aspergillus of the Misiones rainforest

Samples of soil and rotten wood, fruit and tree leaves were collected from the subtropical rainforest of Misiones from June to August 2015. Fungi from rotten materials were isolated by scrapping the material surface and inoculated on 39 gL⁻¹ potato dextrose agar (PDA) with 0.5% (p/v) chloramphenicol to inhibit bacterial growth (Benbow & Sugar, 1999). Fungi from soil were isolated using the method of dilution on agar plates (Mueller, Bills, & Foster, 2004). From all samples, fungal isolates belonging to the genus *Aspergillus* were selected based on their macroscopic and microscopic characteristics using the keys of Pitt and Hocking (1997) and Carrillo (2003). The fungal isolates were deposited in the Culture Collection of the Laboratory of Molecular Biotechnology (LBM, from Spanish *Laboratorio de Biotecnología Molecular*) of the Institute of Biotechnology Misiones and summarized in Table 1

with *Aspergillus* fungi of the LBM Collection isolated by Zini (2013).

2.2. Fungal isolates and maintenance

The 12 *Aspergillus* fungi isolated in this work and the 13 *Aspergillus* of the Institute Collection were screened for their ability to produce xylanases growing on SCB. The strain LBM 117 of *Trichoderma atroviride* was used as a positive control because it is known as a good xylanase producer when grown on SCB (Barchuk et al., 2016). All the fungal isolates were maintained on 39 gL^{-1} PDA medium at 4 °C.

2.3. Feedstock preparation

SCB was sampled from a sugarcane mill in San Javier locality in the province of Misiones, Argentina. SCB was dried at 60 °C overnight and then milled to produce a material retained through a 40-mesh screen. All the experiments were carried out with the same variety of SCB.

2.4. Quantitative screening of xylanolytic-producing isolates without proteases

Screening of fungal isolates for xylanolytic production was carried out incubating the isolates in Czapek minimum medium (2 gL^{-1} NaNO₃, 1 gL^{-1} KH₂PO₄, 0.5 gL^{-1} KCl, 0.5 gL^{-1} MgSO₄·7H₂O, 0.01 gL^{-1} FeSO₄·7H₂0) supplemented with 3 gL^{-1} peptone and 10 gL^{-1} SCB as sole carbon source. Peptone was used due to its ability to support high levels of xylanases (Díaz, Giorgio, Fonseca, Villalba, & Zapata, 2015). Erlenmeyer flasks containing 25 mL of this medium were inoculated with 1 mL of a spore suspension of the fungal isolate. The spore suspension was prepared by aseptically scraping the spores from the surface of the plates and suspending in sterile solution of Tween 80 (0.1% v/v). The spore concentration, 10^7 spores mL^{-1}, was determined by counting the spores in the central squares of a Neubauer camera using optic microscopy (Alves & Faria, 2010). The inoculated Erlenmeyer flasks were incubated in the dark at 28 ± 2 °C at 100 rpm for 20 days. An aliquot of 1 mL of the culture supernatant was extracted every 48 h from each experiment to determine endoxylanase activity.

β-xylosidase, casein protease and gelatin protease activities were then studied to select the isolates with the highest xylanolytic levels and the lowest proteolytic levels. β-xylosidase and proteolytic activities were determined at the day of the highest production of endoxylanases of each fungal isolate.

2.5. Enzyme assays

Endoxylanase activity was performed according to Bailey, Biely, and Poutanen (1992) using beechwood xylan (Sigma-Aldrich, USA) as substrate. Reducing sugars were determined by the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959). Endoxylanase activity was expressed as international units (U) defined as the amount of enzyme needed to produce 1 μmol of reducing sugars per min at 50 °C.

The β-xylosidase activity was assayed according to Ghose and Bisaria (1987) using as substrate 4-nitrophenyl β-D-xylopyranoside (pNPX) (Sigma-Aldrich, USA). One unit (U) of pNPX-hydrolysing activity was defined as the amount of enzyme necessary to release 1 μmol of p-nitrophenol per min at 50 °C.

Casein protease activity was assayed on 10 gL^{-1} skimmed milk agar plates at pH 7 and gelatin protease activity, on 10 gL^{-1} gelatin plates at pH 4.8 (Smith & Goodner, 1958; Vermelho et al., 1996). Plugs of 5 mm diameter were cut in both skimmed milk agar and gelatin plates and filled with 15 μL of the supernatants of the isolates. The filled plugs were incubated at 37 °C for 10 h. Gelatin plates were stained with 1 mL Bradford reagent (Bio Rad) to visualize the degradation halos.

2.6. Statistical analysis

All experiments were carried out in duplicate. To determine the day of the highest endoxylanase production by each fungal isolate, results from experiments were evaluated with the ANOVA and the least significance difference test (95% confidence level) using the software Statgraphics Centurion (StatPoint, Inc. version 15.2.05).

To identify the influence of the xylanolytic and proteolytic enzymes and their correlation with the biodegradation of SCB of the different *Aspergillus* isolates, a principal component analysis (PCA) was performed using the software InfoStat 2017 version (Di Rienzo et al., 2001). The exploratory analysis and the conglomerate analysis were also carried out with the software InfoStat 2017 version.

3. Results and discussion

3.1. Screening and selection of high xylanolytic and low proteolytic-producing isolates

3.1.1. Xylanolytic production

In this work the SCB was suitable as a carbon source for *Aspergillus* isolates growth and as an inducer to produce xylanolytic enzymes, endoxylanases and B-xylosidases. It has been proved that ascomycetes can produce high levels of xylanases growing in SCB
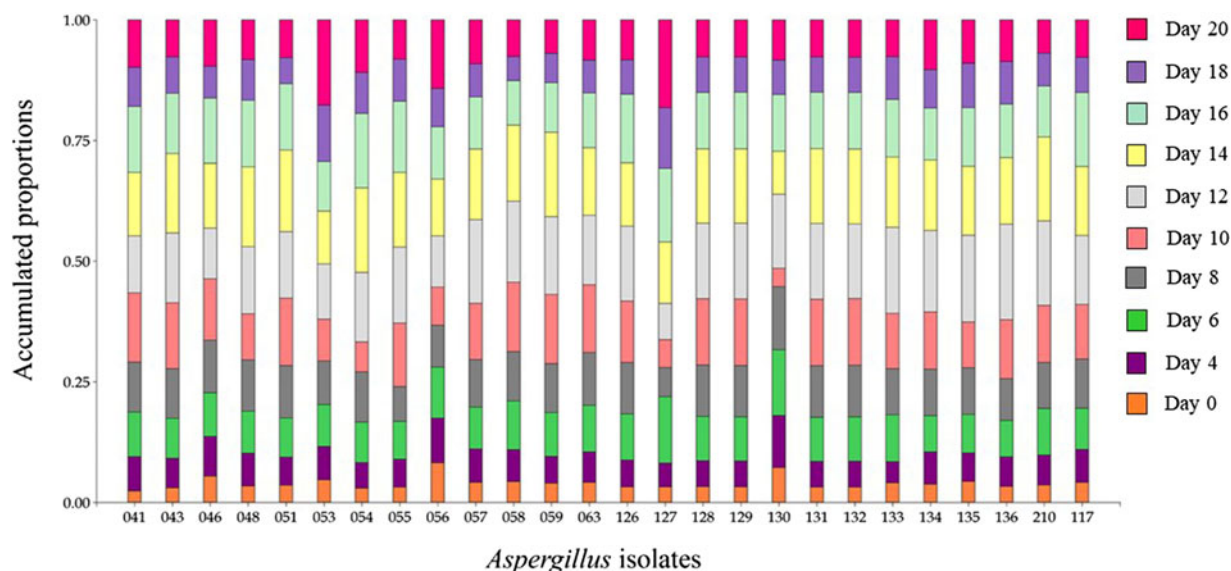
Figure 1. Endoxylanases produced by *Aspergillus* isolates during 20 days of incubation. Each bar represents an isolate and different colours belong to enzyme activity proportion from each day.

without any treatment (Barchuk et al., 2016; Sales, de Moura, da Silva, de Macedo, & Porto, 2011).

Nearly all the *Aspergillus* isolates had a similar profile of the production of endoxylanases during 20 days of treatment (Figure 1). This exploratory analysis showed that most of the isolates produced low quantities of endoxylanase enzymes at the first four-days of incubation, except isolates LBM 056 and LBM 130 which produced the maximal endoxylanase levels at days 20 and 12 of incubation, respectively.

The endoxylanase activity of almost all isolates improved from day 6 to day 16 of incubation, reaching the highest endoxylanase activity in this period. Isolate LBM 041 showed the best enzyme production at day 10. *A. niger* LBM 055, isolates LBM 057, LBM 058, LBM 063, LBM 126, LBM 128, LBM 129, LBM 130, LBM 131, LBM 132, LBM 133, *A. niger* LBM 134, LBM 135 LBM 136 and LBM 210 produced the highest endoxylanase levels at day 12; isolates LBM 043, LBM 048, LBM 051, LBM 054 and LBM 059, at day 14. Isolates LBM 046 and LBM127 and the control *T. atroviride* LBM 117 produced the highest endoxylanase levels at day 16. In almost all cases, after day 16 of incubation, the endoxylanase production decreased dramatically perhaps due to the presence of proteolytic enzymes in the medium which may be a serious problem for the maintenance of enzymatic stability of xylanases (Oliveira et al., 2006). Only isolates LBM 053 and LBM 056 produced the maximal xylanase levels at day 20.

Data from ANOVA and LSD analysis (Table S1 and Table S2, respectively) showed that 60% of isolates produced their highest endoxylanase levels at day 12 of incubation ($p < 0.05$). The 20% of fungal isolates showed the highest endoxylanase activity at day 14; 8% at day 20; 4% at day 10 and 4% at day 16 ($p < 0.05$) (Table 2). Regardless the day of the highest enzyme activity, 68% of isolates produced more than 20 UmL^{-1} of endoxylanase; from these, isolates LBM 043, *A. niger* LBM 055, LBM 131, LBM 132, LBM 133 and *A. niger* LBM 134 showed the highest endoxylanase production of almost 30 UmL^{-1}, even higher than the activity reached by *T. atroviride* LBM 117, a well-known xylanolytic producer (Barchuk et al., 2016). The endoxylanase levels reported in this work were higher than that produced by other ascomycete even when it was incubated using pretreated SCB with 0.25% sulphuric acid solution; that is the case for *Penicillium janthinellum* that reached 23 UmL^{-1} of endoxylanase when it grew on this pretreated SCB (Oliveira et al., 2006).

Aspergillus isolates were also screened for their capacity to produce B-xylosidases at the day of highest endoxylanase activity and two days later, without any statistical difference between them ($p \geq 0.05$). Since xylanases could be affected by the co-production of proteolytic enzymes, the isolates were screened for their capacity of producing casein and gelatin proteolytic enzymes on neutral and acidic pH, respectively (Table 2).

Regarding B-xylosidases, isolates LBM 046, LBM 053, *A. niger* LBM 055, *A. niger* LBM 134 and LBM 135, corresponding to 20% of the isolates, produced more than 0.6 UmL^{-1}. In contrast, 36% of fungal isolates were the lowest producers of B-xylosidases reaching less than 0.1 UmL^{-1}. The rest of the isolates, 44%, produced between 0.1 and 0.6 UmL^{-1} of B-xylosidases. Moreover, there is not a relationship between the production of endoxylanases and B-xylosidases, meaning that isolates which produced the highest endoxylanase levels did not necessarily produce high levels of B-xylosidases.

Table 2. Production of endoxylanase, β-xylosidase and proteolytic enzymes by fungal isolates.

Fungal isolates	Day of endoxylanase determination[a]	Enzymes activities			
		Endoxylanase[b]	β-xylosidase[b]	Proteolytic (nonfat-milk)[c]	Proteolytic (gelatin)[c]
Aspergillus sp. LBM 041	10	15.46 ± 6.59	0.26 ± 0.003	7.48 ± 0.014	10.13 ± 0.032
Aspergillus sp. LBM 043	14	27.70 ± 3.83	0.03 ± 0.001	12.93 ± 1.199	12.67 ± 0.134
Aspergillus sp. LBM 046	16	13.79 ± 5.54	0.66 ± 0.050	7.48 ± 0.032	8.26 ± 0.173
Aspergillus sp. LBM 048	14	24.81 ± 5.57	0.58 ± 0.091	8.65 ± 0.004	7.93 ± 0.178
Aspergillus sp. LBM 051	14	21.97 ± 6.01	0.04 ± 0.006	10.71 ± 0.226	9.12 ± 0.004
Aspergillus sp. LBM 053	20	15.78 ± 2.41	0.65 ± 0.023	7.53 ± 0.035	10.09 ± 0.032
Aspergillus sp. LBM 054	14	22.64 ± 0.04	0.28 ± 0.026	10.48 ± 0.460	± 0.071
A. niger LBM 055	12	29.48 ± 0.21	0.68 ± 0.029	7.55 ± 0.00	10.48 ± 0.014
Aspergillus sp. LBM 056	20	8.77 ± 0.06	0.57 ± 0.034	7.85 ± 0.071	11.67 ± 0.113
Aspergillus sp. LBM 057	12	24.63 ± 0.32	0.56 ± 0.041	7.89 ± 0.014	13.67 ± 0.109
Aspergillus sp. LBM 058	12	22.18 ± 1.22	0.05 ± 0.004	10.06 ± 0.124	13.89 ± 0.028
Aspergillus sp. LBM 059	14	25.36 ± 0.25	0.05 ± 0.003	9.56 ± 0.046	8.76 ± 0.152
Aspergillus sp. LBM 063	12	20.20 ± 6.26	0.05 ± 0.007	13.56 ± 0.025	12.74 ± 0.24
Aspergillus sp. LBM 126	12	19.74 ± 3.50	0.05 ± 0.004	13.26 ± 0.007	13.37 ± 0.007
Aspergillus sp. LBM 127	20	17.43 ± 0.98	0.20 ± 0.003	9.93 ± 0.223	9.24 ± 0.014
Aspergillus sp. LBM 128	12	22.37 ± 2.55	0.07 ± 0.004	14.30 ± 0.184	12.96 ± 0.216
Aspergillus sp. LBM 129	12	22.28 ± 2.69	0.23 ± 0.024	8.17 ± 0.021	7.34 ± 0.177
Aspergillus sp. LBM 130	12	10.99 ± 0.11	0.36 ± 0.009	7.48 ± 0.039	11.71 ± 0.262
Aspergillus sp. LBM 131	12	26.43 ± 0.02	0.07 ± 0.004	11.71 ± 0.216	10.17 ± 0.007
Aspergillus sp. LBM 132	12	29.41 ± 2.32	0.18 ± 0.025	17.06 ± 0.081	12.86 ± 0.014
Aspergillus sp. LBM 133	12	26.98 ± 2.50	0.02 ± 0.001	9.16 ± 0.007	11.56 ± 0.304
A. niger LBM 134	12	27.52 ± 1.70	0.60 ± 0.009	7.37 ± 0.209	7.37 ± 0.173
Aspergillus sp. LBM 135	12	23.82 ± 0.69	0.63 ± 0.039	10.06 ± 0.085	11.55 ± 0.018
Aspergillus sp. LBM 136	12	19.15 ± 4.61	0.29 ± 0.042	7.34 ± 0.177	11.18 ± 0.647
Aspergillus sp. LBM 210	12	22.32 ± 2.48	0.11 ± 0.003	12.90 ± 0.194	11.68 ± 0.004
T. atroviride LBM 117	16	19.15 ± 1.52	0.10 ± 0.02	9.82 ± 0.007	11.02 ± 0.127

aDay of the highest endoxylanase activity for each fungus and for β-xylosidase and proteolytic assays.
bAverage of endoxylanase and β-xylosidase[a] activities (in UmL^{-1}) at the days of highest enzymatic activities.
cAverage of halos (in mm) of proteolytic activities at day of highest endoxylanase activity.

3.1.2. Proteolytic activity

Regarding proteolytic enzymes, 68% of isolates produced casein proteases and 88% produced gelatin proteases (Table 2). Almost all isolates which produced casein proteases, produced gelatin proteases too. Also, isolates produced higher quantities of gelatin proteases than casein proteases.

The proteolytic activities were detected as halos of degradation on both skimmed milk and gelatin agar plates (Figure 2). In the case of skimmed milk agar plates, their halos of degradation were clearly defined (Figure 2a). Nevertheless, in the case of gelatin proteolytic assays, it was very difficult to detect and measure the halos of degradation without the use of a developing agent (Figure 2b). Vijayaraghavan and Vincent (2013) used Bromocresol Green (BCG) reagent to visualize narrow halos of degradation for their proteolytic studies; we instead used Bradford reagent to visualize the diffuse zone of the degradation halos in gelatin plate assay.

Bradford agent was effective in defining the halo of degradation and it is a very simple method. In the present study, the clear zone corresponding to the halo of degradation was clearly observed after the addition of Bradford reagent on the gelatin agar plate. Coomassie dye of Bradford reagent bound to the unhydrolysed protein molecules in the gelatin agar plate (Bradford, 1976) making this area sharply blue. In contrast, the degraded proteins zone became distinctly white because this reagent could not bind to the hydrolysed gelatin proteins. These results indicate that Coomassie dye is a useful method to detect extracellular gelatin proteases produced by fungi.

Almost every supernatant presented proteolytic activity on either skimmed milk or gelatin assays or on both, and the proteolytic enzymes produced different sizes of halos. Supernatants from isolates LBM 043, LBM 063, LBM 126, LBM 128, LBM 132 and LBM 210 produced very big halos of degradation on both skimmed milk and gelatin agar plates which means that these isolates have produced large quantities of casein and gelatin proteases which act at neutral and acidic pH, respectively. On the other hand, supernatants from isolates LBM 041, LBM 046, LBM 053, *A. niger* LBM 055, LBM 056, LBM 057, LBM 130, *A. niger* LBM 134 and LBM 136 showed no halos of degradation on skimmed milk plates indicating that these isolates did not produce neutral casein proteases, or this technique was not sensitive enough for these enzyme quantities. However, isolates LBM 041, LBM 053 and LBM 130 showed large halos of degradation on gelatin agar plates, demonstrating that they had high gelatin proteolytic activity at acidic pH. The other fungi studied, isolates LBM 048, LBM 051, LBM 054, LBM 058, LBM 059, LBM 127, LBM 129, LBM 131, LBM 133, LBM 135, LBM 136 and *T. atroviride* LBM 117 produced moderate amounts of casein proteases since they showed mild halos of degradation. Almost all these isolates showed large halos of degradation on gelatin agar plates, except isolates LBM 059, *A. niger* LBM 134 and LBM 136 with halos that were smaller, and isolate LBM 048 that did not produce a halo of degradation. Isolates
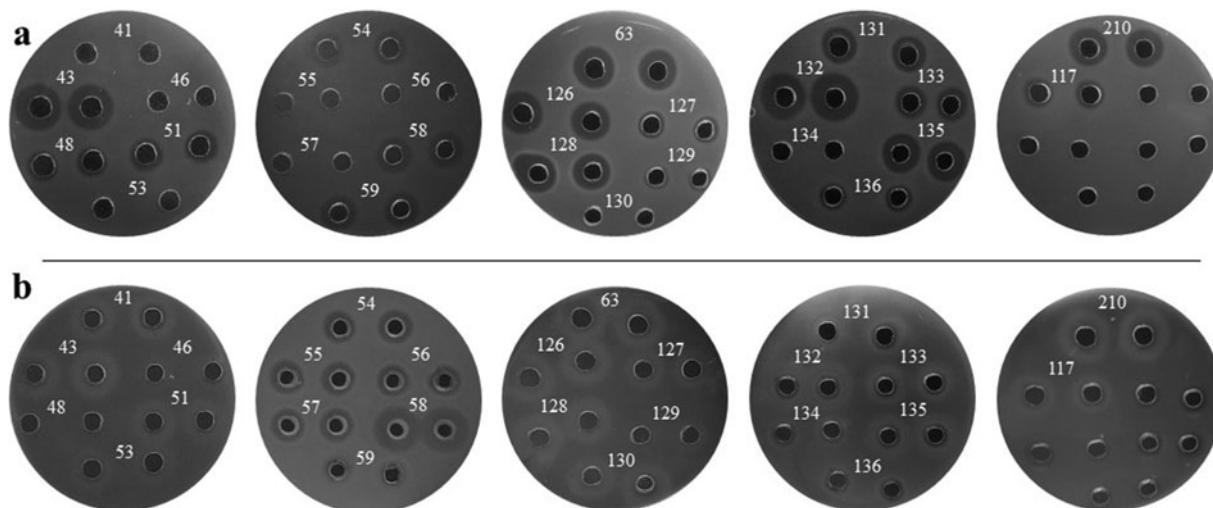
Figure 2. Proteolytic activities of *Aspergillus* supernatants. (a) Casein proteases on skimmed milk plate assay and (b) gelatin proteases on gelatin plate assay.

that produced gelatin proteases did not produce casein proteases, and vice versa. This outcome could be because the expression of protease genes is highly variable according to different times and the physical and nutritional conditions of the experiments. In this sense, our results indicated that the differences of pH affected the proteolytic activities and confirmed that different *Aspergillus* isolates have unique enzymatic profiles. Thus, the search for new fungi and the bioprospection analysis for classifying their enzymatic profiles are indispensable in obtaining commercially viable enzymes.

3.2. General trends in the enzymatic activity in individual Aspergillus isolates

To find the best xylanolytic-producing isolates with no proteolytic activity we used a principal component analysis (PCA) (Figure 3). Figure 3a shows the score plot, whiis a map of the isolates (observations) and how they are situated with respect to each other based on the variables (Nilsson, Andreas, & Lagerkvist, 2016) in the loading plot (Figure 3b). The loading plot shows the influence of the variables (endoxylanase, β-xylosidase and proteolytic activities) on fungal isolates and displays the correlation structure of the variables. Two plots help to analyse the correlation between the observations and the variables (Bohacz, 2017).

The statistical analysis of the main components on the enzyme production by *Aspergillus* fungi showed that the first principal component (PC1) was responsible for explaining 45.1% of the results' variability, and the second main component (PC2) was responsible for 26.9% variation, resulting in 72% of total variation of the experimental results. That 72% indicates that PCA represents adequately the relationships between the isolates and the variables

(Balzarini, Bruno, Córdoba, & Teich, 2015). Regarding PC1 analysis, endoxylanase activities at any time and the casein protease activity presented a positive correlation meaning that when one variable increased, the other did the same. Both enzyme activities showed negative correlation with β-xylosidase and gelatin protease activities. When variables were analysed in the PC2, the proteolytic activities had negative correlation with β-xylosidase and endoxylanase activity at day 20. PC1 showed also a contrasted behaviour of endoxylanase activities at all days with positive weight versus β-xylosidase activity with little negative weight. Casein and gelatin protease activities did not have inertia on PC1, since their projections on the x axis were close to 0; however, in the PC2 both proteolytic activities had positive weight and served to differentiate those isolates that produced very high levels of these enzymes from those with lower levels. Moreover, in the PC2 it could be observed that β-xylosidase and endoxylanase activity at day 20 had negative weight and the rest of the endoxylanase activities had negligible weight on this PC. This means that those fungi having high endoxylanase and casein protease activities are likely to have high scores since in the PC1 these enzymes had positive scores, and this explains 45.1% of the experimental results.

From this multivariate analysis, *A. niger* LBM 055 and *A. niger* LBM 134 produced the highest endoxylanase and β-xylosidase levels, with negligible proteolytic production, resulting in their placement in the right lower quadrant (positive for PC1 and negative for PC2). In contrast, isolates LBM 056 and LBM 130 were placed in the top left quadrant since they had the lowest endoxylanase production and high levels of proteolytic enzyme activities, showing medium levels of β-xylosidase activity. Isolates LBM 041, LBM 046, LBM 053 and LBM 127 were located
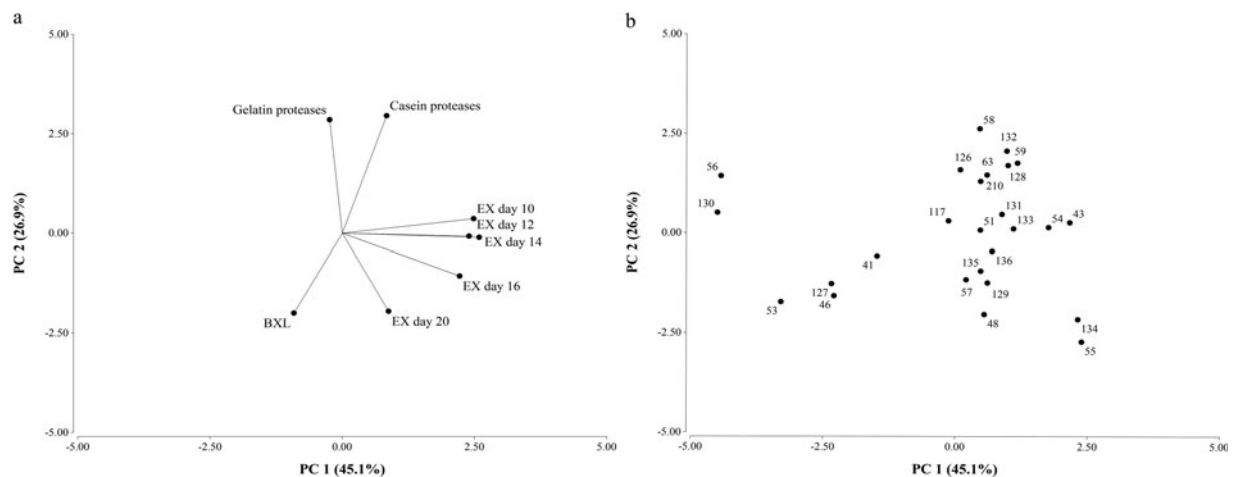
Figure 3. Principal component (PC) analysis of enzymatic activities for all *Aspergillus* isolates. (a) Loadings plot of the first and second principal components for total endoxylanase activity at day 2 (EX day 2), at day 4 (EX day 4), at day 6 (EX day 6), at day 8 (EX day 8), at day 10 (EX day 10), at day 12 (EX day 12), at day 14 (EX day 14), at day 16 (EX day 16), at day 18 (EX day 18), at day 20 (EX day 20); β-xylosidase activity (BXL) and both gelatin and casein proteases. The plan formed by these components reproduces 72% of the original information. (b) Scores of *Aspergillus* isolates using non-treated SCB as substrate in the plane formed by the first and the second principal components. The isolates located in the same part of the loading plot means the higher impact of the variables (xylanolytic and proteolytic activities) on the observations.

on the left lower quadrant indicating low levels of endoxylanase and no casein protease activities and presented different behaviour on β-xylosidases and gelatin proteases activities. The rest of the isolates were placed far on the left half of the graph, indicating they had good levels of endoxylanase activity and different behaviours with regard to β-xylosidase, casein and gelatin activities.

With the aim of classifying the isolates and forming homogeneous groups based on their levels of enzyme activities we carried out an analysis of conglomerates. The dendrogram formed is shown in Figure 4 with a cophenetic correlation of 0.779. Six groups were formed;the best one included *A. niger* LBM 055 and *A. niger* LBM 134. These are promising candidates for carrying out an efficient hydrolysis of xylan from SCB since they had high levels of endoxylanase and β-xylosidase activities and low proteolytic enzyme levels. A second group included good xylanolytic-producing fungi, isolates LBM 048, LBM 057, LBM 129, LBM 135 and LBM 136; however, they also produced high quantities of proteolytic enzymes. Hence, their biotechnological application in the hydrolysis of SCB became difficult since their xylanases activities could be unstable due to the high proteolytic levels. However, they are applicable for other biotechnological purposes (Polizeli et al., 2005), or the enzymes can be used with the addition of a proteolytic inhibitor to preserve the xylanolytic enzymes but the cost of such enzymatic production increases. The third group formed by isolates LBM 043, LBM 051, LBM 054, LBM 117, LBM 131 and LBM 133 had not only high levels of proteolytic enzymes, but also, low β-xylosidase activity. Fourth and fifth groups were formed by fungi showing medium

levels of endoxylanase enzymes. Isolates LBM 58, LBM 59, LBM 63, LBM 126, LBM 128, LBM 132 and LBM 210 formed the fourth group and presented low β-xylosidase activities and super high levels of proteolytic enzymes. Isolates from the fifth group, LBM 041, LBM 046, LBM 053 and LBM 127, had different levels of β-xylosidase enzymes and high levels of gelatin proteases. The sixth group formed by isolates LBM 056 and LBM 130 showed the lowest levels of endoxylanase activity and high levels of gelatin proteases. Hence, fungi belonging to the last three groups are not considered as good xylanolytic producers, and they were discarded for future studies.

Although there are several studies on fungal xylanolytic enzymatic screening, this work shows the significance of a multivariate exploratory investigation including a principal component and conglomerates analysis to categorize effectively xylanolytic producing and non-producing fungi associated with low levels of proteolytic activities. It served as a good tool to elucidate that *A. niger* LBM 055 and *A. niger* LBM 134 were the best producers of endoxylanases and β-xylosidases, while being the lowest proteolytic enzyme producers.

4. Conclusion

In this work, we assayed the xylanolytic and proteolytic producing-ability of 25 *Aspergillus* fungi using an easy-access, efficient and low-cost substrate such as SCB. Each isolate showed different enzyme production profiles and formed six groups based on their enzymatic levels: from a group with the highest endoxylanase and β-xylosidase activities and the
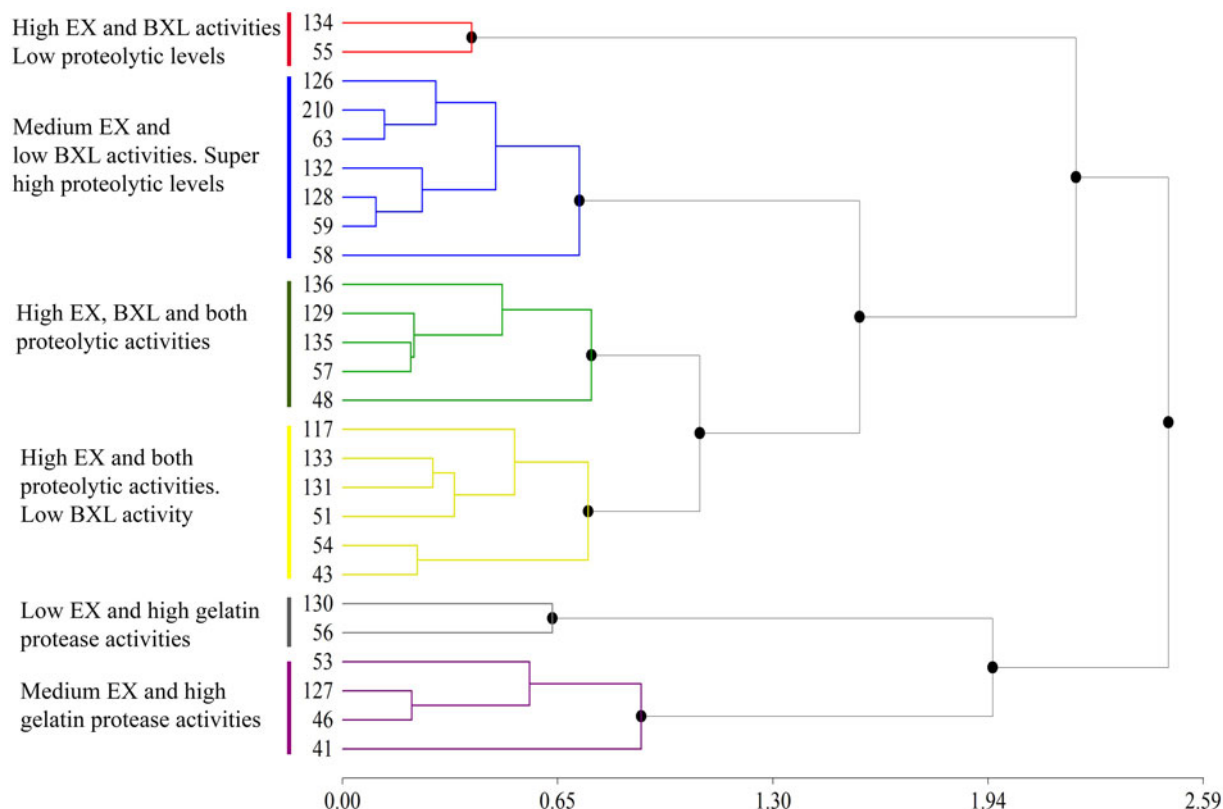
Figure 4. Groups of *Aspergillus* isolates formed by the Conglomerate analysis. The classification was based on their enzymatic levels and six clusters with different specificities were formed.

lowest protease levels up to a group with the lowest xylanolytic and high proteolytic activities. *A. niger* LBM 055 and *A. niger* LBM 134 were the highest xylanolytic enzymes producing isolates, reaching 29 UmL^{-1} and 27 UmL^{-1} of endoxylanase activity, respectively, producing 0.6 UmL^{-1} of β-xylosidase enzymes and with no casein protease activity. For this reason, we considered them as the most promising xylanolytic producers for the hydrolysis of xylan presented in SCB.

Furthermore, our results indicate that Bradford reagent is useful for detecting extracellular proteases from fungi on gelatin plate assay with well-defined halos of degradation. This is a sensitive, simple and easy technique for screening fungal microbial proteases.

Disclosure statement

The authors declare that they have no conflict of interest.

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