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RESEARCH ARTICLE

Raw sugarcane bagasse as carbon source for xylanase production by *Paenibacillus* species: a potential degrader of agricultural wastes

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Abstract *Paenibacillus* species isolated from a variety of natural sources have shown to be important glycoside hydrolases producers. These enzymes play a key role in bio-refining applications, as they are central biocatalysts for the processing of different types of polymers from vegetal biomass. Xylanase production by three native isolates belonging to the genus *Paenibacillus* was approached by utilizing mineral-based medium and agricultural by-products as a convenient source to produce biocatalysts suitable for their degradation. While varieties of alkali pretreated sugarcane bagasse were useful substrates for the strains from *Paenibacillus* genus evaluated, raw sugarcane bagasse was the most effective substrate for endoxylanase production by *Paenibacillus* sp. AR247. This strain was then selected to further improvement of its enzyme production by means of a two-step statistical approach. It was determined that the carbon source, provided as an inexpensive agro-waste, as well as phosphate and magnesium were the culture media components that most influenced the enzyme production, which was improved three times compared to the screening results.

Keywords *Paenibacillus* spp. · Xylanase production · Sugarcane bagasse · Biorefinery

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Introduction

The comprehensive exploitation of vegetal biomass and even agricultural residues is an important factor for the progress of biorefineries. In this context, big efforts are engaged to improve the use of agricultural wastes as widely available and renewable sources (Carvalho et al. 2015). Sugarcane (*Saccharum* spp.) is a tropical crop primarily used for sucrose and ethanol production, generating sugarcane bagasse and straw as main by-products after harvesting. Most of the sugarcane produced is burned for energy cogeneration; however, it can be an important source as a start material for a wide variety of bioproducts (Gao et al. 2013). In order to be used, lignocellulosic biomass requires physical, chemical, or physicochemical pretreatments that allow a better availability of the carbohydrate components for subsequent biotechnological utilization within a comprehensive managerial approach. This must consider technological, environmental, and economic aspects of processes (Maroušek et al. 2014) that often involve plant cell wall degrading enzymes. Among them, glycoside hydrolases are able to act synergistically to release lignocellulose components, such as oligosaccharides, fermentable sugars, and other molecules as *building blocks* to produce biofuels and a wide variety of value-added product (Linares-Pastén et al. 2014). The enzymatic repertoire needed to promote the hydrolysis of lignocellulosic biomass include xylanases; an important group important not only to increase

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the cellulose availability to enzymes, but also to produce xylan-derived valuable products, such as low calories sugar, prebiotics, and biofuels (Collins et al. 2005; Uday et al. 2016).

As a source of microbial enzymes, *Paenibacillus* genus is well-known as extracellular catalysts producers to efficiently degrade the complex matrix of lignocellulosic materials. They are widely distributed in nature, with a great potential to produce glycoside hydrolases suitable for many industrial applications (Grady et al. 2016). *Paenibacillus* spp. are frequently strong xylanase producers, as was reported for *P. thailandensis* S3-4AT, *P. nanensis* MX2-3 T (Khianngam et al. 2009) and *Paenibacillus* sp. XJ18 (Kurrataa'Yun and Meryandini 2015), as well as for *Paenibacillus* species which enzymes have shown to degrade plant carbohydrates (Dheeran et al. 2012; St. John et al. 2012; Zheng et al. 2014).

In this work, the production of extracellular xylanases by three *Paenibacillus* spp. strains was evaluated using agricultural by-products as carbon source. These results will contribute to the wide variety of biocatalysts that promote the exploitation of agricultural wastes in the perspective of the growing world demand for food, biofuels and other bioproducts.

Materials and methods

Microorganisms and culture conditions

The strains AR247, AR460–1, and AR489 were previously isolated and identified as members of *Paenibacillus* genus according to their 16S rDNA partial sequences, deposited under the accession numbers LN829577, LN829578, and LN829580 (Manfredi et al. 2015). These sequences were analyzed using the EzTaxon-e server to taxonomically relate the isolate to close strains (Kim et al. 2012) and aligned by using the SINA from SILVA database (Pruesse et al. 2012). Evolutionary distances were computed using the Kimura 2-parameter method (Kimura 1980), and the phylogenetic tree was reconstructed using MEGA 6 (Tamura et al. 2013).

For general purposes, all strains were grown at 30 °C for 48 h in a half diluted Tripticase Soya Broth (DTSB, Manfredi et al. 2016). Liquid cultures were incubated in an orbital shaker at 200 rpm, and spores and vegetative cells were maintained in 20% glycerol at −20 °C. Morphology of the colonies was observed directly on agar media; strains features evaluated were size, shape, color, and texture. Cell and colony morphology were observed using an optical microscope and a scanning electron microscope (SEM) SUPRA™ 55VP (Carl Zeiss, Germany).

A mineral-based medium was used for general growth and also for enzyme production. It contained, in g/L: 0.35, Na_2HPO_4; 0.10, KH_2PO_4; 0.50, $(NH_4)_2SO_4$; 0.50, $MgCl_2.6H_2O$; 0.10, $NaNO_3$; 0.30, $CaCl_2$; and 0.10, yeast extract (YE). As carbon source, the following commercial substrates and agricultural by-products derived from the local agroindustry were utilized at a final concentration of 10 g/L: (i) low viscosity carboxymethyl cellulose (CMC) and birchwood xylan, both purchased from Sigma; (ii) sugarcane bagasse (SCB) and derivatives: alkali-pretreated sugarcane bagasse (OH-SCB) obtained as was previously described (Manfredi et al. 2015) and hemicelluloses extracted from SCB (HC-SCB) according to Breccia et al. (1995); (iii) other raw agro-based substrates: wheat bran (WB) and oat bran (OB).

Enzymatic assays and non-denaturing-PAGE

Samples of crude extracts were recovered after centrifuging the cultures (10000×*g*, 10 min) to test enzyme activity and total protein content. Xylanase and endoglucanase (as CMCase) were assayed by determining the reducing sugars from the hydrolysis of 1% (wv) birchwood xylan or CMC, respectively, in 100 mM phospate buffer pH 6.5, using the dinitrosalicylic acid (DNS) following Miller (1959) method as was described (Martínez et al. 2005; Manfredi et al. 2015). One international unit (IU) of enzyme activity is defined as the amount of enzyme that releases 1 μmol of reducing sugars, equivalent to xylose, per minute under the assay conditions that were 50 °C and 30 min, or otherwise indicated. All samples were analyzed in triplicate, and mean values and standard deviations were calculated.

Non-denaturing polyacrylamide gel electrophoresis (PAGE) was performed on 10% polyacrylamide gels according to Laemmli (1970) at constant voltage (75 V) during 3 h. After silver staining, the apparent molecular weight (MW) of the bands was estimated using a HMW 669–66 kDa marker (GE Healthcare Life Sciences). Zymographic profiles were revealed with Congo Red staining after incubation of the gels at 50 °C for 30 min in 100 mM phosphate buffer pH 6.5 added with 1% (*w*/*v*) xylan from birchwood (Teather and Wood 1982).

Characterization of extracellular xylanases

Xylanolytic activity was evaluated at temperatures ranging from 4 °C to 80 °C and at pH within the range of 2.2 to 10.0 units, using the DNS method as was indicated. The substrate buffers at 100 mM final concentration containing birchwood xylan 1% (*w*/*v*), were: glycine-HCl (pH 2.2–3.0); sodium citrate-citric acid (pH 4.0–6.0), sodium phosphate (pH 6.0–7.5); Tris-HCl (pH 7.5–8.8); and glycine-NaOH (pH 8.8–10.0).

The influence of the following metal ions on the xylanolytic activity of all strains was evaluated by their addition to the reaction mixture at a final concentration of 5 mM: NaCl, KCl, $MgSO_4.7H_2O$, $CaCl_2$, $ZnSO_4.7H_2O$, $CuSO_4.5H_2O$, $FeSO_4.7H_2O$, $FeCl_3.6H_2O$, $MnCl_2.H_2O$, $CoSO_4$, and $BaCl_2.2H_2O$. The effect of PEG (MW 4000 and 6000), SDS,

Table 1. Plackett-Burman design matrix and the evaluated response, xylanase production by *Paenibacillus* sp. AR247

Run	Coded Values [a]									Xylanase Activity (IU/mL)
	A	B	C	D	E	F	G	H	I	
1	-1	-1	+1	+1	+1	-1	+1	+1	-1	3.02
2	-1	-1	-1	+1	+1	+1	-1	+1	+1	3.56
3	+1	-1	+1	-1	-1	-1	+1	+1	+1	1.24
4	0	0	0	0	0	0	0	0	0	7.05
5	+1	+1	-1	+1	+1	-1	+1	-1	-1	7.15
6	-1	+1	+1	+1	-1	+1	+1	-1	+1	4.39
7	+1	+1	+1	-1	+1	+1	-1	+1	-1	6.86
8	+1	+1	-1	+1	+1	-1	+1	-1	-1	7.54
9	+1	-1	-1	-1	+1	+1	+1	-1	+1	6.93
10	0	0	0	0	0	0	0	0	0	7.22
11	+1	-1	+1	+1	-1	+1	-1	-1	-1	2.34
12	0	0	0	0	0	0	0	0	0	7.31
13	+1	-1	-1	-1	+1	+1	+1	-1	+1	7.28
14	-1	+1	-1	-1	-1	+1	+1	+1	-1	5.32
15	-1	-1	-1	-1	-1	-1	-1	-1	-1	2.93
16	+1	-1	+1	+1	-1	+1	-1	-1	-1	2.59
17	+1	-1	+1	-1	-1	-1	+1	+1	+1	1.35
18	-1	-1	+1	+1	+1	-1	+1	+1	-1	2.79
19	-1	+1	+1	-1	+1	-1	-1	-1	+1	5.46
20	-1	-1	-1	-1	-1	-1	-1	-1	-1	3.50
21	+1	+1	-1	+1	-1	-1	-1	+1	+1	8.86
22	-1	+1	-1	-1	-1	+1	+1	+1	-1	5.44
23	+1	+1	-1	+1	-1	-1	-1	+1	+1	8.24
24	-1	-1	-1	+1	+1	+1	-1	+1	+1	3.57
25	-1	+1	+1	+1	-1	+1	+1	-1	+1	4.90
26	-1	+1	+1	-1	+1	-1	-1	-1	+1	5.22
27	0	0	0	0	0	0	0	0	0	7.35
28	+1	+1	+1	-1	+1	+1	-1	+1	-1	7.26

[a] The medium components were: SCB [A] concentration levels of -1/0/+1, were: 4.0 – 7.0 – 10.0 g/L; Na_2HPO_4 [B]: 0.35 – 1.93– 3.50 g/L, KH_2PO_4 [C]: 0.1 – 0.55– 1.0 g/L; $(NH_4)_2SO_4$ [D]: 0.25 –0.5 – 0.75 g/L; $MgCl_2.6H_2O$ [E]: 0.5 – 2.75 – 5.0 g/L; $NaNO_3$ [F]: 0.0 – 0.05 – 0.1 g/L; $CaCl_2$ [G]: 0.1 - 0.55 – 1.0 g/L; YE [H]: 0.1 - 0.55 – 1.0 g/L and Tween 80 [I]: 0.1-0.55 – 1.0 g/L

Table 2. Box Behken matrix and values of the xylanolytic activity to optimize the evaluated response, xylanase activity from *Paenibacillus* sp. AR247

Run	Coded Values [a] (g/L)			Xylanase Activity (IU/mL)
	X_1	X_2	X_3	
1	-1 (8)	0	-1 (1.5)	7.57
2	+1 (12)	-1	0 (3)	7.34
3	0 (10)	0	0 (3)	8.81
4	0 (10)	+1	+1 (4.5)	6.79
5	0 (10)	0	0 (3)	8.88
6	-1 (8)	0	+1	6.94
7	-1 (8)	+1	0 (3)	6.70
8	0 (10)	0	0 (3)	8.73
9	-1 (8)	0	-1 (1.5)	7.62
10	0 (10)	0	0 (3)	8.99
11	-1 (8)	0	+1 (4.5)	7.10
12	0 (10)	-1	+1 (4.5)	8.43
13	0 (10)	-1	-1 (1.5)	5.97
14	+1 (12)	0	-1 (1.5)	7.37
15	+1 (12)	0	+1 (4.5)	8.29
16	-1 (8)	+1	0 (3)	6.65
17	+1 (12)	-1	0 (3)	7.46
18	0 (10)	0	0 (3)	8.78
19	-1 (8)	-1	0 (3)	7.10
20	+1 (12)	0	-1 (1.5)	7.31
21	+1 (12)	+1	0 (3)	8.01
22	0 (10)	-1	-1 (1.5)	6.11
23	0 (10)	0	0 (3)	8.96
24	+1 (12)	0	+1 (4.5)	8.32
25	-1 (8)	-1	0(3)	7.25
26	+1 (12)	+1	0 (3)	7.97
27	0 (10)	+1	-1 (1.5)	7.54
28	0 (10)	-1	+1 (4.5)	8.36
29	0 (10)	+1	+1 (4.5)	6.82
30	0 (10)	+1	-1 (1.5)	7.71

[a] Coded values were: X_1 = SCB; X_2 = phosphates ratio (Na_2HPO_4/KH_2PO_4 = 4:1) and X_3 = Mg^{2+}

EDTA, Tween (20 and 80), and Triton X − 100, all added at 10 mM final concentration, was also evaluated.

The thermal stability of the enzymatic cocktail produced by *Paenibacillus* sp. AR247 was carried out at temperatures between 30 °C and 80 °C during 3 h. The pH stability profile was assessed after suitable dilutions of the crude extracts which were preincubated at different pH values for 1 h at room temperature, as was described by Manfredi et al. (2016).

Enzymatic reactions were performed after all treatments at 50 °C and pH 6.5 for the strains AR460–1 and 489, while for *Paenibacillus* sp. AR247, temperature and pH conditions determined as optimal were used. Results were referred to a control without any treatment and taken as 100% of the activity.

Screening of culture media components influencing xylanase production

Systematic experimental designs were performed in order to improve the enzymatic production with 1% raw sugarcane bagasse as carbon source. For this purpose, we used the strain that showed the highest xylanase

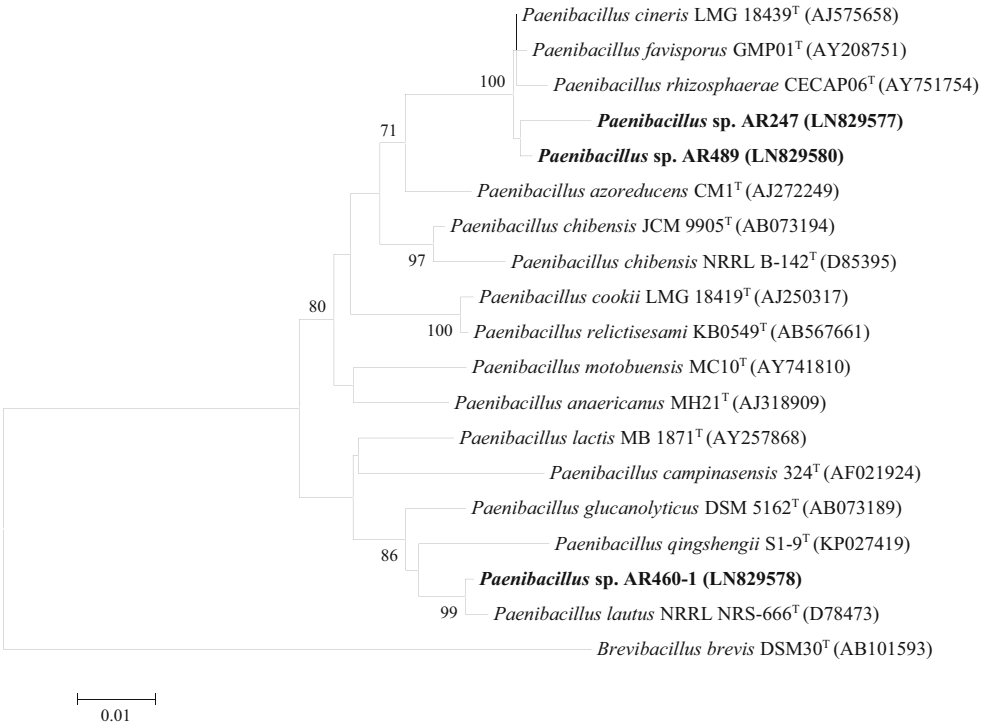

Fig. 1 Evolutionary relationships of the strains AR247, AR460–1, and AR489 and related *Paenibacillus* species, inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) is shown. The analysis involved 19 nucleotide sequences (accession numbers between parenthesis), with a total of 756 positions in the final dataset. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted with MEGA6

activity, *Paenibacillus* sp. AR247 that produced 2.99 ± 0.048 IU/mL in SCB 1% mineral medium. Firstly, a Plackett-Burman design through Minitab® 17 statistical software was used to find factors significantly affecting the xylanase production using an IV resolution grade design. Nine factors were evaluated in two levels, (+1) and (−1), as was indicated in Table 1. Central points were also included to better adjust the model data. All experiments were performed in duplicate at 200 rpm and 30 °C during 96 h, using 20 mL of liquid culture in 125-mL flasks. The evaluated responses were extracellular xylanase activity.

Once defined variables with statistically significant influence, a Box-Behken design was applied considering both the phosphorous salts as one combined factor, called PP. The selected factors were evaluated in levels minimum (−1), maximum (+1) as well as central points in duplicate experiments at identical operational conditions. For estimation of the RSM model errors, central points were repeated six times, thus the matrix of the BB design resulted in 30 culture media combinations (Table 2).

Statistical analysis

Statistical analysis of data was performed using Minitab® 17 statistical software through analysis of variance (ANOVA). Results are given as the mean of 3 replicates ±SD; significant differences were observed for $p < 0.05$. Associations between variables were assessed using Tukey's HSD test ($p < 0.05$).

Results

Characterization of *Paenibacillus* species strains

Three previously isolated bacterial strains belonging to *Paenibacillus* genus were evaluated in order to assess their potential as xylanase producers (Manfredi et al. 2015). Under a light microscope observation (1000×) all strains were rod shaped and motile spore forming bacteria and reacted in a positive manner to Gram staining, except for the strain AR460–1 that showed a variable response (data not shown). Evolutionary relationships with related type strains indicated that all evaluated strains clustered unequivocally within the *Paenibacillus* genus (Fig.1). The SEM observation of the colonies of *Paenibacillus* sp. AR247 obtained after 48 h of cultivation in mineral medium amended with raw SCB, revealed palisade arranged spore-forming rods that presented a substantial variation of size, with a length ranging from 1.5 to 3.0 μm and a diameter within 0.3 to 0.8 μm (Fig. 2).

The three strains were able to grow efficiently in both, nutritive and mineral-based media, reaching O.D. 600nm ≥ 1.0 after 48 h of incubation at 30 °C and 200 rpm. However, xylanase and cellulase production was only detected when the mineral broth was used.

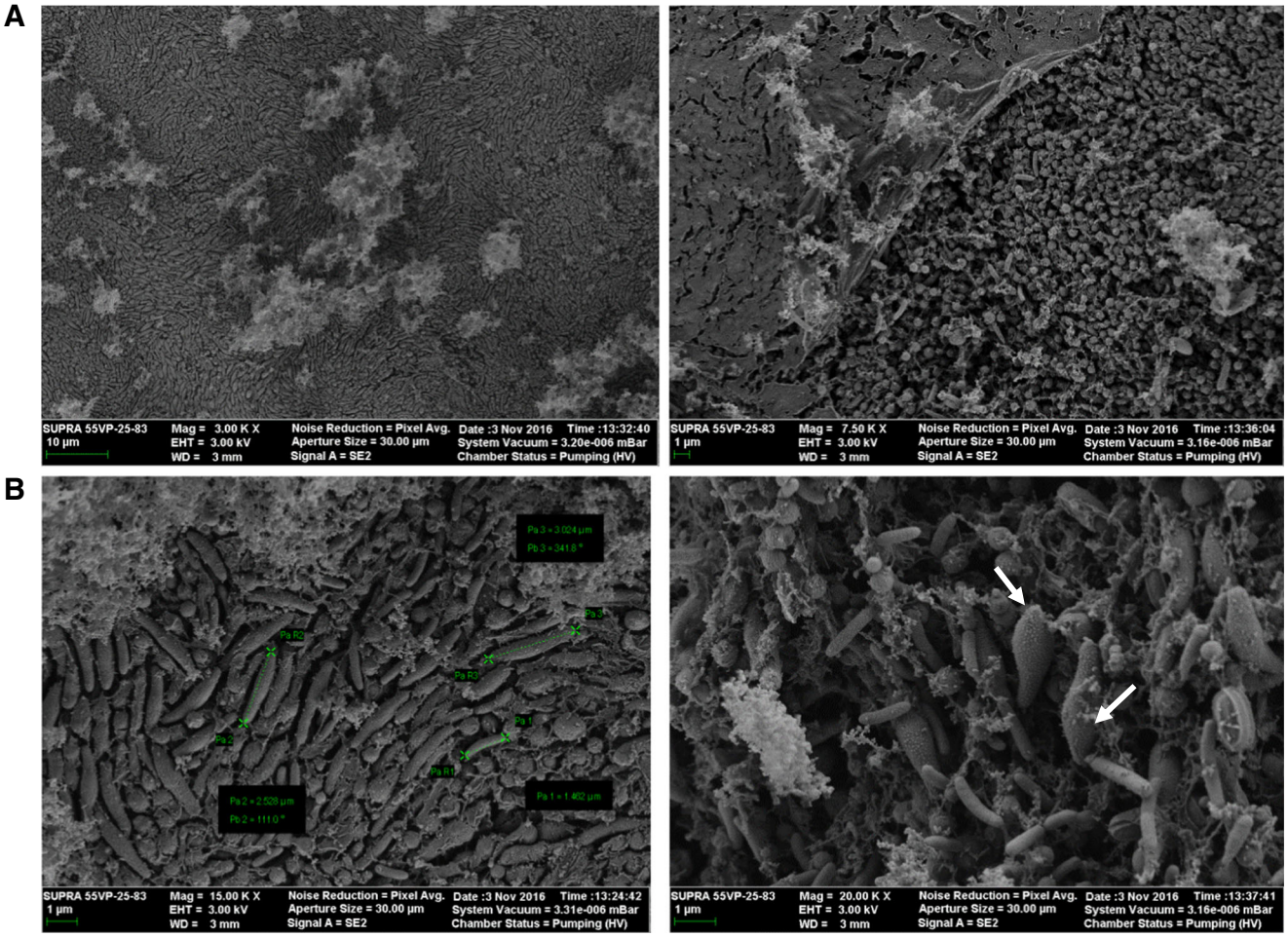

Fig. 2 SEM observation of *Paenibacillus* sp. AR247 colonies at 48 h of cultivation grown onto raw SCB mineral medium agar plates. **(A)** 3 K × (left) and 7.5 K × (right) magnification of colonies, where palisade arranged rods corresponding to a colonial pattern from the strain AR247 are observed. **(B)** 15 K × (left) and 20 K × (right) magnification. *Arrows* indicate sporulated bacteria. Typical rods size was of 5.0–3.0 µm and a diameter of 0.3–0.8 µm

Evaluation of different carbon sources for extracellular enzyme production

A variety of substrates were evaluated as potential source for endoxylanase production using a mineral-based culture broth. The use of 1% (*w*/*v*) CMC, WB, and OB produced xylanase values below 0.65 IU/mL after 4 days of cultivation, except for the strain AR247 that reached 1.80 ± 0.01 IU/mL when it was cultured into OB-MM (Supplementary material). In addition, raw SCB was the most effective substrate for endoxylanase production by this strain, reaching near 3.00 ± 0.05 IU/mL after 4 days of cultivation. Otherwise, the strains AR489 and AR460–1 produced less than 0.40 IU ml^{-1} of xylanases with SCB (Fig. 3). The preferred substrates were HC-SCB for the strain AR460–1 (1.90 ± 0.06 IU/mL) and OH-SCB for the strain AR489 (1.60 ± 0.05 IU/mL) after 96 h. However, it should be noticed that the alkali pretreated SCB (OH-SCB) showed to be a useful substrate for the three

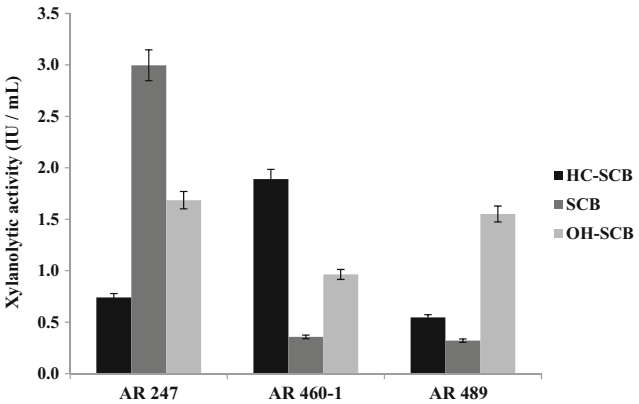

Fig. 3 Extracellular xylanase activity of *Paenibacillus* sp. strains AR247, AR460–1, and AR489 after 4 days of cultivation, using sugar cane related substrates. *Error bars* represent the standard deviation calculated from at least three independent experiments. *SCB* sugarcane bagasse, *OH-SCB alkaline treated SCB*, *HC-SCB* hemicellulose from SCB

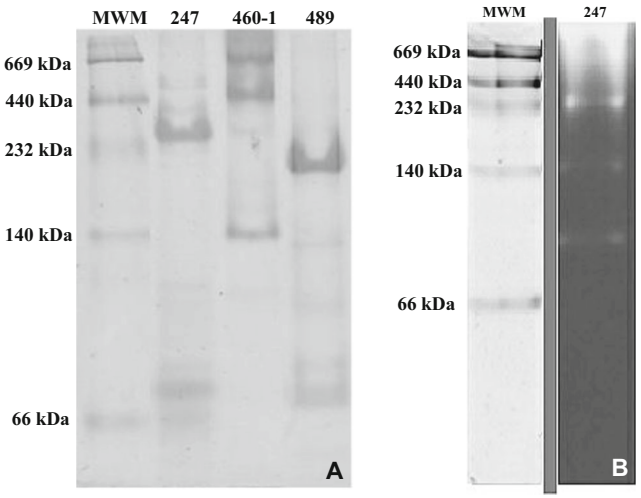

Fig. 4 Non denaturing-PAGE of extracellular proteins of *Paenibacillus* sp. strains AR247, AR460–1, and AR489. (**A**) Protein profiles from the culture supernatants after 96 h of cultivation. (**B**) Zymographic profile of *Paenibacillus* sp. AR247 obtained with OH-SCB as susbtrate. Lane *MWM* molecular weight marker

strains assayed, producing xylanolytic titles between 1.00 and 1.70 IU/mL (Fig. 3).

On the other hand, endoglucanase activity of all the evaluated cultures showed values up to 0.20 IU/mL when OH-SCB was used as substrate and the lowest values were observed when CMC and OB were used (Supplementary material).

Native PAGE analysis and effect of ions and other additives on the xylanolytic cocktails

Extracellular protein profiles of the three *Paenibacillus* strains grown using OH-SCB showed different protein patterns within the molecular weight range analyzed after silver staining **(**Fig. 4a). Their corresponding zymograms obtained by means of a Congo Red staining also revealed different enzymatic profiles among the three strains. *Paenibacillus* sp. AR247 presented three main bands with apparent MW of approximately 100 kDa; 140 kDa and >200 kDa (Fig. 4a). The strains AR460–1 and AR489 showed a similar pattern among them with three bands with xylanolytic activity, respectively (data not shown).

Most of the metal ions assayed into the xylanolytic reaction mixture did not show significant effect on the enzymatic activity from extracts obtained with OH-SCB-MM (Table 3). However, the hydrolysis of xylan was negatively influenced by Ca^{2+} on the enzymes produced by the strains AR460–1and AR489. The extracellular xylanase activity from the strain AR489 was improved when Ba^{2+} and Zn^{2+} were present into the reaction mixture, but it was reduced around 12 and 31% by the addition of Mg^{2+} and Fe^{2+} salts, respectively. A strong inhibitory effect of Cu^{2+} was observed on the xylanolytic activity from the strains AR247 and AR489. In contrast, this cation did not significantly influence the crude extract from the strain AR460–1.

Table 3 Effect of cations and additives on the xylanolytic activity from the *Paenibacillus* strains evaluated

Cations [5 mM] Additives [10 mM]	Relative activity (%)		
	AR247	AR460-1	AR489
Control [a]	100	100	100
Na^+	99	102	98
K^+	99	101	103
Mg^{2+}	102	101	88
Ca^{2+}	93	57	72
Ba^{2+}	81	80	111
Mn^{2+}	88	81	97
Fe^{2+}	100	93	69
Fe^{3+}	77	85	87
Co^{2+}	92	78	80
Cu^{2+}	50	94	46
Zn^{2+}	88	102	114
PEG_{4000}	92	84	97
PEG_{6000}	100	62	93
SDS	63	59	59
EDTA	80	84	45
TW80	85	77	97
TW20	90	79	105
TX-100	94	88	76

[a] The activity of the enzymes assayed under the optimum condition in the absence of additives was defined as 100%. Data are presented as means ± SD (n = 3)

The effect of surface-active agents on the enzymatic cocktails was also shown in Table 3. It can be seen that the xylanase produced by the strain AR247 evidenced a remarkable tolerance to most of the assayed additives with the exception of SDS, which abolished 37% of its activity. The presence of EDTA led to a moderate reduction of the xylanolytic activity from the strains AR247 and AR460–1, yet the xylanase produced by AR489 was almost halved inhibited (~55%) by this chelating agent.

Culture media components influencing xylanase production by *Paenibacillus* sp. AR247

Considering that *Paenibacillus* sp. AR247 was the best xylanase producer in presence of most of the assayed substrates, it was selected for further assessment of the medium components in order to improve the enzyme production. The screening of the culture medium components which was realized by means of the Plackett-Burman design showed that the highest xylanolytic

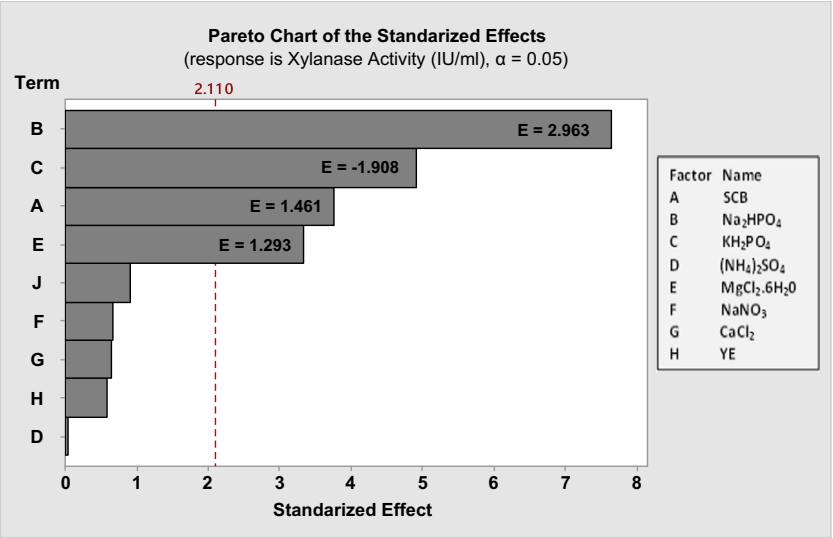

Fig. 5 Pareto chart with value of main effects of the culture medium components affecting xylanase production by *Paenibacillus* sp. AR247

activity was registered in the duplicates 21/23, with a mean value of 8.50 ± 0.44 IU/mL. The Pareto chart analysis (Fig. 5) showed three main effects with significant and positive influence on the evaluated response, corresponding to the factors sodium phosphate, SCB, and Mg^{2+}. As the potassium phosphate had, on the contrary, a significant but negative influence on the hemicellulases production, it was then evaluated around its lower level assayed. Thus, it was considered for the following RSM design with the sodium salt as an only combined factor called phosphate source (PP), in a ratio of 4:1 between Na_2HPO_4/KH_2PO_4, as can be observed in Fig. 5. Accordingly, the xylanolytic activity was improved around three times compared to the initial screening (~3.0 IU/mL at 96 h, Fig. 3).

The results were then fitted into a Box-Behken design to further analyze the components of the medium which resulted significant: raw SCB, Mg^{2+}, and phosphate salts (PP). The 30 combinations were evaluated via a response surface methodology (RSM). The enzyme production ranged from 6.00 to 9.00 IU/mL, with a maximum recorded for one the central point's replica (8.99 ± 0.04 IU/mL; Table 2). Analysis of the variance showed that both the SCB and Mg^{2+} were statistically significant factors for the lineal model ($p = 0.000$), while all of variables were significant terms for both the quadratic and interaction model with $p = 0.000$ (Table 4).

A multiple regression analysis on the experimental data is shown in the following equation:

$$Y = -7.15 + 2.772\ X_1 + 0.244\ X_2 + 0.734\ X_3 - 0.1497\ X_1^2 - 0.9481\ X_2^2 - 0.3086\ X_3^2 + 0.1355\ X_1X_2 + 0.1279\ X_1X_3 - 0.5292\ X_2X_3$$

Where Y is the predicted response (xylanolytic activity); and the medium componentes levels are X_1 for SCB; X_2 for phosphates ratio; and X_3 for Mg^{2+}. The R^2 was 97.6%, meaning that

Table 4 Effect of variable and statistical analysis of the response xylanolytic activity from *Paenibacillus* sp. AR247 using a Box-Behken design

Code	Variable	Level (g/L)			Xylanase Activity (IU/mL)		
					Effect (*E*)	Statistical Significance	
		-1	0	+1		*t* test	*p* value
X_1	SCB	8	10	12	0.6431	8.01	0.000
X_2	PP [A]	(1/0.25)	(2/0.50)	(3/0.75)	0.0229	0.29	0.778
X_3	$MgCl_2$	1.5	3	4.5	0.4828	6.01	0.000
X_1^2	$(SCB)^2$				-1.1978	-10.14	0.000
X_2^2	$(PP)^2$				-1.8962	-16.05	0.000
X_3^2	$(MgCl_2)^2$				-1.3886	-11.75	0.000
X_1*X_2	SCB*(PP)				0.5420	4.77	0.000
X_1*X_3	SCB*$MgCl_2$				0.7671	6.76	0.000
X_2*X_3	(PP)*$MgCl_2$				-1.5877	-13.98	0.000

[a] Code values as were indicated in Table 2 footnote

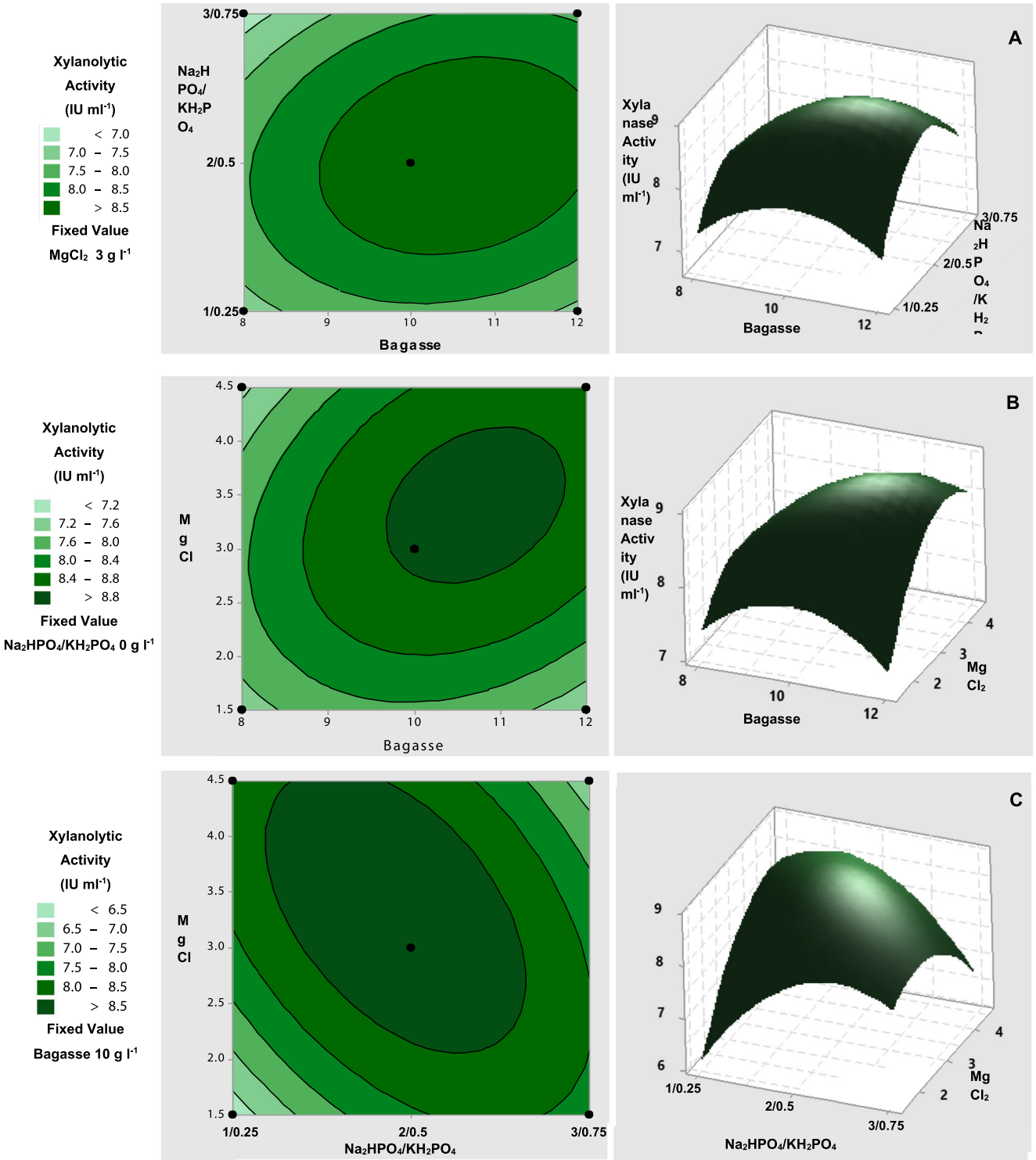

Fig. 6 Response surface and contour plots of the combined effects on xylanase production **(A)** of SCB and phosphorous source concentrations; **(B)** of magnesium salt and carbon (SCB) concentrations; **(C)** of magnesium salt and phosphorous source concentrations

more than 97% of the response variability could be explained by the statistic model. The three-dimensional response and their corresponding contour plots, drawn on the basis of the model equation, showed that the objective function is unimodal in nature with an optimum at the central area (Fig. 6). As a result, the statistical analysis indicated that the medium to maximize the xylanase production by this strain was composed as follows, in

g/L: 10.70, sugarcane bagasse; 3.50, $MgCl_2.6H_2O$; 2.00, Na_2HPO_4; 0.50 KH_2PO_4; 0.25, $(NH_4)_2SO_4$; 0.10, $CaCl_2$; and 0.10, YE.

3.5. Effect of temperature and pH on extracellular xylanase activity and stability

The crude xylanolytic cocktail produced by *Paenibacillus* sp. AR247 showed an optimal activity at 60 °C. Aditionally, these xylanases retained over 90% of its activity at 50 °C and around 50% of the maximum value between 40 °C and 70 °C (Fig. 7a). The assayed thermal stability profile showed over 80% of preservation of the enzymatic activity after 3 h of preincubation at 30 and 40 °C. However, the activity of the crude extract was almost completely abolished after being preincubated for 3 h at temperatures ≥50 °C (Fig. 7a).

Maximum hydrolysis of birchwood xylan by the enzymes from *Paenibacillus* sp. AR247 was observed at pH 6.0 with no significant differences between pH 5.0 and 7.0. Near 45% of the xylanase activity was retained at pH values between 4.0 and 8.0. Also, the crude enzymatic cocktail was remarkably stable in a broad range of pH, including both the neutral-acid and the neutral-alkaline region (from 3.0 to 9.0 units of pH) (Fig. 7b).

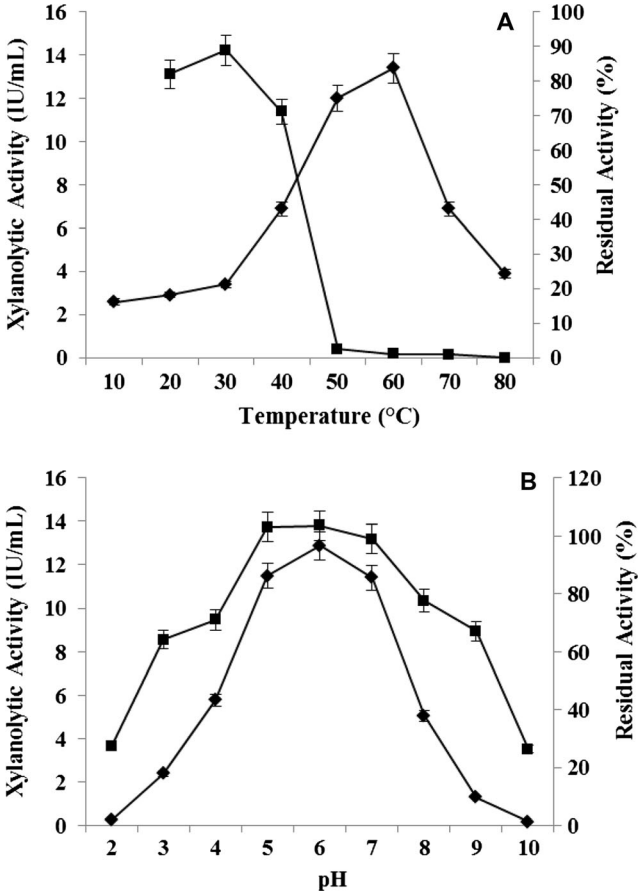

Fig. 7 Xylanolytic enzymes from *Paenibacillus* spp. AR247. (**A**) *Black diamonds* indicate optimal temperature and *black squares* indicate thermal stability. (**B**) Black diamonds indicate optimal pH and black squares indicates pH stability. *Error bars* represent the standard deviation calculated from at least three independent experiments. Samples were concentrated (2×) by diafiltration with a cut-off of 10 kDa

Discussion

Paenibacillus genus is one of the eight genera of the family *Paenibacillaceae*, where it is the most diverse one and includes many species with increasingly important roles in sustainable agriculture and biotechnology (Grady et al. 2016). In this work, three locally isolated bacterial strains were taxonomically related to *Paenibacillus* genus into a monophyletic group (Fig. 1), despite the paraphyletic nature of the *Paenibacillaceae* family (Dsouza et al. 2014). Figure 2 shows colonies of *Paenibacillus* sp. AR247 presenting a typical colonial pattern, which is frequently observed in related species (Ingham and Ben Jacob 2008).

Insoluble substrates, including mixtures of plant polymers, have shown to induce high-yield hemicellulases production (da Silva et al. 2017). Among them, lignocellulosic waste is a topic of global studies in order to take advantage given increasing demands for bioproducts. In this context, the utilization of raw or pretreated sugarcane bagasse for xylanase production which then proved to be appropriate for agro-waste processing was reported for *Penicillium echinulatum* 9A02S1 (Camassola and Dillon 2014). Therefore, by-products from sugarcane and other crops were utilized as carbon source to grow *Paenibacillus* spp. strains in order to produce a repertoire of enzymes potentially suitable to degrade them. Our results showed that the extracellular enzyme production was more efficient when sugarcane bagasse derived substrates compared to the enzymatic titles obtained with commercial substrates (Fig. 3; Supplementary material). It is worth to mention that the promising results observed utilizing OB as substrate will be further approached to evaluate the potential of the enzymes produced for the production of xylooligosaccharides as prebiotics, as was reported for Zheng et al. (2014). Whereas *Paenibacillus* sp. AR460–1 and AR489 performed better when HC-SCB and OH-SCB were used as substrate, *Paenibacillus* sp. AR247 xylanase production was best promoted when milled SCB was used as carbon supply (Fig. 3). The alkali-based pretreatments used for their generation are aimed to weaken the structure of lignocellulose, thus increasing the accessibility of the enzymes to the hemicellulose from plant cell walls (Gao et al. 2013). Nevertheless, it is interesting to notice that OH-SCB and HC-SCB were the less efficient substrates for AR247 strain.

The protein profile of the three isolated strains showed differences by means of a native PAGE analysis (Fig. 4a). Samples used were from cultures grown with OH-SCB as substrate for comparative purposes since that substrate

allowed to a proper enzyme production by all strains. The band of ~140 kDa observed for the strain AR247 (Fig. 4b) could correspond to an enzyme belonging to GH10 family (Collins et al. 2005). It might be related to that reported for *Paenibacillus* sp. JDR-2 (St. John et al. 2012) as was evidenced by a preliminary examination of the secretome from *Paenibacillus* sp. AR247 (data not shown). Still, other bands detected need to be studied to further understand the hemicellulolytic activity of the strains considered. In addition, most of the cations assayed into the reaction mixtures of the crude extracts of all strains did not affect the enzymatic activity (Table 3). Copper was found to inhibit xylanase activity (Dheeran et al. 2012). Interestingly, the enzymatic cocktail produced by *Paenibacillus* sp. AR460–1 was weakly affected by Cu^{2+}, feature which may worth a deeper investigation to assess the potential of the enzymes produced by the strain AR460–1. Considering agro-industrial wastes contain numerous metal ions and other impurities that can potentially inhibit enzyme activity, the tolerance of the hemicellulolytic cocktails is a desirable feature (Manfredi et al. 2016).

The ability of *Paenibacillus* sp. AR247 to grow and to produce xylanase using untreated sugarcane bagasse was further assayed to improve the enzymatic titles obtained by means of a statistical approach, which has proven to be useful for identifying the effect of individual variables and for seeking the optimum conditions for a multivariable system efficiently (Cui and Zhao 2012; Wang et al. 2014). A Plackett-Burman design applied indicated that the variables (medium components) which showed a significant effect on the xylanase production were SCB, Mg^{2+}, and sodium phosphate at their maximum tested level, while potassium phosphate at its lowest evaluated concentration (Fig. 5). As a result, a ratio (4:1) between the two phosphate salts (PP) was considered as one variable for posterior assays according to a Box-Behnken matrix (Table 4). The effect of the linear, quadratic, and cross product significant terms illustrated in Fig. 6 indicated that the xylanase production tends to increase along with gradual increments of the carbon source concentration, with maximum values registered within the range from 10 to 12 g/L of the factor SCB. In the same way, the response was improved with the PP ratio at the range of 0 to 1 (coded value). The RSM analysis of interactions evidenced that the effect of Mg^{2+} was negligible when both the SCB and Mg^{2+} levels were in their lowest assayed concentrations (Fig. 6b). However, the highest xylanase production was observed when the concentration of Mg^{2+} was at its highest level, between 3 to 4 g/L (Fig. 6c). Accordingly, Cui and Zhao (2012) suggested that the positive influence of magnesium on the xylanase production might be favor through a stabilizing effect on ribosomes and cellular membranes.

The crude enzymatic extract obtained showed properties suitable for agro-wastes processing within an acidic-neutral pH range and at mild temperature conditions (Fig. 7); conditions that are potentially useful for simultaneous saccharification and fermentation processes for second generation ethanol production. Although members of the genus *Paenibacillus* were described as thermostable glycoside hydrolases producers (Dheeran et al. 2012), several reports also showed extracellular enzymes that frequently performed better at a moderate temperature range, such as those from *Paenibacillus* sp. 2S-6 (Ko et al. 2011), *P. favisporus* (Padilha et al. 2014), *Paenibacillus* sp. DG-22 (Lee and Lee 2014), and *Paenibacillus* sp. IHB B 3084 (Dhar et al. 2015).

Concluding remarks

Three *Paenibacillus* spp. strains studied in the present work efficiently produced extracellular xylanases with potential towards the degradation of agricultural wastes. Here, we selected *Paenibacillus* sp. AR247 to evaluate the medium components influencing the enzyme production. Results of this study indicated that the culture media components that played a pivotal role in enzyme production were carbon and phosphorous sources and the presence of magnesium into the culture broth. Further steps towards the optimization of the culture medium were performed taking advantage of the ability of *Paenibacillus* sp. AR247 to grow and to produce xylanase using raw sugarcane bagasse. This substrate presented the improvement of not requiring any pretreatment further than a simple milling process to be successfully used. Although the crude enzymatic extract produced showed promising properties with potential for biorefinery applications utilizing agro-waste material, additional assays including the evaluation of parameters such as pH, aeration, and temperature would improve the enzyme production. Finally, the results obtained also provided data to approach upcoming studies on enzyme production and lignocellulose degradation by using proteomic and genomic tools, in order to reveal insights on the development of functional biocatalysts from *Paenibacillus* species.

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