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Agrowastes as Feedstock for the Production of Endo-β-Xylanase from *Cohnella* sp. Strain AR92

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Paenibacillaceae · *Cohnella* · Sugarcane bagasse · Hemicellulose degradation

Abstract
Members of *Cohnella* sp. isolated from a variety of environments have been shown to be glycoside hydrolase producers. Nevertheless, most evaluations of members of this genus are limited to their taxonomic description. The strain AR92, previously identified as belonging to the genus *Cohnella*, formed a well-supported cluster with *C. thailandensis* and *C. formosensis* (>80% bootstrap confidence). Its growth and xylanase production were approached by using a mineral-based medium containing alkali-pretreated sugarcane bagasse as the main carbon source, which was assayed as a convenient source to produce biocatalysts potentially fitting its degradation. By means of a two-step statistical approach, the production of endoxylanase was moderately improved (20%). However, a far more significant improvement was observed (145%), by increasing the inoculum size and lowering the fermentation temperature to 25°C, which is below the optimal growth temperature of the strain AR92 (37°C). The xylanolytic preparation produced by *Cohnella* sp. AR92 contained mild temperature-active endoxylanase (identified as redundant GH10 family) for the main activity which resulted in xylobiose and xylo-oligosaccharides as the main products from birchwood xylan.

Introduction

Easily available lignocellulosic materials are substrates of interest for the development of integrated biorefinery. For this goal, the search and description of novel enzymes and microorganisms able to degrade plant biomass comprise a key strategy in for developing environment-friendly bioprocesses [Capolupo and Faraco, 2016]. Lignocellulosic biomass degradation is carried out by cooperative action among microorganisms to achieve the complete deconstruction of the plant cell wall. To this end, a panel of enzymes, including glycoside hydrolases (GHs), esterases, and ligninases, cleave polymeric complex substrates acting synergistically. Hemicellulases create better access to other hydrolytic enzymes within the

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Table 1. Phenotypic characteristics of *Cohnella* sp. AR92 and related species

	AR92	*C. formosensis*	*C. thailandensis*	*C. thermotolerans*
Growth in/at				
Temperature range, °C	15–45	20–50	20–50	20–55
pH range	6–9	6–8	6–9	5–9
3% NaCl	weak	–	+	weak
Gram staining	+	+	+	+
Spore formation	+	+	+	+
Hydrolysis of				
Xylan	+	+	+	+
CMC	+	–	+	+
DNA G + C content, %	56.0	58.3	53.3	59.0

Strains: *Cohnella* AR92 (this study); *C. formosensis* CC-Alfalfa-35^T [Hameed et al., 2013]; *C. thailandensis* KCTC 22296^T [Khianngam et al., 2010]; *C. thermotolerans* DSM 17683^T [Kämpfer et al., 2006]. CMC, carboxymethyl-cellulose.

cellulose microfibrils embedded into plant biomass; they therefore play a critical role by heightening the efficiency of lignocellulose decomposition [Dondelinger et al., 2016; Hu et al., 2013].

As GH producers, prokaryotes contribute actively to the depolymerization of hemicelluloses and cellulose in natural environments. Numerous reports have highlighted the extensive and unexplored diversity of the prokaryotic enzymatic systems involved, thereby contributing to unravelling the role of bacterial taxa in the decomposition of plant polysaccharides [Berlemont and Martiny, 2013; López-Mondéjar et al., 2016].

Here, we report on a new xylanolytic bacterium belonging to the *Cohnella* species. Members of this genus were isolated from a variety of environments, such as different soils, fresh water, and samples from the rhizosphere or endophytic compartments of plants [Kämpfer et al., 2006; Lee et al., 2015]. Though potential new species are listed in the NCBI database, besides the recent proposal of *C. lubricantis* sp. nov [Kämpfer et al., 2017], at the time of writing, there are 25 species recognized in the genus [Euzéby, 1997]. Several *Cohnella* species have been described as xylanolytic bacteria, but no further description of xylanase production exists to date. The literature is limited with regard to the production and characterization of enzymes from members of this genus. Among the few current reports, an intracellular GH10 xylanase from *C. laeviribosi* HY-21 was cloned and characterized by means of site-directed mutagenesis [Kim et al., 2010]; a thermophilic lipase from *Cohnella* sp. A01 was also cloned and characterized after purification of the recombinant product [Golaki et al., 2015]; and the production of chitinase from the A01 strain was recently published [Aliabadi et al., 2016].

This work aims to describe the production of the endoxylanolytic system from *Cohnella* sp. strain AR92, utilizing hemicellulose-rich substrates derived from agrowaste materials. The results are expected to contribute to the description of the *Cohnella* genus, particularly as a producer of GH potentially useful for developing a comprehensive exploitation of agricultural wastes.

Results

Description of Cohnella *sp. AR92*

Cohnella sp. AR92 is a gram-positive xylanolytic bacterium that grew optimally at 37°C and pH 7.0–8.0 in a tryptic soy broth (TSB) medium (approx. 10^8 CFU/mL at 24 h) (Table 1). After 2 days of incubation on tryptic soy agar (TSA) at 30°C, the strain developed circular colonies with regular margins, 0.5–1.0 mm in diameter, creamy white, convex and semitranslucent. The observation under a light microscope (×1,000) revealed motile cell rods with subterminal endospores, which were confirmed by scanning electron microscopy (SEM) (Fig. 1). The observation of flagella was difficult to confirm due to the presence of extracellular filamentous fibers that may be related to the formation of biofilm, although they lack typical characteristics (Fig. 1b).

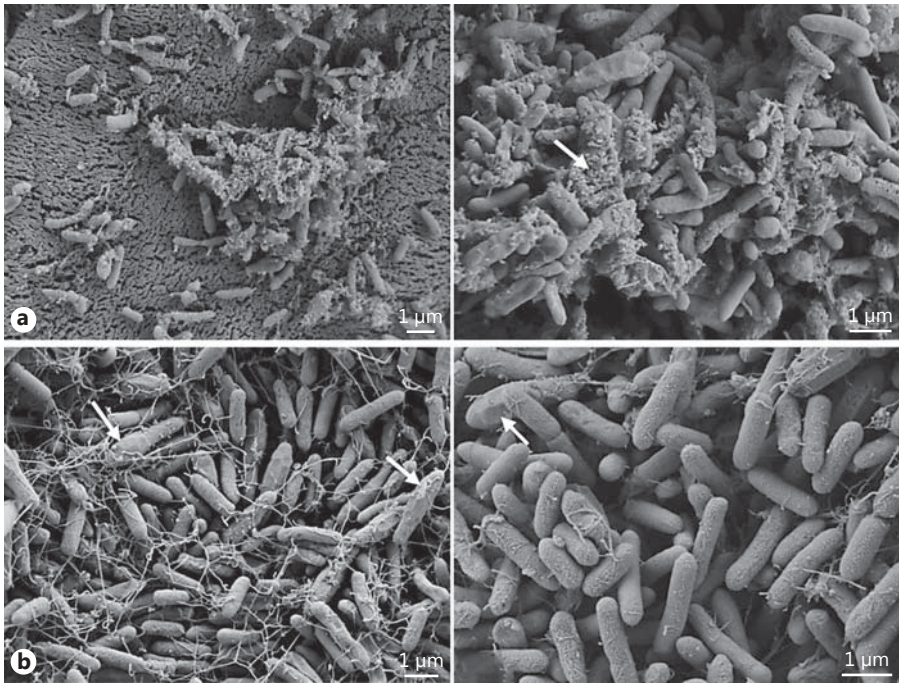

Fig. 1. SEM observation of *Cohnella* sp. AR92 cells at 72 h of cultivation. **a** Magnification of cells recovered from M02-OH-SCB medium. Arrow indicates amorphous structures probably corresponding to the hemicellulose fraction of the pretreated substrate. ×15,000 (left); ×25,000 (right). **b** Magnification of colonies from M02-OH-SCB agar plates. Arrows indicate sporulated bacteria. ×20,000 (left); ×30,000 (right). Typical rod size was 2.2–2.5 μm/0.4–0.5 μm.

The 16S rDNA sequence of the strain AR92 (GenBank accession No. FJ976043.1) was used to update its taxonomic position via an EzTaxon-e search. *Cohnella* sp. AR92 was placed within a monophyletic group containing all of the current type strains of the genus according to all phylogenetic trees reconstructed, where the studied strain formed a distinct phyletic lineage closely associated with *C. formosensis* CC-Alfalfa-35^T and *C. thailandensis* KCTC 22296^T with >80% bootstrap confidence, thus indicating a stable phylogenetic neighborhood (Fig. 2; online suppl. Fig. 1; for all online suppl. material, see www.karger.com/doi/10.1159/000480541).

Endo-β-1,4-Xylanase Production by Cohnella *sp. AR92*

Different carbon and nitrogen sources for xylanase production were first evaluated using the mineral medium M02 during a fermentation period of 192 h. By amendment with simple sugars, the enzyme production was nearly suppressed (≤0.1 IU/mL). In contrast, the enzyme production was effectively promoted in media containing complex carbohydrates as a carbon source. Compared to xylan from birchwood (5.0 ± 0.3 IU/mL for 96 h), the extracellular xylanase production was improved when wheat bran (WB) (6.4 ± 0.4 IU/mL for 96 h) and sugarcane bagasse (SCB) derivatives were used (Fig. 3). Maximum enzymatic activities were detected with alkali-pretreated SCB (OH-SCB) (13.1 ± 0.6 IU/mL for 96 h) and hemicellulose fractioned from SCB (HC-SCB) (13.2 ± 0.4 IU/mL for 96 h). In both media, the activity remained with no significant changes until 192 h of cultivation; and the Tukey test showed no statistical differences between xylanase activities obtained with the agro-based substrates OH-SCB and HC-SCB.

With regard to the nitrogen source, ammonium sulfate resulted in maximum biomass and enzyme production (25% increment) in comparison with the organic nitrogen sources assayed (online suppl. Fig. 2). As a result, OH-SCB and $(NH_4)_2SO_4$ as the source of carbon and nitrogen, respectively, and a fermentation period of 96 h, were selected for subsequent enzyme production assays.

Identification and Optimization of Key Variables Affecting the Enzyme Production

On the basis of a Plackett-Burman design (online suppl. Table 1), the medium components with a significant effect on xylanase and biomass production ($p < 0.05$) were yeast extract (YE), Na_2HPO_4/KH_2PO_4 (P), and OH-SCB (C), but $(NH_4)_2SO_4$ (N) and $CaCl_2 \times 2H_2O$ (Ca) did not exert any influence ($p > 0.05$). $MgCl_2 \times 6H_2O$ (Mg) showed a slightly significant effect, but only on the enzyme production (online suppl. Table 2). In order to maximize the xylanase production, the components C, P, and YE were subjected to a Box-Behnken design (online suppl. Table 3), where Mg and N were fixed at their maximum levels. The

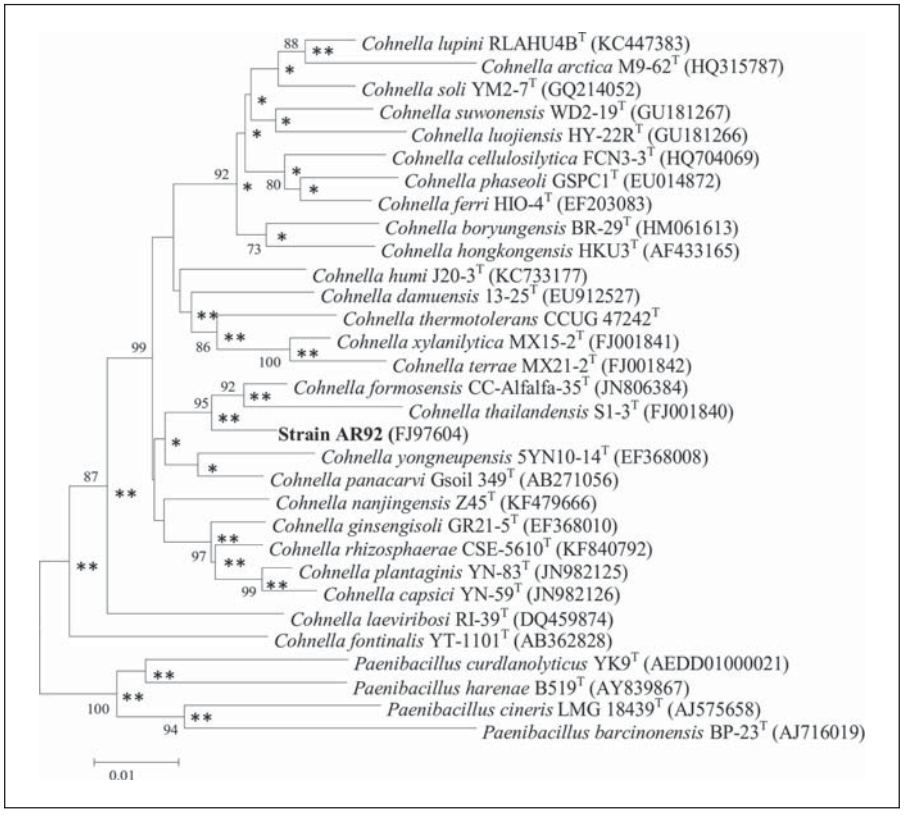

Fig. 2. Neighbor-joining phylogenetic tree based on 16S rDNA sequences, showing the position of the strain AR92 and related taxa. Bootstrap values are shown as percentages of 100 replicates; only values >70% are shown. Nodes marked with 1 asterisk were supported by one of the other applied treeing methods (maximum likelihood or maximum parsimony); 2 asterisks indicate nodes supported in trees calculated with both other applied treeing methods. Four species of the genus *Paenibacillus* were used as an outgroup. Bar, 0.01 substitutions per nucleotide position. The accession Nos. appear after the names.

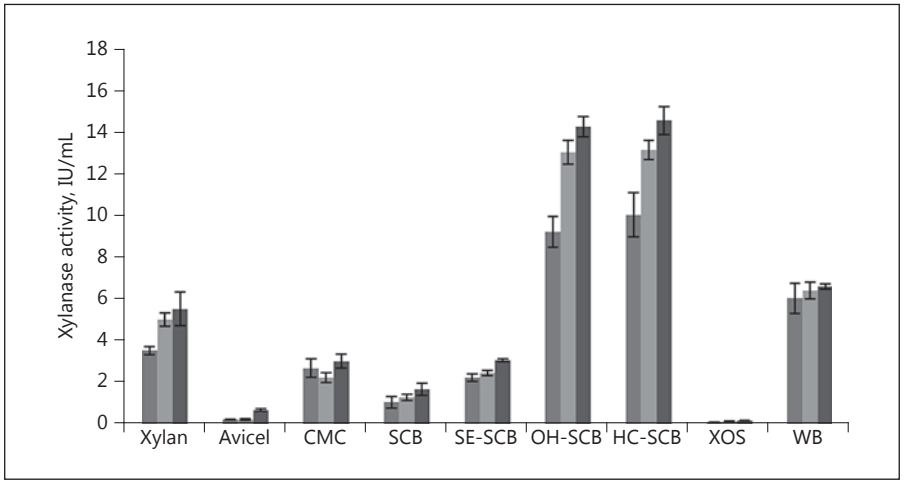

Fig. 3. Extracellular xylanase activity of *Cohnella* sp. AR92 grown on various carbon sources at 48, 96, and 192 h. Error bars represent the SD calculated from at least 3 independent experiments. Xylan, birchwood xylan; CMC, carboxymethyl-cellulose; SCB, sugarcane bagasse; SE-SCB, solid fraction from the steam explosion of SCB; OH-SCB, alkali-treated SCB; HC-SCB, hemicellulose from SCB; XOS, liquid fraction from steam explosion of SCB; WB, wheat bran.

analysis of variance for the model expressed below is shown in online supplementary Table 4. By considering the significant terms, the experimental results were fitted to a second-order polynomial equation (R^2 = 98.2%):

$$EA = -4.11007 + 2.48328 \times C + 15.655 \times YE + 0.0225 \times P - 0.181155 \times C^2 + 0.367862 \times C \times YE - 7.33395 \times YE^2 + 0.845 \times YE \times P - 1.14026 \times P^2,$$

where EA is the response variable (xylanase activity, IU/mL), and C, YE, and P are the concentrations of alkali-pretreated SCB, YE, and phosphates, respectively. The analysis of 3-dimensional response plots and their corresponding contour plots demonstrated that the objective function is unimodal in nature and showed an optimum at the boundaries (online suppl. Fig. 3).

Table 2. Influence of operational parameters on the xylanase production of *Cohnella* sp. strain AR92

Parameter	Xylanase activity, IU/mL	Protein, μg/mL	Specific activity, IU/mg	Biomass, CFU/mL
Initial biomass[a] (approx.)				
5×10^5 CFU/mL	7.6±1.2	108.0±8.0	71.1±15.9	$3.2 \pm 0.7 \times 10^6$
5×10^6 CFU/mL	15.3±0.6	170.0±7.0	90.3±6.8	$2.6 \pm 0.5 \times 10^7$
5×10^7 CFU/mL	30.7±0.4	181.0±5.0	169.3±7.2	$2.4 \pm 0.9 \times 10^7$
5×10^8 CFU/mL	32.0±1.7	213.0±12.0	150.4±16.8	$4.0 \pm 1.1 \times 10^7$
Agitation[b]				
100 rpm	11.2±0.8	137.0±10.0	81.6±11.8	$2.7 \pm 0.7 \times 10^7$
200 rpm	29.8±1.1	178.0±6.0	167.2±11.4	$3.6 \pm 0.5 \times 10^7$
300 rpm	32.2±1.0	195.0±6.0	165.3±9.9	$2.7 \pm 0.3 \times 10^7$
Initial pH[c]				
6.0	18.6±1.1	139.0±5.0	133.8±12.7	$1.1 \pm 0.6 \times 10^7$
7.0	31.4±1.1	182.0±8.0	172.1±13.3	$3.0 \pm 0.7 \times 10^7$
8.0	30.5±1.1	178.0±5.0	170.9±11.1	$2.2 \pm 0.3 \times 10^7$
9.0	3.1±0.2	97.0±5.0	31.6±3.5	$1.3 \pm 0.3 \times 10^7$
Temperature[d]				
25°C	46.3±1.0	160.0±7.0	289.6±18.0	$1.2 \pm 0.20 \times 10^7$
30°C	32.2±1.9	185.0±6.0	173.7±16.3	$2.9 \pm 0.1 \times 10^7$
37°C	11.5±0.6	191.0±9.0	59.9±6.3	$3.9 \pm 0.9 \times 10^7$

The values corresponded to 96 h of cultivation and are the means ± SD of 3 independent replicates.
[a] 200 rpm; pH = 7.0; 30°C. [b] 5×10^7 CFU/mL; pH = 7.0; 30°C. [c] 5×10^7 CFU/mL; 200 rpm; 30°C.
[d] 5×10^7 CFU/mL; 200 rpm; pH = 7.0.

The expected xylanase activity of 16.2 IU/mL was estimated for the predicted optimal medium, which contained: OH-SCB, 8.2 g/L; YE, 1.3 g/L; and Na_2HPO_4/KH_2PO_4, 4.4/1.2 g/L. Its experimental verification resulted in 15.8 ± 0.6 IU/mL of xylanase at 96 h of fermentation, a value that denoted a 20.6% increment compared to the xylanase activity obtained with the initial medium composition (13.1 ± 0.6 IU/mL) (Fig. 3).

With regard to the fermentation parameters, the xylanase production by *Cohnella* sp. AR92 was higher, in the range of pH 7.0–8.0 and 200 rpm (Table 2). Otherwise, raising the initial biomass concentration to 10^7–10^8 CFU/mL rendered an improvement of 100% of the enzyme activity (approx. 30 IU/mL). The fermentation temperature also had an important effect on xylanase production, increasing from 32.2 ± 1.9 IU/mL at 30°C to 46.3 ± 0.9 IU/mL at 25°C at 96 h. This implied an additional increment of approximately 45% of the extracellular activity obtained (Table 2).

Xylanase Preparation

The hemicellulolytic preparation from *Cohnella* sp. AR92 contained endo-β-1,4-xylanase as the primary enzyme activity implicated in xylan conversion. The activity of other enzymes was also detected in the crude extract, including that of endoglucanase, mannanase, avicelase, arabinofuranosidase, acetyl esterases, glucosidase and xylosidase (Table 3). The main enzymatic products

Table 3. Extracellular enzyme activities detected in the xylanolytic cocktail produced by *Cohnella* sp. AR92 grown on optimum mineral medium with OH-SCB at 96 h

Substrate	Enzyme activity, IU/mL	Specific activity, IU/mg
Xylan	46.3±0.9	289.6±18.0
*p*NFβ-D-xylopyranoside	0.12±0.01	0.76±0.11
*p*NFα-L-arabinofuranoside	0.13±0.01	0.82±0.12
*p*NFβ-D-glucoside	0.11±0.02	0.69±0.12
*p*NF acetate	0.10±0.01	0.61±0.09
CMC	0.31±0.03	1.92±0.27
Avicel	0.05±0.01	0.34±0.06
Locust bean gum	0.12±0.01	0.75±0.09

Values are expressed as means ± SD. OH-SCB, alkali-pretreated SCB; *p*NF, *p*-nitrophenyl; CMC, carboxymethyl-cellulose.

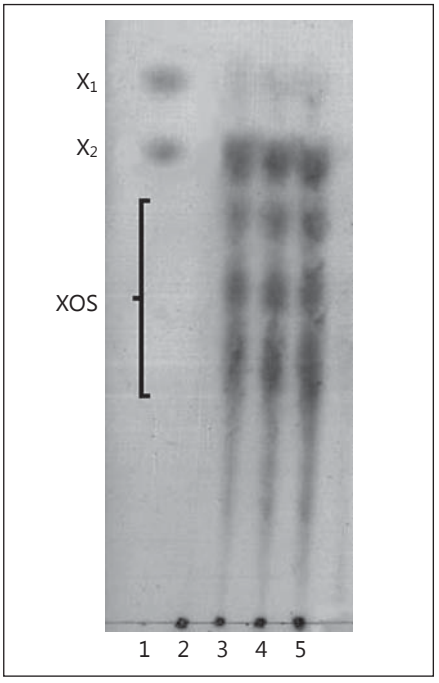

Fig. 4. Products generated from birchwood xylan by the xylanolytic cocktail. Reaction mixtures contained 10 IU of xylanase, 2% (w/v) of xylan at 40 °C and pH 6. Lanes: 1, xylose (X_1) and xylobiose (X_2) standards; 2, xylan control without enzyme; 3–5, products obtained after 3, 8, and 24 h of incubation, respectively.

released, demonstrated by thin-layer chromatography analysis after hydrolysis on birchwood xylan after 3, 8, and 24 h of reaction, were xylobiose and xylo-oligomers, while minor amounts of monomeric xylose were also detected (Fig. 4).

Silver staining of native polyacrylamide gel electrophoresis (PAGE) showed a pattern of the extracellular proteins spread between 50 and 600 kDa (Fig. 5a). The zymographic analysis showed an enzyme profile that changed along the time course of cultivation. From 72 h onwards, at least 6 bands of xylanase activity appeared, with the molecular weight (MW) ranging from 120 to 230 kDa (Fig. 5b). Given the apparent MW of xylanolytic bands observed, 4 bands were retrieved from a replica PAGE stained with Coomasie blue. They were identified by mass spectrometry as endo-β-xylanases belonging to the GH10 family of high MW (>100 kDa) using the UniProt database (Table 4). The matching sequences corresponded to multimodular xylanases belonging to *Paenibacillus* sp.

The xylanolytic preparation exhibited the maximum activity at 60°C and retained 56 and 83% of the maximum at 40 and 50°C, respectively (Fig. 6a). The xylan hydrolysis at different pH values produced a bell-shaped response, with an optimum at pH 6.0, and presented >65%

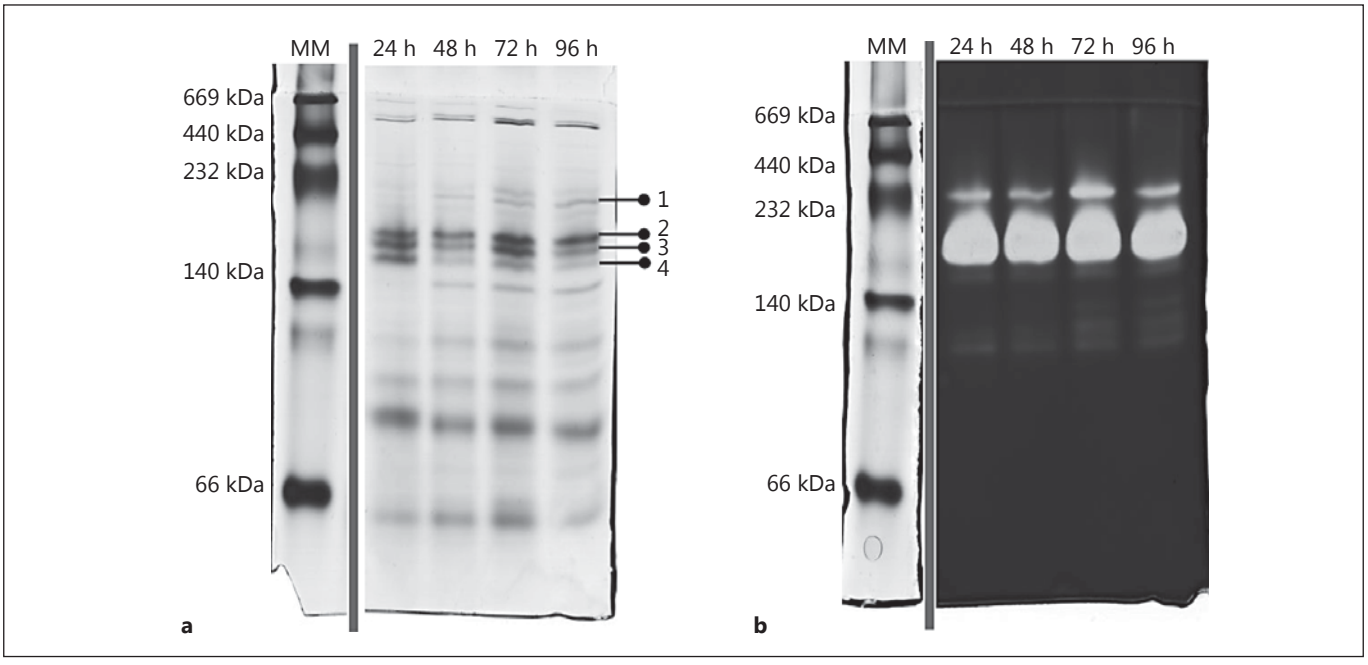

Fig. 5. Extracellular protein profile and zymographic analysis of *Cohnella* sp. AR92 culture. Native ND-PAGE silver staining (**a**) and zymogram (**b**) using birchwood xylan as a substrate. Black lines with dot indicate bands recovered for protein identification by mass spectrometry. MM, molecular markers.

of its activity within the pH range of 5–7 (Fig. 6b). The thermal stability profile, after 8 h of incubation, showed total preservation of the enzyme activity in the range of 30–40°C. Moreover, activity was fully preserved after preincubation at 30°C for 72 h (Fig. 6c). The xylanolytic preparation retained 100% of its activity after 6 weeks of storage at 4–8°C (data not shown). In contrast, after 1 h incubation at 50°C, the residual activity decreased considerably and was absent after treatments at 60–80°C. A remarkable pH stability of the xylanolytic activity (>70%) was observed within pH 4.0–10.0, though near full stability was found between pH 5.0–7.0 (Fig. 6b).

Xylanase activity was assayed under standard conditions in the presence of several metal ions (Table 5). Cations Mg^{2+}, Mn^{2+}, and Co^{2+} slightly stimulated the activity, Cu^{2+} and Fe^{3+} had an inhibitory effect, and Hg^{2+} nearly inactivated the enzyme activity. Triton X-100 did not affect the xylanolytic preparation, and residual values ≥84% were observed in the presence of Tween 80 and PEG 8000, but SDS triggered an important inhibition of the xylanase activity. Concerning ionic strength, NaCl concentrations of up to 1.5 M NaCl did not show any influence on the enzyme activity, which retained >60% of its endoxylanase activity in the presence of 3.0 M NaCl. Finally, values of 82 and 57% of the enzyme activity were conserved at ethanol concentrations of 5 and 10% (v/v), respectively (Table 5).

Discussion

The strain AR92 was confirmed as a member of the *Cohnella* genus on the basis of the 16S rDNA, and forming a well-supported cluster with *C. formosensis* CC-Alfalfa-35^T and *C. thailandensis* KCTC 22296^T (Fig. 2; online suppl. Fig. 1). In addition, these 3 xylanolytic strains share several phenotypic and biochemical characteristics (Table 1).

Table 4. Identified peptides from proteins isolated from ND-PAGE

Band No.	Identification	UniProt accession No.	Theoretical MM, kDa	Matched peptides with high confidence
1	β-xylanase from *Paenibacillus* sp. soil 787	A0A0Q9TWX1	109.6	1. QLIQGVGMQEHDGLGTNPSNTR 2. AGNSVTVTR 3. ATDDNYVEQAFK 4. TAQAAQYAR 5. NITYYIDNFVVK
	β-xylanase from *Paenibacillus* sp. P46E	A0A1Q5XUT6	135.4	1. VRYDGFASVDAGMTDNQGIATK 2. DINDRWALDHPGK
2	β-xylanase from *Paenibacillus* sp. IHB B 3415	A0A0B2FBF4	144.4	1. LYYNDYNDDNQNK 2. LLIDGVGMQAHYNINTNPDNVK
	β-xylanase from *Paenibacillus* sp. W-61	Q8GHJ4	142.7	1. LYYNDYNDDNQNK 2. EINENYAK
	β-xylanase from *P. rhizosphaerae*	A0A1R1EKF7	144.3	1. YQMAWQGATGTAK 2. ASSNPLLFDK
3	β-xylanase from *P. rhizosphaerae*	A0A1R1EKF7	144.3	1. EVLDDHPDWDIK 2. YQMAWQGATGTAK 3. ASSNPLLFDK 4. VTFWGMDDGTSWR
	β-xylanase from *P. odorifer*	A0A1R0ZJB3	160.7	1. MHHNVATAGNAMKPDALQPTK 2. ASSNPLLFDKNLQAK 3. ASSNPLLFDK
4	β-xylanase from *P. rhizosphaerae*	A0A1R1EKF7	144.3	1. YQMAWQGATGTAK 2. VTFWGMDDGTSWR

Bands correspond to GH10 xylanase enzymes secreted by *Cohnella* sp. AR92 grown on optimum mineral medium with alkali-pretreated SCB at 96 h. MM, molecular mass.

Table 5. Effect of additives, salt and ethanol concentration on the xylanolytic activity of *Cohnella* sp. AR92

Additive	Relative activity, %	Tukey test
Control	100.0±3.8	d, e
Na^{+}	93.3±2.8	d–f
K^{+}	95.6±2.2	d–f
Mg^{2+}	112.8±5.3	b, c
Zn^{2+}	101.4±0.5	c–e
Ca^{2+}	98.5±1.0	d, e
Ba^{2+}	104.1±2.4	b–d
Cu^{2+}	41.7±2.4	g
Fe^{3+}	43.6±2.0	g
Fe^{2+}	84.2±5.9	f
Hg^{2+}	3.7±0.3	i
Co^{2+}	114.6±7.0	b
Mn^{2+}	129.2±4.1	a
Triton X-100	100.0±4.0	d, e
EDTA	85.1±1.1	f
PEG 8000	89.5±0.5	e, f
Tween 80	84.1±5.0	f
SDS	28.0±0.9	h
NaCl concentration		
0.0 M	100.0±1.8	a
0.1 M	101.0±2.2	a
0.5 M	97.4±4.3	a
1.0 M	94.5±4.7	a
1.5 M	91.2±1.9	a, b
2.0 M	83.1±0.6	b, c
2.5 M	72.6±1.8	c, d
3.0 M	65.1±3.5	d
Ethanol concentration		
0.0 M	100.0±4.4	a
5.0 M	81.9±1.0	b
10.0 M	56.7±5.2	c
15.0 M	26.6±4.4	d
20.0 M	20.3±2.3	d

Values are expressed as means ± SD. Data from triplicates were grouped according to the Tukey test ($p < 0.05$): equal letters, no significant differences from each other. The enzymatic assays were performed at 60°C and pH 6 for 30 min and 100% activity corresponded to approximately 55.0 IU/mL.

The utilization of simple carbohydrates as the sole carbon source in the mineral-based medium did not allow extracellular xylanase production by *Cohnella* sp. AR92, probably due to catabolic repression as also observed for the related *Paenibacillus* sp. [Dheeran et al., 2012; Fukuda et al., 2010]. In contrast, the lignocellulosic substrates OH-SCB and HC-SCB were the most effective sources for enzyme production by *Cohnella* sp. AR92 (Fig. 3). The usefulness of feedstocks for enzyme production was also reported for members of the *Paenibacillaceae* family [Arora et al., 2014; Rakotoarivonina et al., 2012]. *Cohnella* sp. AR92 showed a continuous enzyme production during a prolonged stationary phase and a low sporulation rate when grown on such lignocellulosic material as the carbon source (Fig. 1). Similarly, it was suggested that the growth of *Thermobacillus xylanilyticus* on lignocellulosic materials promoted the extracellular accumulation of proteins and simple sugars during the exponential growth phase, which extended the viability of the microorganism as well as a low degree of sporulation [Rakotoarivonina et al., 2012]. These findings might also be related to the result of a physiological adaptation to complex microbial niches related to lignocellulosic recycling in nature. Accordingly, Rhee et al. [2014] hypothesized that the aptness of complex substrates for the enzyme production of *Bacillus subtilis* 168 might be due to a higher affinity to oligomers than to the single molecules (monomers) of the ABC sugar transporters.

By means of a Plackett-Burman design, the carbon source, the YE, and the phosphorous source were identified as the main factors affecting the xylanase production by *Cohnella* sp. AR92 whereas a Box-Behnken approach showed that the enzyme production was improved along with gradual increments of these nutrients up to the optimum values (online suppl. Fig. 3). The importance of the carbon source concentration for extracellular GH production was previously targeted, as growing concentrations of the corresponding carbohydrates could effectively promote an increased enzymatic production by fungi and bacteria [Bano et al., 2013; Cui and Zhao, 2012]. The poor growth of *Cohnella* sp. AR92 observed at the lowest assayed level of filtered YE suggested auxotrophy regarding some B vitamins (online suppl. Table 1). Lemos et al. [2001] reported the significant influence of filtered YE on xylanase production by *Aspergillus awamori* NRRL 3112, but attributed it to the ion content of the YE. Finally, the salts combination used as phosphorous source had the lowest influence on the enzyme production. While the optimum medium defined conducted to a moderate increment of the enzyme production (20%), a far more significant improvement was observed (145%) by increasing the inoculum size and lowering the fermentation temperature to 25°C (Table 2). A similar outcome was reported for extracellular chitinase production by *Cohnella* sp. A01 [Aliabadi et al., 2016]. In our study, a final endoxylanase titer of 46.3 ± 1.0 IU/mL was achieved, implying an overall 9-fold improvement, with respect to the screening medium containing birchwood xylan as a carbon source (5.0 ± 0.3 IU/mL for 96 h).

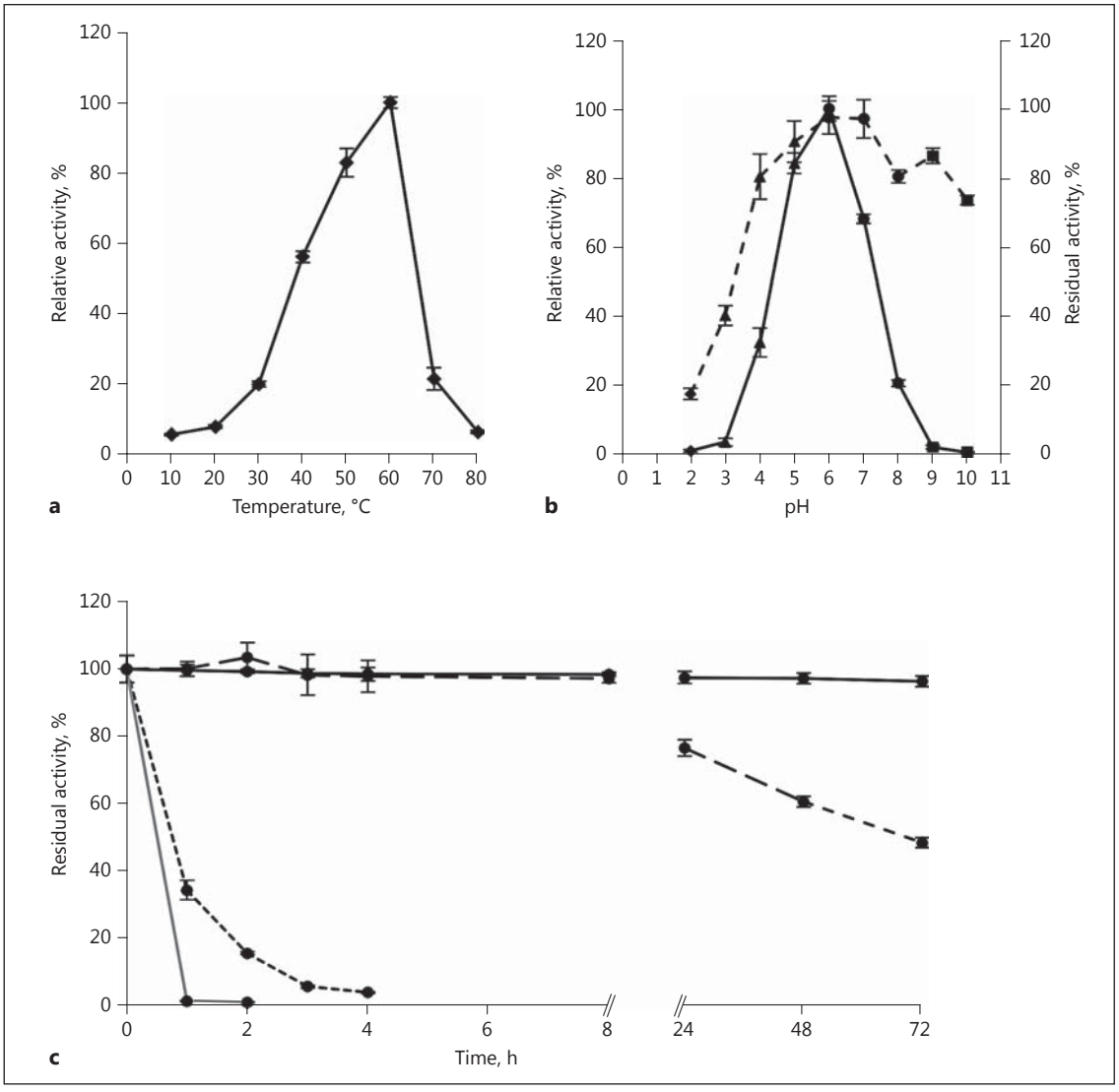

Fig. 6. a Effect of the temperature on endoxylanase activity. **b** Effect of the pH on endoxylanase activity (solid line) and stability (dashed line). Buffers were: (◆) glycine-HCl; (▲) sodium citrate-citric acid; (●) sodium phosphate; (■) sodium carbonate-sodium bicarbonate. **c** Thermal stability of the enzyme preparation, pre-incubation temperatures: solid line 30°C; long-dashed line 40°C; short-dashed line 50°C, and grey line 60–80°C. The *x* axis represents the pre-incubation time. One hundred percent activity corresponds to 55.8 ± 0.89 IU/mL (**a**), 54.3 ± 1.14 IU/mL (**b**) and 57.1 ± 1.9 IU/mL (**c**). Error bars represent the standard deviation calculated from at least three independent experiments.

With regard to the xylanolytic cocktail produced, the main products from xylan degradation were xylobiose and XOS, while xylose was also detected. This indicated the presence of endo-β-xylanase in the major activity and beta-xylosidase as a minor component of the enzymatic preparation (Table 3; Fig. 4). This conclusion was supported by our preliminary analysis of the whole-genome sequence of the studied strain where sequences encoding potential extracellular GH10 and GH43 were detected (data not shown). Furthermore, endo-β-xylanase was the main hemicellulase produced, confirmed by the sequence analysis of dominant bands recovered from non-denaturing (ND)-PAGE identified as GH10 by mass spectrometry (Table 4). Redundant codifying sequences were also observed in related taxa having multidomain GHs of high MW (>100 kDa) with tandem-repeated carbohydrate-binding domains and S-layer homology (SLH) domains [Sawhney et al., 2016; WGS RefSeq:

NZ_AZXQ00000000.1]. Cells growing attached to the debris of lignocellulosic substrates were reported for members of the *Paenibacillaceae* family, with extracellular surface layer-associated GHs [López-Mondéjar et al., 2016; Pason et al., 2006; St John et al., 2012]. In agreement, the cells of the strain AR92 were observed growing attached to insoluble OH-SCB, frequently imbibed into amorphous structures (Fig. 1a).

The endo-β-xylanase preparation produced was stable at 30 and 40°C, and within the acid-to-neutral range of pH (5.0–7.0), and it was also tolerant of ethanol in the reaction mixture (Table 3), conditions that makes it potentially useful for simultaneous saccharification and fermentation processes for second-generation ethanol production. The xylanolytic cocktail exhibited salt tolerance, observed up to 3.0 M NaCl, and even to cations such as Mg^{2+}, Mn^{2+}, and Co^{2+}, which is desirable for agrowaste processing. It also displayed a high storage stability (at 8°C for 6 weeks), a feature that could be related to the low protease activity which is a common characteristic of this genus [Cho et al., 2007]. The low protease activity has facilitated technological applications for these bulk enzymatic preparations; as was proposed for protein enrichment of corn-cob heteroxylan by *Bacillus stearothermophilus* [Ugwuanyi et al., 2008].

Comparable properties were observed for a recombinant hemicellulases from the *C. laeviribosi* HY-21 and *Paenibacillus* sp. E18 [Kim et al., 2010; Shi et al., 2010] strains as well as for the cellulases from *Bacillus* sp. AR03, a strain isolated from the same niche [Manfredi et al., 2016].

Concluding Remarks

A systematic approach was applied to improve the enzyme production by *Cohnella* sp. AR92 employing abundant and inexpensive feedstocks, in order to evaluate the production of biocatalysts potentially useful for agrowaste processing.

Alkali-pretreated SCB was the carbon source that better promoted the xylanase production, which was also influenced by the YE and the phosphate source. However, the factors that significantly enhanced the enzyme production were the inoculum size and the cultivation at a suboptimal temperature (25°C). The bulk of the enzymatic preparation produced contained endo-β-1,4-xylanase, which belongs to the GH10 family, as the primary enzyme involved in the activity implicated in xylan conversion, with xylobiose and XOS as the main products.

To date, *Cohnella* sp. AR92 is one of a few isolates of the genus being evaluated with regard to its growth and enzyme production. Its xylanolytic repertoire and behavior makes this strain interesting for further study, motivating ongoing genomic and proteomic analysis.

Experimental Procedures

Characterization of the Strain AR92 and Phylogenetic Analysis

The strain AR92 was cultured in TSB or TSA (Laboratorios Britania S.A., Argentina) at 30°C for 48 h. Colonies were morphologically examined and a gram reaction was performed according to Murray et al. [1994]. Cell morphology, endospores, and motility were observed using an Olympus CX31 optical microscope and the Supra 55VP for SEM. Growth at different temperatures (15–55°C) and pH (4.0–10.0), tolerance to NaCl (0.5–5%) were performed in TSB at 24 and 48 h of cultivation. Semiquantitative tests for xylan and cellulose degradation were carried out in TSA containing 1% (w/v) birchwood xylan or 1% (w/v) carboxymethylcellulose (CMC) (Sigma-Aldrich, Argentina); after 48 h microbial growth, the Petri dishes were developed with 2 g/L Congo red solution (5 mL), and transparent halos on a red background indicated hydrolytic activity [Teather and Wood, 1982].

The 16S rDNA sequence (GenBank accession No. FJ976043.1) was fitted into an EzTaxon-e server to taxonomically relate the isolate to close strains [Kim et al., 2012]. The alignment of the sequences selected from the previous analysis was built by using the SINA service from the SILVA database [Pruesse et al., 2012], and edited to remove gaps and ambiguous nucleotides. Evolutionary distances were calculated using the Tamura-Nei method and phylogenetic trees were reconstructed by employing the NJ, MP, and ML methods using MEGA 6 [Tamura et al., 2013]. Confidence values of the branches of the trees were determined by using bootstrap analyses based on 100 resamplings. The phylogenetic trees were checked for consistency and conserved-nodes trees were marked by asterisks.

Growth and Enzyme Production

A based medium, designed as M02, was employed for xylanase production: $(NH_4)_2SO_4$ 0.5 g/L; Na_2HPO_4 3.5 g/L; KH_2PO_4 1 g/L; $MgCl_2 \times 6H_2O$ 0.5 g/L; $CaCl_2 \times 2H_2O$ 0.1 g/L; YE 1 g/L; and pH 7.2. Different carbon sources (5.0 g/L) were used to evaluated enzyme production: (i) simple sugars: glucose, fructose, xylose, sucrose, and maltose; (ii) polymers: CMC, Avicel-cellulose and birchwood xylan; and (iii) lignocellulosic materials: SCB, OH-SCB, steam-explosion pretreated SCB (SE-SCB), HC-SCB [Breccia et al., 1995], and WB. The SCB was obtained from a local pulp and paper plant, washed thoroughly, dried at 45°C up to constant weight, and then milled to obtain a semifine powder (passing through a 1-mm-mesh sieve). Different substrates were produced from SCB being washed but not milled: OH-SCB was produced according to Manfredi et al. [2015]; the steam explosion treatment of SCB (179°C for 10 min) rendered the solid fraction SE-SCB and the liquid fraction rich in xylo-oligosaccharides (XOS) [Manfredi, pers. commun.]. All the SCB-derived substrates and the WB were dried and milled in the same way as SCB. YE, sterilized by filtration, was used as the source of mineral and growth factors (e.g., vitamins) while 0.5 g/L of $(NH_4)_2SO_4$, casein peptone, or soya pep-

tone (Britania) were assayed as the nitrogen source. The inocula were prepared by growing the microorganism in TSB medium at 30°C in a rotary shaker (200 rpm) for 14 h, which routinely produced motile cells that were not sporulated, as checked by microscopic evaluation. Cells were washed twice with NaCl 0.9% (w/v) to inoculate with a 10% (v/v) cellular suspension, adjusting an initial cell concentration of approximately 10^6 CFU/mL. Samples of 1 mL were taken at regular intervals for CFU/mL determination and to separate the cell-free supernatants by centrifugation (10,000 rpm for 10 min) to test the xylanase activity.

Medium Components and Fermentation Parameters Affecting Xylanase Production

A Plackett-Burman design was used to screen the culture media components that influence growth and xylanase production by *Conhella* sp. AR92: C, YE, P, N, Ca, and Mg. The phosphorous source was utilized as a combination of both salts, upholding a constant ratio of 3.5/1.0 (online suppl. Table 1). Analysis of the effects of each independent variable was carried out with the Statgraphics Centurion XVI software. Significant variables were selected on the basis of the *F* test and the *p* value at a 95% significance level. Furthermore, response surface methodology optimization of the screened components for xylanase production was carried out via a Box-Behnken experimental design. Factors C, YE, and P were evaluated at 3 levels including 4 replicates at the center point. The design generated 16 treatment combinations (performed in duplicate), which were assayed in 2 blocks (online suppl. Table 3). Data from the design was subjected to a second-order multiple regression analysis with Statgraphics to obtain the parameter estimators of the mathematical model.

The fermentation parameters inoculum size, agitation, initial pH, and temperature were analyzed by means of a one-factor-at-a-time approach utilizing the optimal medium composition obtained. Inocula were prepared to adjust initial biomass values of 10^5–10^8 CFU/mL and the agitation of the flasks was assayed in the range of 100–300 rpm. The initial pH of media was adjusted to between 6 and 9. Finally, cultivation at different temperatures was carried out at 25–37°C. The condition of each parameter that showed maximum enzyme activity was used for assaying the next one. Samples were taken after 96 h of incubation.

Enzymatic Activity Determinations

Xylanase, endoglucanase, avicelase, and mannanase activity was determined using birchwood xylan, CMC, Avicel-cellulose, and mannan, respectively. The reaction mixture contained 0.45 mL of 1% (w/v) suitable substrate in 100 mM of sodium phosphate buffer, pH 6.0, and 50 µL of sample. After incubation for 30 min at 50°C, the released reducing sugars were measured by the dinitrosalicylic acid method (DNS) [Miller, 1959]. One unit of enzyme activity was defined as the amount of enzyme required to release 1 µmol of reducing sugars (xylose or glucose) per minute.

β-Glucosidase, β-xylosidase, α-L-arabinofuranosidase, and acetyl-esterase activities were determined using *p*-nitrophenyl β-D-glucopyranoside, *p*-nitrophenyl β-D-xylopyranoside, *p*-nitrophenyl α-L-arabinofuranoside and *p*-nitrophenyl acetate (Sigma), respectively. Reactions were performed at pH 6 and 50°C for 30 min as described by Pinheiro et al. [2016].

Protein concentration was determined by the method of Bradford [1976], using bovine serum albumin as standard, and specific activity was calculated as IU/mg of total protein contained.

The degradation products resealed from the xylan reaction after 3, 8, and 24 h of incubation (at 40°C and pH 6.0) were separated by thin-layer chromatography according to Rhee et al. [2014]. Plates were dried and sprayed with a solution containing 90 mL of ethanol with 0.2 g of anthron in 10 mL of sulfuric acid, and then heated at 100°C to reveal the resolved components.

Xylanolitic Cocktail Characterization

Xylanase activity was measured at different temperatures (10–80°C) and different pH values (2.2–10.0). These assays and enzyme stability were performed according to Manfredi et al. [2016]. The effect of the following additives (5 mM) on the enzyme activity was assayed in the reaction mixture: NaCl, KCl, $CaCl_2 \times 2H_2O$, $BaCl_2 \times H_2O$, $MgSO_4 \times 7H_2O$, $MnSO_4 \times H_2O$, $CuSO_4 \times 5H_2O$, $ZnSO_4 \times 7H_2O$, $CoSO_4$, $FeSO_4 \times 7H_2O$, $FeCl_3$, $HgCl_2$, EDTA, SDS, PEG 8000, Triton X-100, and Tween 80. Salt concentration (0.1–3.0 M) and ethanol concentration (5–20% (v/v)) were also evaluated. Assays were performed under conditions determined as optimal, i.e., 60°C and pH 6.0. Controls, considered as 100% reference values of xylanase activity, were performed by simultaneously running samples under identical conditions but in the absence of supplements.

Native PAGE and Protein Identification

Three gels were used for ND-PAGE, performed 3 times in parallel at 8% (w/v) polyacrylamide according to Laemmli [1970] using a high-MW (66–669 kDa) marker kit (GE Healthcare, Life Sciences). One gel was used for zymographic analysis and treated with 1% (w/v) birchwood xylan (pH 6.0 for 30 min at 50°C) and then stained with Congo red [Teather and Wood, 1982]. The other 2 gels were stained with Coomassie blue and silver staining, respectively. Four bands from the Coomassie blue-stained gel were recovered, digested with trypsin, and analyzed with nanoHPLC coupled to an Orbitrap mass spectrometer, at CEQUIBIEM (http://cequibiem.qb.fcen.uba.ar/). The sequence information obtained was processed using Proteome Discover v1.4 software, against a protein file containing the 24,123 xylanases extracted from the UniProtKB/Swiss-Prot database (http://www.uniprot.org).

Statistical Analysis

Data were analyzed using Minitab® 17 software (PA, USA, Minitab Inc.) by means of analysis of variance (ANOVA). All results are presented as the mean of 3 replicates ± SD. Differences were significant when $p < 0.05$. Associations between variables were assessed using the Tukey HSD test ($p < 0.05$).

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Disclosure Statement

We confirm that we have no conflicts of interest to declare.

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