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Selection of *Trichoderma* Strain to Enhanced Cellulase-Poor Xylanase Production Using Sugarcane Bagasse as Sole Carbon Source under Light

Mónica Lucrecia BARCHUK*, Gabriela Verónica DÍAZ, Pedro Agustín Francisco COLL[1], Juan Ernesto VELAZQUEZ, María Isabel FONSECA, Laura Lidia VILLALBA, Pedro Darío ZAPATA

Instituto de Biotecnología Misiones Maria Ebe Reca (INBIOMIS). Facultad de Ciencias Exactas, Químicas y Naturales. Universidad Nacional de Misiones, Argentina

*Corresponding Author E-mail: lucre_barchuk@hotmail.com

ABSTRACT

Species from fungal genus Trichoderma were found to produce culture broths rich in xylanase activity, an enzyme which plays an essential role for efficient and complete hydrolysis of lignocellulosic biomass. This research aimed to enhance cellulase-poor xylanase production using a low-cost medium. Firstly, several native Trichoderma strains isolated from subtropical environments of Misiones (Argentina) were evaluated based on its xylanase producing potential. Among these, T. atroviride LBM 117 produced the highest cellulase-poor xylanase enzymes, 29 U mL^{-1} and 28 U mL^{-1} in experiment with and without light, respectively, using sugarcane bagasse as a sole carbon source. Besides, when experiments were carried out with light, the highest xylanase production occurred in shorter incubation period than experiments without it. Xylanase activity was characterized in both supernatants showing the maximum xylanase activity at 50 °C and pH 4.8. Results of electrophoresis on polyacrylamide gels showed two isoenzymes of 105 and 150 KDa in supernatants from experiment with light and only one isoenzyme of 105 KDa from experiment in darkness.

Keywords: *Trichoderma, xylanases, sugarcane bagasse (SCB), light.*

INTRODUCTION

Xylanases (1,4-β-D-xylanhidrolases, EC 3.2.1.8.) are the most important hemicellulolytic enzymes on lignocellulose degradation (Devia Ulloa, 2014). These enzymes hydrolyze the 1,4-β-D-xylosidic linkages in xylan, the major constituent of hemicellulose [1,2]. A good deal of attention has been paid to xylanases produced by *Trichoderma* species due to their high production level and extracellular secretion of these enzymes. Xylanases have immense potential in a large number of biotechnological applications; the most promising application of these enzymes has been on biobleaching process in pulp and paper industry since the discovery of Viikarri *et al.* [3]. [1,4,5,6,7]

However, the production cost is the major factor limiting the industrial application of xylanases, indicating the need for low cost production systems. In the last few years, researchers have turned to the search for renewable raw material that serves as a good substrate for the production of industrially xylanases [8,9]. Sugarcane bagasse (SCB) is the largest agro-industrial residue produced from sugar plants and comparing to others agricultural residues, SCB can be considered as a rich carbon

source due to its high yields and annual regeneration capacity [10]. South America is the second most producing sugarcane producer [10], hence, SCB is widely available for its use. In view of biotechnological and industrial application, the aim of this work focused on the evaluation of the xylanolytic ability in subtropical *Trichoderma* strains native from Misiones (Northeast, Argentina) and the enhancement of cellulase-poor xylanase production using a low-cost medium. In addition, the light effect on xylanase production was evaluated and the enzyme activity was characterized in supernatants.

MATERIAL AND METHODS

Fungal strains and maintenance

Twenty *Trichoderma* strains isolated from the Misiones rainforest (Argentina) were used in this study. They were deposited at the culture collection of the Instituto de Biotecnología Misiones "María Ebe Reca", Universidad Nacional de Misiones, Argentina. Stock cultures were maintained by periodic sub-culturing on 39.5 g L^{-1} potato dextrose agar (PDA) and kept at 4 °C.

Qualitative screening of xylanase producing strains

To identify xylanase producing strains, an initial screening in agar plates was performed on Mandels medium [11] supplemented with 0.1% (w/v) beechwood xylan (Sigma-Aldrich, USA). Petri plates were inoculated with the fungal strains and incubated at 28 ± 2 °C with light until the mycelium covered 80% of the surface dish. Xylanolytic activity was detected with Congo red technique. [12]

Quantitative screening of xylanolytic-producing strains

Xylanase producing strains were grown on agar plates with beechwood xylan. The spores were scraped aseptically from the surface and suspended in sterile Tween 80 aqueous solution (0.1% v/v) to obtain a spore suspension (10^7-10^8 spores mL^{-1}). Erlenmeyer flasks containing 20 mL of Mandels medium containing 1% (w/v) beechwood xylan were inoculated with a 1 mL of spore suspension and incubated at 28 ± 2 °C with light at static conditions during 27 days. An aliquot of 1 mL of the culture supernatant was

extracted every 72 h to determine xylanase activity.

Enzyme assay

Xylanase activity assay was performed according to Bailey *et al.* [13] using beechwood xylan (Sigma-Aldrich, USA) as substrate. Carboxy methyl cellulases (CMCases) activity was determined using 0.5% (w/v) carboxy methyl cellulose (CMC) as substrate. Filter paper (FPAase) activity was assayed based on method developed by Mandels *et al.* [14] using Whatman No. 1 filter paper as substrate. Enzyme assays were performed at 50 °C during 60 min and liberated reducing sugars were determined using the 3,5-dinitrosalicylic acid reagent (DNS) method [15]. Protein concentration was determined by the method of Lowry *et al.* [16] with Bovine serum albumin (BSA) as the standard.

Comparative evaluation for xylanase activity using SCB vs. beechwood xylan

SCB was collected from a local sugar mill. It was prepared by exhaustive washing with distilled water, dried at 80 ℃ for 24-48h and milled (40 mesh).

The best xylanase producing strains were selected and cultured in 100 mL Erlenmeyer flasks containing Mandels medium supplemented with different SCB concentrations, 5, 10 and 15 g L^{-1}. In addition, a medium containing 1% (w/v) beechwood xylan was carried out as control of xylanase activity. All Erlenmeyer flasks were inoculated with 1 mL of spore suspension (10^7-10^8 spores mL^{-1}) and incubated at 28 ± 2 ℃ and 100 rpm during 27 days in two different trials, with and without light. An aliquot of 1 mL of the culture supernatant was extracted every 72 h to determine xylanase activity.

Evaluation of cost/effectiveness of SCB and xylan media

The cost/effectiveness of the media which supported the major xylanase activity was evaluated based on the average market FOB prices of nutritional components of media. *T. atroviride* LBM 117 grown with and without light in media containing 15 and 10 g L^{-1} of SCB were compared to beechwood xylan as substrate.

Characterization of xylanase activity of *T. atroviride* LBM 117 supernatants

Xylanase activity was characterized in supernatant from the experiments with the highest xylanase enzymes yield, in *T. atroviride* LBM 117.

Effect of pH on enzyme activity and stability

To assay the optimum pH, xylanase activity was determined at 50 ℃, at different pH values, using citrate buffer (pH 3), 0.05 M sodium acetate buffer (pH 4, 4.8 and 5) and 0.05 M sodium phosphate buffer (pH 6,7 and 8) and 0.05 M tris-glicine buffer (pH 9 and 10). pH stability at 30 ℃ was assayed by pre-incubating the supernatant at pH 3, 4.8, 7 and 10 at 1, 24, 48 and 72 h. Remaining xylanase activity in percentage was determined.

Effect of temperature on enzyme activity and stability

Optimum temperature was determined by incubating the reaction mixture at 4, 10, 30, 40, 45, 55, 60 and 70 ℃ and assaying the activity at the pH determined as optimum. Thermal stability was assayed by pre-incubating the supernatant at 30, 50 and 70 ℃. Afterwards remaining xylanase activity was determined and expressed as percentage.

Polyacrylamide gel electrophoresis

To determine xylanase isoenzymes molecular weight (MW), an electrophoretic separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 7.5% w/v) with 1% (w/v) xylan beechwood (Sigma-Aldrich, USA) was performed. The gel was renatured and xylanase activity was determined according to [17]. Xylanases isoenzymes MW were compared to a MW marker (Kaleidoscope, BioRad).

Statistical analysis

All experiments were conducted in duplicate. Experimental results from quantitative screening were analyzed with the software Infostat/P 2008 version.

Analysis of variance for xylanase activities of all experiments were estimated using the software Statgraphics Centurion (StatPoint, Inc.) and Graph Pad Prism 5th version.

RESULTS

Screening of xylanase producing strains

Eleven out of the twenty *Trichoderma* strains tested were found to be xylanolytic producers on the basis of sharp and distinct zone around the colonies on the xylan agar plates. These strains were *Trichoderma* sp. LBM 192, *T. atroviride* LBM 112, *T. atroviride* LBM 117, *T. stilbohypoxyli* LBM 120, *T. harzianum* LBM 122, *Trichoderma* sp. LBM 193, *T. brevicompactum* LBM 095, *T. harzianum* LBM 100, *T. pleuroticola* LBM 093, *T. harzianum* LBM 094 and *T. harzianum* LBM 103 (Fig. 1).
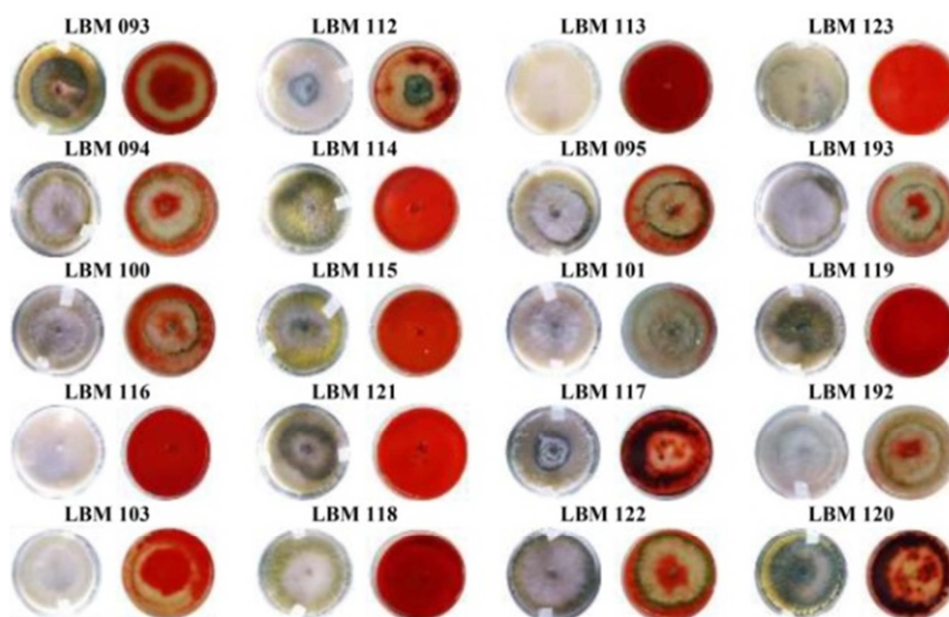

Figure 1. Qualitative screening of *Trichoderma* strains for xylanolytic activity on solid medium (PDA) with 0.1 % (w/v) beechwood xylan. Plates with strains (left) were stained with Congo red (right) to detect xylanase producing strains.

Xylanase producing strains were conducted in a submerged fermentation to distinguish the most efficient xylanolytic strains. As shown in Figure 2, *Trichoderma* sp. LBM 193, *T. atroviride* LBM 117, *T. harzianum* LBM 103, *T.*

harzianum LBM 094, *Trichoderma* sp. LBM 192 showed the highest xylanase activity, over 20 U mL^{-1}, with a cophenetic correlation of 0.907.
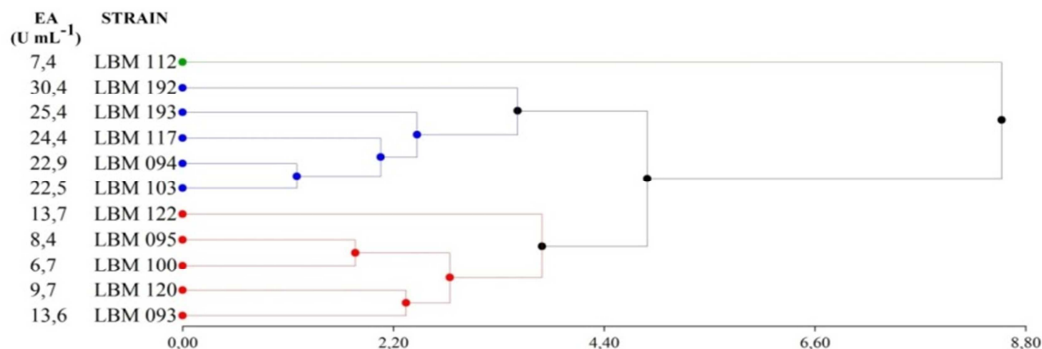

Figure 2. Conglomerate dendrogram of quantitative screening of eleven strains of *Trichoderma* growing in medium with beechwood xylan. Fungi grouped in blue means the best xylanase producing strains. EA, highest enzyme activity, U mL^{-1}, detected for each strain.

SCB vs beechwood xylan as substrate

At this stage of the work, the five strains selected in the quantitative screening were used to evaluate the SCB as substrate for xylanase production. In Figure 3 it can be observed the xylanase production profile of each strain when were incubated with three concentrations of SCB and beechwood xylan as control under two conditions, with and without light. In this experiment, the medium containing SCB yielded higher xylanase enzymes in most of the strains compared to the medium with beechwood xylan ($p < 0.05$). *T. atroviride* LBM 117 reached the

highest xylanase activity, 29 U mL^{-1} in the experiment with 15 g L^{-1} SCB and light on day 21. In contrast, when the fungus was grown on beechwood xylan, the maximum xylanase activity yielded was 23 U mL^{-1}. The other strains, *T. harzianum* LBM 103, *T. harzianum* LBM 122 and *Trichoderma* sp. LBM 192 showed also the maximum xylanase activity when were grown on 15 g L^{-1} SCB with light. Only, *Trichoderma* sp. LBM 193 produced its major xylanase activity, 16.7 U mL^{-1}, when was incubated with beechwood xylan without light on day 18.
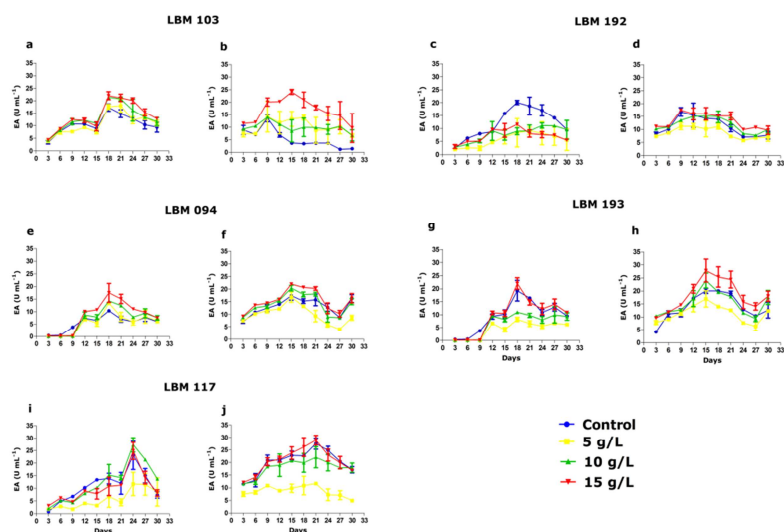

Figure 3. SCB vs. beechwood xylan as substrate. Xylanase activity profiles of five strains in medium whit beechwood xylan (blue) and SCB (yellow, green and red) in experiment with (**a, c, e, g, i**) and without light (**b, d, f, h, j**).

T. atroviride LBM 117 reached the highest xylanase activity (p < 0.05) in experiment with light and in experiment carried out with 10 g L^{-1} SCB without light (28 U mL^{-1}). Hence, both media were analyzed in terms of cost/effectiveness comparing them with the

medium containing the commercial substrate, beechwood xylan (Table 1). The results showed a positive marginal profit up to 96 and 97% of the value of SCB 10 and 15 g L^{-1}, respectively, over commercial substrates.

Table 1. Cost/effectiveness evaluation of *T. atroviridae* LBM 117 media containing 15 g L^{-1} SCB (1), 10 g L^{-1} SCB (2) and 10 g L^{-1} beechwood xylan (3).

Components (g L^{-1})	Medium 1		Medium 2		Medium 3	
	Composition (%)	Cost (U$D)	Composition (%)	Cost (U$D)	Composition (%)	Cost (U$D)
$(NH_4)_2SO_4$	0.1380	0.1316	0.1373	0.1309	0.1380	0.1316
K_2HPO_4	0.1971	0.7148	0.1961	0.7113	0.1971	0.7148
$CaCl_2$	0.0394	0.4050	0.0392	0.4031	0.0394	0.4050
$MgSO_4$	0.0296	0.0995	0.0294	0.0990	0.0296	0.0995
Urea	0.0296	0.1245	0.0294	0.1238	0.0296	0.1245
Yeast extract	0.0246	0.0817	0.0245	0.0813	0.0246	0.0817
$FeSO_4.7H_2O$	0.0005	0.0011	0.0005	0.0011	0.0005	0.0011
$MnSO_4.7H_2O$	0.0002	0.0010	0.0002	0.0010	0.0002	0.0010
$ZnSO_4.7H_2O$	0.0001	0.0006	0.0001	0.0006	0.0001	0.0006
SCB	**0.9856**	**0.0001**	**1.4711**	**0.0002**	**-**	**-**
Xylan	**-**	**-**	**-**	**-**	**0.9856**	**39.4369**
Water	98.5554	0.0662	98.0721	0.0659	98.5554	0.0662
Total	100	1.6261	100.	1.6182	100	41.0629
Productivity (U mL^{-1})	27.3		29.0		22.83	
Standarized total cost (U$D/10,000 U)	**0.5956**		**0.5580**		**17.9872**	

To reveal levels of cellulose activity, CMCase and FPAase enzyme production from *T. atroviride* LBM 117 were determined. Results reported that *T. atroviride* LBM 117 had very

low cellulose enzyme activity demonstrating that the fungus produced cellulase-poor xylanases (Table 2).

Table 2. Production of xylanase, CMCase and FPAase enzymes by *T. atroviride* LBM 117 on SCB with and without light.

LBM 117 supernatant	Protein concentration	Xylanase activity (U mL^{-1})	Specific xylanase activity (U mg^{-1})	CMCase (U mL^{-1})	FPAase (U mL^{-1})
15 g L SCB with light	1.88	29	15.45	0.32	0.25
10 g L SCB without light	1.48	28	18.86	0.12	0.10

Characterization of xylanase activity in *T. atroviride* LBM 117 supernatants

Xylanase activity was characterized in *T. atroviride* LBM 117 supernatants which reached the highest xylanase enzymes. pH 4.8 (p < 0.05) at 50 °C was found to be the optimal pH supporting 27.84 and 27.31 U mL^{-1} for

supernatants from experiment with and without light, respectively. Xylanase activity decreased at higher and lower pH than optimal (Fig. 4a). Once determined the optimal pH, xylanase activity was studied at different temperatures. Maximum xylanase activity was detected at 50 °C (p < 0.05) at pH 4.8, being 28.91 and 25.31 U

mL^{-1} for supernatants from experiments with and without light, respectively (Fig. 4b).

pH stabilities of supernatants were determined at different pH (Fig. 4c). The conditions of best xylanase stability were pH 4.8 and light, upholding 90% of enzyme activity after 72 h. At pH 7, remained 70% of enzyme activity after 72 h in supernatants from experiments with and without light. At pH 3 the xylanases kept 50% of the initial activity at 48 h and 72 h in supernatants from experiment with and without light, respectively. At pH 10 xylanase activity decreased 50% after 24 h in

both experiments. Thermal stabilities were determined at 30, 50 and 70 °C in the absence of substrate and showed that xylanase activity had good stability at 30 and 50 °C (Fig. 4d). At 30 °C xylanase enzymes retained more than 50% of the activity after 48 h and 72 h in supernatants from experiments with and without light, respectively. At 50 °C xylanase activity behavior is similar for both experiments, showing the 50% of activity after 8 and 12 h for experiments with and without light, respectively. At 70 °C, the xylanase activity decreased rapidly for both experiments.
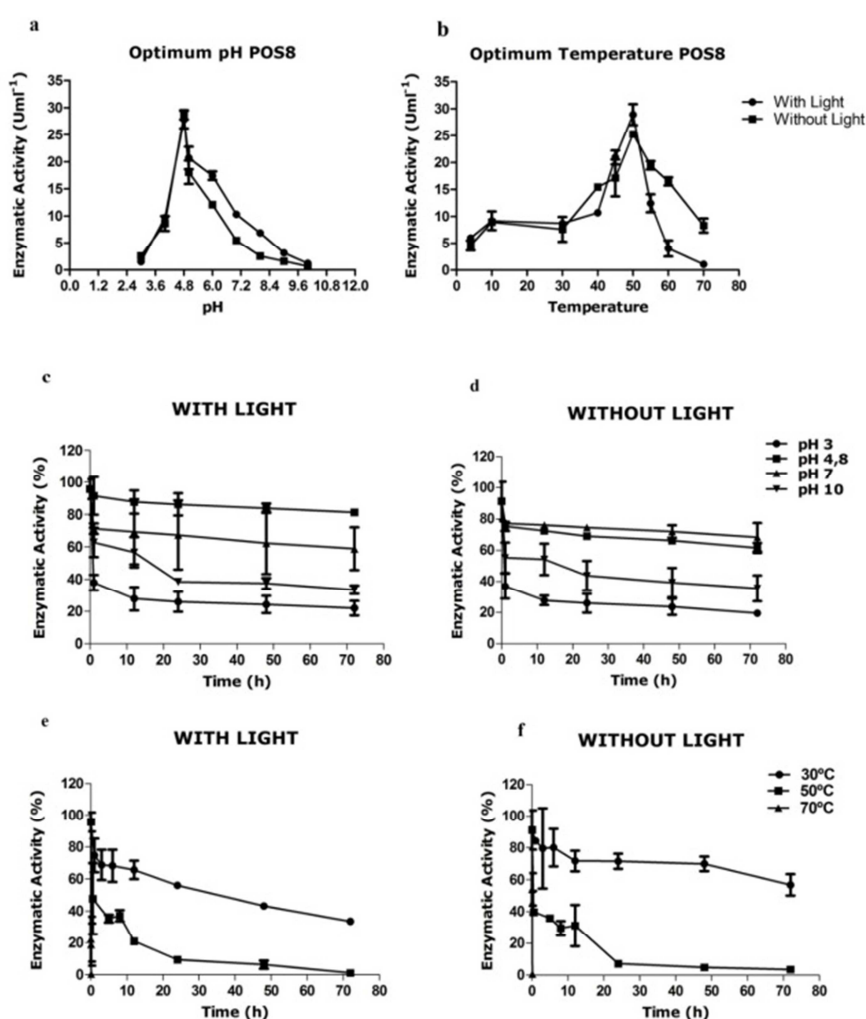

Figure 4. Characterization of xylanase activity of supernatants from *T. atroviridae* LBM 117. Effect of pH on xylanase activity, **4a**, and xylanase stability, **4c** and **4d**, of experiments carried out with and without light, respectively. Effect of temperature on xylanase activity, **4b**, and xylanase stability **4e** and **4f** of experiments with and without light, respectively.

Results from electrophoresis on SDS-PAGE of supernatants from *T. atroviride* LBM 117 exhibited hydrolyzing bands that appeared as clear zones. In supernatant from experiment with

light was found two xylanase isoforms of 105 and 215 KDa while in supernatant from experiment without was revealed only one isoenzyme of 105 KDa.
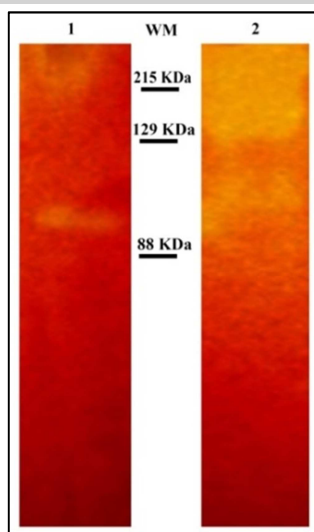
Figure 5. SDS gel electrophoresis of *T. atroviride* LBM 117 supernatant from experiments with (right) and without light (left). It showed two isoenzymes of 105 and 215 KDa.

DISCUSSION

Qualitative screening using solid medium containing xylan as the sole carbon substrate is a well-known method to identify xylanase producers based on the diffusion principle where the Congo red has specific link and binds strongly with xylan [12,18]. The halo detection is an easy and fast way of conducing screening and selecting strains even for the same species of microorganisms [19,20]. This technique allowed us to clearly identify eleven subtropical xylanase producing strains of *Trichoderma*. As *Trichoderma* species sporulate too much in presence of light and spread over the plate [21] making difficult the result interpretation when the mycelium covered all plate surface. Therefore, its growth needed to be monitored daily until they covered 80% of the plate. Once detect xylanase producing strains, a quantitative method was carried out to identify the higher xylanolytic producers. Xylanase production is affected by the substrate because it serves as carbon source and enzyme inducer [22]. Therefore, the quantitative screening was performed with commercial beechwood xylan, to guarantee an efficient xylanase production [23,24]. At the end of this experiment, *Trichoderma* sp. LBM 193, *T. atroviride* LBM 117, *T. harzianum* LBM 103, *T. harzianum* LBM 094, *Trichoderma* sp. LBM 192 grouped together in the conglomerate chart indicating that there was no significant difference between them. These fungi were the best xylanase producing strains yielding over 20 U mL^{-1} enzymes.

Although beechwood xylan could support high xylanase production, in this work it was observed that medium containing SCB yielded higher xylanase enzymes in most of the selected strains, particularly in *T. atroviride* LBM 117. This fungus reached the highest xylanase activity, when was incubated on SCB with light. Further, when fungi were incubated with light, they produced the highest xylanase activities in shorter periods than without light. The effect of light as a trigger on conidiation for several species of *Trichoderma* was already described [25,26]. Also, the increase transcription of cellulase genes in constant light compared with constant darkness was demonstrated [27,28]. However, this behavior has not been clearly described for transcription of xylanase genes. In this work, the positive effect of constant light on xylanase production was evident, the enzyme activity occurred earlier than in experiments carried out with darkness.

The xylanase activities yielded by *Trichoderma* strains using SCB, in particularly, *T. atroviride* LBM 117 reached at this point were much higher than the capacity of other species of Trichoderma. *T. atroviride* LBM 117 could produce 29 U mL^{-1} of xylanase enzymes, a 12-fold activity than the maximum xylanase production reported for *T. viride,* 2,45 U mL^{-1}, using maize straw as lignocellulosic material in submerged fermentation [29]. When was

compared with other lignocellulosic wastes, SCB supported xylanase activity in *T. atroviride* LBM 117 compared to wheat bran, (20.6 U mL), rice bran (21.8 U mL) and sawdust (10.7 U mL) as carbon sources using for xylanase production in *Penicillium* sp. SS1 [30]. However, SCB supported much higher xylanase activity in *T. atroviride* LBM 117 respect to *Aspergillus niger* ANL 301 when was incubated with wheat bran (6.47 U mL^{-1}), sugarcane pulp (0.95 U mL^{-1}), sawdust 0.65 (U mL^{-1}). [31]

In any industrial production process, the cost of the substrate plays a crucial role. Easy and cheap enzyme overproduction is important for an efficient biotechnological application [6]. Therefore, a low-value medium as a lignocellulosic waste is always preferred before a commercial substrate for enzyme production at industrial scale [22, 32]. In this study, the cost/effectiveness analysis indicated that xylanase production by *T. atroviride* LBM 117 is much cheaper using SCB than commercial xylan. This clearly demonstrate the economic advantage of using this agro-industrial waste as a suitable substrate. Besides, the extra value of this work is that it was conducted in the northeast of Argentina, where SCB is produced in large quantities and represents a waste, causing the deterioration of environment. In this sense, SCB is a widely available source for enzyme production. The obtained information serves for making decisions regarding the development of industrial technologies that allows full utilization of this waste and decreases costs and negative environmental impact. Tuck *et al.* [33] reviewed the opportunities for diverting residual biomass and affirmed that most abundant prospective feedstocks are carbohydrates and lignocellulosic biomass, providing the basis of biorenewable chemical production without the need to allocate land for specific crops dedicated to this aim.

In many applications, xylanase enzymes must be free of cellulose activity or have a very low traces of it, particularly in biobleaching; that is to avoid negative effects on the bioprocess as a poor paper quality caused by the hydrolysis of cellulose [34,35,36,37]. Supernatants from *T. atroviride* LBM 117 revealed very low cellulase enzyme activity demonstrating that the fungus

produced cellulase-poor xylanases. Furthermore, biobleaching process is strongly influenced by physic conditions as pH and temperature [1,6]. Hence, xylanase characterization for this type of application is required. In many studies the enzymes are purified and characterized [38]; however, characterizing xylanase activity in the supernatant could achieve an optimal enzyme yield and reduce the cost of enzyme purification [39]. In this work, *T. atroviridae* LBM 117 showed the best xylanase activity in supernatants at pH 4.8 and the stability was higher at pH 4.8 and 7, after 72 h. Then, the supernatant from *T. atroviride* LBM 117 could be used in technological applications where acidic and basic xylanases are required. The literature discloses that most of the fungi produce higher xylanase activity when pH is towards the acidic side from 4 to 7 [39,40,41,42]. On the other hand, optimal temperature for *T. atroviride* LBM 117 xylanase activity in supernatants was 50 °C and presented a good stability at 30 and 50 °C. Similarly, most of the reported fungal xylanases show their higher activity near to 50 °C [38,43]. In the literature, several methods have been described for xylanase activity detection in gels [18,44]. In this work, enzyme activity has been detected through the clear bands that appear against a bright red background stained with Congo red, showing different profiles for both experiments, with and without light.

CONCLUSION

The screening tests showed that eleven *Trichoderma* strains native from Misiones presented xylanolytic ability and five among them produce high enzyme activity of over 20 U mL^{-1}, when they were grown on commercial beechwood xylan as substrate. These strains were not only good xylanase producers when they were incubated in low-cost medium as SCB, but also yielded more xylanase activities than the ones obtained using beechwood xylan. *T. atroviride* LBM 117 was the highest xylanase producer on SCB and produced very low levels of cellulase enzymes. Hence, SCB can be considered as valuable renewable energy source for cellulase-poor xylanase production in this subtropical *Trichoderma* strain.

Xylanase enzymes in supernatants from *T. atroviride* LBM 117 demonstrated its

maximal performance at pH 4.8 and 50 °C while the enzyme activity was stable in a wide pH range and moderate temperatures. Profiles for both experiments with and without light were different, showing two isoenzymes of 105 and 215 KDa for the first one and only the isoenzyme of 105 KDa of the second one. As the aim of the work was to apply cellulase-poor xylanase in the biobleaching processing, immobilization of the enzyme is the principal purpose for the future work in order to obtain reusable cellulase-poor xylanases more stables at extremal pH and temperatures.

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