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XYLANASES AND PECTINASES OF *Aspergillus flavus* CECT-2687 ON DIFFERENT CARBON SOURCES AND INITIAL pH VALUES

XILANASAS Y PECTINASAS DE *Aspergillus flavus* CECT-2687 EN DIFERENTES FUENTES DE CARBONO Y VALORES DE pH INICIAL

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Abstract
Aspergillus flavus is a saprophytic opportunistic plant pathogen that infects crops and stored grains, in a process assisted by the action of enzymes, such as xylanases and pectinases. The enzymatic potential of *A. flavus* has not been addressed in detail, even when this knowledge could be useful for the understanding of the colonization dynamics during the grains spoilage by fungi, in order to find of strategies to control it. The effect of carbon source and pH on enzyme production was evaluated by growing *A. flavus* CECT-2687 in liquid cultures on different monosaccharides, polysaccharides or agricultural residues, at several initial pH values. The results showed a significant effect of pH, carbon source and the interaction of both factors. Xylanases were produced on monosaccharides, although the highest xylanolytic activities were obtained on agroindustrial xylan-rich residues. Exopectinases titers were similar on polysaccharides and agricultural residues, and were not observed on monosaccharides. Pectin lyases were produced on pectin and agricultural residues; and a pectin lyase of 27 kDa was identified in crude extracts of lemon peel cultures at initial pH of 8. The highest enzymatic productions were obtained on cultures with initial pH of 8.
Keywords: xylanases, exopectinases, pectin lyases, agroindustrial residues, *Aspergillus flavus* CECT-2687.

Resumen
Aspergillus flavus es saprofito y patógeno oportunista de cultivos y granos almacenados, en un proceso asistido por la acción de enzimas como xilanasas y pectinasas. El potencial enzimático de *A. flavus* no se ha abordado en detalle, incluso cuando este conocimiento podría ser útil para comprender la dinámica de colonización de granos por hongos y encontrar estrategias para su control. El efecto de la fuente de carbono y el pH sobre la producción de enzimas se evaluó cultivando *A. flavus* CECT-2687 en cultivos líquidos en diferentes monosacáridos, polisacáridos o residuos agrícolas, a diferentes valores de pH. Se encontró un efecto significativo del pH, la fuente de carbono y su interacción. Las xilanasas se produjeron en monosacáridos, las actividades más elevadas se obtuvieron en residuos agroindustriales ricos en xilano. Los títulos de exopectinasas fueron similares en polisacáridos y residuos agrícolas y no se observaron en monosacáridos. Las pectin liasas se produjeron en pectina y residuos agrícolas; y se identificó una pectina liasa de 27 kDa en extractos crudos de cultivos de cáscara de limón a pH inicial de 8. La mayor producción de todas las enzimas se observó en los cultivos con pH inicial de 8.
Palabras clave: xilanasas, exopectinasas, pectin liasas, residuos agroindustriales, *Aspergillus flavus* CECT-2687.

1 Introduction

The genus *Aspergillus* is composed by around 350 different species, and it is one of the most abundant all over the world. Some of these species, as *A. fumigatus*, *A. flavus* or *A. ochraceous*, are responsible for human and animal diseases, as well as food spoilage. But also some of them, as *A. niger* and *A. oryzae*, are widely employed for the production of valuable products that can be used in food industry and health (Sheikh-Ali *et al*., 2014; Alonso *et al*., 2015; Reyes *et al*., 2017; de Vries *et al*., 2017).

As the most of *Aspergillus* species, *A. flavus* is broadly distributed in nature. It is classified

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as a saprophyte fungus, but it can also colonize plant tissues, so it could be also considered as an opportunistic plant pathogen. Furthermore due to its ability to produce aflatoxins its presence in foodstuff can be quite dangerous (Perrone and Gallo, 2017). So, the spoiled crops and stored grains by *A. flavus* have a very high impact on both health and economy (Bhatnagar-Mathur *et al*., 2015).

It is well acknowledged that fungi have the ability to produce different sets of cell wall degrading enzymes (CWDE) that make them able to degrade the polysaccharides contained on complex substrates like plant tissues, bagasse, cereals and some fruit peels (Kubicek *et al*., 2014). Many of these enzymes have been studied and fully characterized in several *Aspergillus* species. However, the enzymatic potential of *A. flavus* has received less attention, even when this knowledge could be useful to prevent the grains spoilage.

On the other hand, the number of genes and the corresponding enzymes identified and characterized for *A. flavus* is still low, compared to other *Aspergillus* strains (*A. niger* or *A. oryzae*, for example). From these, only pectinases P1, P3, P2c, and pectin esterase have been studied in more detail and characterized to some extent: P1 and P3 are sensitive to catabolic repression, and P2c is expressed constitutively (Cleveland and McCormick, 1987; Cleveland and Cotty, 1991; Whitehead *et al*., 1995) and has been identified as virulence factor, as some mutants lacking P2c gene are less infective (Liu *et al*., 2017).

Recently, new information about genes related to pectin degradation in several *Aspergillus* genomes was published, showing that there are 73 such genes in *A. flavus*, of which 19 belong to PL CAZY families (de Vries *et al*, 2017). This means that there are more pectin/ pectate lyase enzymes still to be discovered and described. Besides pectinases, the ability of *A. flavus* for producing other enzymes like proteases, amylases and xylanases has been also addressed (Duran *et al*., 2014; Mellon *et al*., 2015).

There are also some reports about the production and purification of pectin lyases (Yadav *et al*., 2013; Pedrolli and Carmona, 2014). However, just a few of the probable pectic enzymes according to the open reading frames were identified in *A. flavus* (de Vries *et al*., 2017).

Therefore, we are interested in knowing how the CWDE produced by *A. flavus* are involved in the process of grain invasion, specifically the effect of pH and carbon source on this enzyme production. This is because depending on the material that the fungus invades, it will encounter different carbon sources and environmental pH, which will determine the enzymes that it must produce to favor the invasion process. So, the aim of this work was to identify the xylanases and pectinases produced by *A. flavus* CECT-2687 growing on monosacharides, polysaccharides and agroindustrial residues at different initial pH values, using a submerged culture in batch mode.

2 Materials and methods

2.1 Microorganism

Aspergillus flavus CECT-2687 was obtained from Colección Española de Cultivos Tipo, Universidad de Valencia. This is a toxigenic strain, classified as aflatoxin B1 and B2 producer (www.cect.org).

2.2 Media and culture conditions

The axenic culture was cultivated and maintained on Sabouraud agar from Difco (USA), with periodic reseeds. Conidia were scrapped from five-day old potato dextrose agar (PDA) slants with sterile saline solution (0.9% w v^{-1} of NaCl) and 0.05% (v v^{-1}) Tween 80. All the experiments were developed under aseptic conditions in 500-ml Erlenmeyer flasks each containing 100 ml of sterile mineral medium with 1% (w v^{-1}) of the corresponding carbon source. Monosaccharides (glucose, xylose or galacturonic acid), polysaccharides (xylan or pectin) and complex carbon sources as agroindustrial residues (corn cob, wheat bran or lemon peel) were employed as carbon source. Inoculum was prepared on several five days-old potato dextrose medium agar plates. Conidia were dispersed in saline solution (0.9 % w v^{-1} NaCl). Spores were counted in a Neubauer counting chamber. Flasks were inoculated with one-milliliter of spore suspension at the appropriate dilution to reach a final concentration of 1×10^6 spores per milliliter of culture medium, and incubated at 37°C and 300 rpm on a rotatory incubator shaker. Mineral medium contained, in % (w v^{-1}): KH_2PO_4, 0.2; K_2HPO_4, 0.2; and $(NH_4)_2SO_4$, 0.5. Several initial pH values were used: 3.5, 6.0, 8.0 or 9.0 (see Tables 1 and 2 at Results section). For adjusting pH, 1 M NaOH or H_2SO_4 were used before sterilization. Samples were undertaken from cultures each 24 h, and the filtrate obtained after filtration trough a Millipore filtration system with a membrane of 5.0 μm, was maintained

under refrigeration until the analysis.

2.3 Enzyme assay

For xylanases and exopectinases quantification, 0.1 ml of each cell free filtrate was added to a test tube containing 0.9 ml of either 1% (w v^{-1}) xylan or pectin solution prepared on 0.1 M of an acetate-buffer pH 5.0. After incubation of each reaction mixture at 45 ºC for 20 minutes, reaction was stopped by the addition of 1 ml of the Dinitro-salicylic acid (DNS) reagent and the reducing sugars released by the enzyme were quantified at 575 nm. Xylose or galacturonic acid (1 mg ml^{-1}) were used as reference standards, respectively. One unit of enzyme activity was defined as the amount of enzyme that releases 1 μmol of reducing sugar, as xylose or galacturonic acid, per milliliter under standard assay conditions.

Pectin lyase activity was quantified by the increase in the absorbance at 235 nm, after the incubation of 1 ml of a 1% (w v^{-1}) pectin solution, prepared with a 0.1 M Tris-HCl buffer pH 9.0 with 0.1 ml of each filtrate during 1 h. One unit of pectin lyase activity was defined as the amount of enzyme that increases one unit in the absorbance at 235 nm at the standard conditions.

2.4 SDS-PAGE

For sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), five ml of the enzymatic filtrate were dialyzed against distilled water and Tris-HCl 10 mM pH 7.5, for one hour each. Dialyzed samples were frozen under liquid nitrogen, lyophilized and resuspended with 50 μL of buffer 0.025 M TRIZMA-BASE, 0.192 M glycine, 0.1% SDS, pH 8.3.

SDS-PAGE was developed on a 10% acrylamide gel, 1.0 mm tick, at constant current (15mA/gel) during 1 h in a Mini-PROTEAN Tetra Cell (Bio-Rad Laboratories, Inc.). Gels were stained with a mixture of 0.025% Coomassie blue R-250, 40% methanol and 7% acetic acid during 1h at 40 rpm, and de-stained with 10% (w v^{-1}) acetic acid solution.

For zymograms, acrylamide gels were first incubated in renaturing buffer (50 mM Tris-HCl buffer pH 7.5) at 37°C during 1 h, after in 100 M Tris-acetate buffer (pH 8.0) at 37°C during 30 min, and finally in a 1% (w v^{-1}) citrus pectin (73% esterification) solution with 100 mM Tris-acetate buffer (pH 8.0), at 37°C during 1 h. After this the excess of substrate was eliminated and the gel was rinsed with distilled water. The clearing zones due to the enzyme action were identified by staining the non-degraded substrate in the gel with 0.05% (w v^{-1}) of Ruthenium red in constant agitation.

2.5 Statistics

All the experiments were done in triplicate, reporting the corresponding average with the standard deviation on the corresponding tables. ANOVA, linear regression and Least Significant Difference (LSD) analysis were done in SAS® software.

For Pareto charts, the effect of every factor (carbon source, initial pH or joint effect of carbon source::initial pH) was obtained after calculating the mean value of all data obtained at every specific condition. For example, to calculate the effect of initial pH of 3.5, the mean value of all the experiments developed at this initial pH -irrespective of carbon source- was used as "standardized effect" on Figures 1 and 2. All those values were compared with mean production of each experiment.

3 Results and discussions

A. flavus CECT-2687 was grown on mineral medium with different carbon sources, showing great versatility for producing xylanases and pectinases. Hydrolytic activities obtained on monosaccharides were very low, which suggests that these enzymatic activities have a constitutive nature in this strain, as was also reported for exopectinases of *A. flavipes* FP-500 (Martínez-Trujillo *et al*, 2009), xylanases produced by *Fusarium* sp., *Mucor racemosus* and *Penicillium miniolutenum* (Ramos-Ibarra *et al*., 2017), and polygalacturonases of *A. terreus* (Sethi *et al*., 2016). A similar behavior of constitutive basal level of xylanase activity on media contained glucose has been reported for Pleurotus ostreatus growing on solid state culture (Álvarez-Cervantes *et al*., 2016).

The corresponding statistical analysis showed that regarding xylanolytic production, xylose was the only monosaccharide with a significant effect on xylanases production; while for polysaccharides, the main effect was observed with xylan. On both cases, pH was significant when it was around 8.0 at the beginning of the culture. However, the main effect was observed with the interaction of xylan at pH 8.0 (Figure 1a).

Xylanolytic production on polysaccharides and agroindustrial residues showed the highest titers with corncobs or wheat bran (Fig 1b).

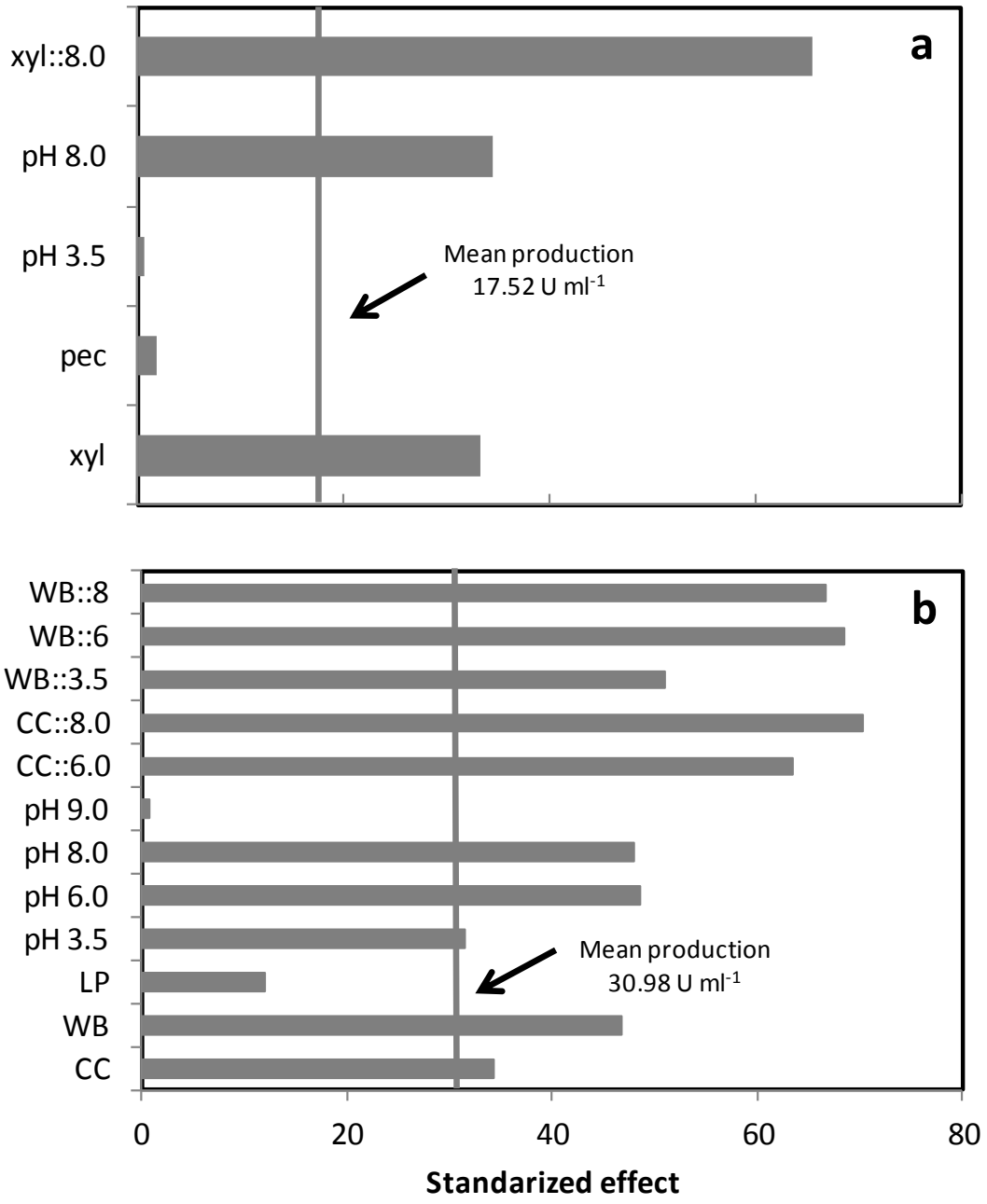

Fig. 1. Pareto chart of standardized effects for each factor and the significant interaction among carbon source::pH for xylanases production after the 72 hours of fermentation on different polysaccharides (a) and agroindustrial residues (b). All effects higher than those corresponding to mean production (shown by the solid gray line) indicate that the corresponding factor is significant for xylanases production. Regarding the interaction effect, the figure shows only those with values higher than mean. Pec, pectin, Xyl, xylan, LP, lemon peel, CC, corn cobs, and WB, wheat bran. The two dots couple (::) depicts the interaction between two different factors.

Table 1. Xylanases production on different carbon sources and initial pH values by *Aspergillus flavus* CECT-2687.

Exp		Initial pH	Carbon source	Xylanases*	
				Production (U ml^{-1})	Productivity (U ml^{-1} h^{-1})
1	Polysaccharides	3.5	Xylan	0.85 ± 0.02^{J}	0.035 ± 0.0009^{L}
2			Pectin	0.28 ± 0.01^{J}	0.005 ± 0.0002^{L}
3		8.0	Xylan	65.59 ± 1.23^{C}	1.307 ± 0.0088^{E}
4			Pectin	3.35 ± 0.35^{I}	0.139 ± 0.0144^{J}
5	Agroindustrial residues	3.5	Corn cobs	2.94 ± 0.30^{I}	0.110 ± 0.0053^{K}
6			Wheat bran	50.91 ± 0.76^{E}	1.158 ± 0.0148^{F}
7			Lemon peel	26.32 ± 0.33^{F}	0.547 ± 0.0041^{G}
8		6	Corn cobs	63.49 ± 2.46^{D}	2.751 ± 0.1051^{C}
9			Wheat bran	68.51 ± 0.6^{B}	2.713 ± 0.0610^{D}
10			Lemon peel	13.71 ± 0.15^{G}	0.189 ± 0.0036^{I}
11		8	Corn cobs	70.38 ± 1.65^{A}	2.907 ± 0.0090^{A}
12			Wheat bran	66.64 ± 0.86^{C}	2.817 ± 0.0391^{B}
13			Lemon peel	6.69 ± 0.02^{H}	0.204 ± 0.0683^{H}
14		9	Corn cobs	0.41 ± 0.014^{J}	0.006 ± 0.0002^{L}
15			Wheat bran	0.71 ± 0.01^{J}	0.027 ± 0.0028^{L}
16			Lemon peel	1.06 ± 0.06^{J}	0.022 ± 0.0012^{L}
LSD				0.8614	0.0314

*Results with different capital letters were significantly different compared with their correspondent LSD value.

This can be attributed to the fact that both materials have a high percentage (40-43 %) of xylan (Beaugrand *et al* 2004; Yang *et al*., 2005). Moreover, xylanolytic production was considerably low when pectin or lemon peel was used as carbon source (Figures 1a and 1b). Initial pH had a positive influence on xylanases production, which means that higher titers can be obtained at higher initial pH values (Figure 1b). This explains why in all the cases the highest xylanolytic activities were obtained on media with initial pH of 8.0, although this production decreased almost to be null when the initial pH of the medium was 9.0 (Figure 1). On the other hand, the highest xylanolytic activities were obtained with lemon peel at pH of 9 (Table 1), however this production was not significant in relation to the mean production (Figure 1b).

Maximum xylanolytic activities were comparable with those reported for other species of industrial importance with the same substrates: xylanolytic activities produced on corncobs were slightly lower than those reported for *A. niger* SS7 (Bakri *et al*., 2008), but considerably higher than those reported for *A. terricola*, *A. ochraceus* (Michelin *et al*., 2010) and *A. flavus* (de Souza, 1999) on the same carbon source. Activities obtained on wheat bran also resulted higher than the production reported for *A. niger* SS7 (Bakri *et al*., 2008) and *A. flavus* (de Souza, 1999) in this carbon source. This could explain the devastating effect of *A. flavus* when invading this type of materials; but it also opens the possibility of using the enzyme produced by this strain in the saccharification process of different materials in order to obtain fermentable sugar that can be further fermented for obtaining bioethanol and another biofuels (Sheikh-Ali *et al*, 2014).

After analyzing the evolution of enzymatic production and the corresponding productivities during the culture time of each experimental condition, it was observed that xylanases obtained on xylan at pH 8.0 were almost comparable with those of corn cobs and wheat bran, both at pH 6 and 8; however, the corresponding productivities were lower than those of these agroindustrial residues. The highest xylanase production and productivity values were obtained with corncobs at pH of 8.0, while xylanolytic activities produced on lemon peel were significant lower (Table 2).

In reference to exopectinases, among polysaccharides only the titer obtained on pectin was higher than mean value; although the main effect on the production of this pectinolytic activity was with the interaction between pectin and pH 8.0 (Figure 2a). For what concerns exopectinases production on agroindustrial residues, these were similar at the acidic pH values (3 and 6), and the lowest values were observed with the alkaline pH condition, mainly at pH 9.0 (Table 2).

Table 2. Exopectinases and pectin lyases production on polysaccharides and agroindustrial residues by *A. flavus* CECT-2687 at different initial pH values.

Exp		Initial pH	Carbon source	Xylanases*		Pectin lyases*	
				Production (U ml^{-1})	Productivity (U ml^{-1} h^{-1})	Production (U ml^{-1})	Productivity (U ml^{-1} h^{-1})
1	Polysaccharides	3.5	Xylan	0 ± 0^{G}	0 ± 0^{H}	0 ± 0^{H}	0 ± 0^{H}
2			Pectin	1.35 ± 0.06^{E}	$0.056 \pm 0.003^{F,G}$	0 ± 0^{H}	0 ± 0^{H}
3		8.0	Xylan	3.45 ± 0.06^{D}	0.149 ± 0.005^{E}	0 ± 0^{H}	0 ± 0^{H}
4			Pectin	9.48 ± 0.03^{A}	0.390 ± 0.008^{A}	5.63 ± 0.14^{C}	0.211 ± 0.002^{C}
5	Agroindustrial residues	3.5	Corn cobs	$6.59 \pm 0.89^{B,C}$	0.082 ± 0.0008^{E}	0 ± 0^{H}	0 ± 0^{H}
6			Wheat bran	6.24 ± 0.28^{D}	$0.062 \pm 0.002^{E,F}$	0 ± 0^{H}	0 ± 0^{H}
7			Lemon peel	7.89 ± 1.01^{B}	$0.149 \pm 0.009^{C,D}$	1.11 ± 0.06^{G}	0.045 ± 0.001^{G}
8		6	Corn cobs	$6.03 \pm 1.22^{B,C}$	0.084 ± 0.017^{E}	4.77 ± 0.4^{D}	0.135 ± 0.008^{E}
9			Wheat bran	6.39 ± 1.32^{C}	0.120 ± 0.028^{D}	2.19 ± 0.13^{F}	$0.047 \pm 7E\text{-}4^{G}$
10			Lemon peel	7.21 ± 0.44^{B}	0.156 ± 0.012^{C}	6.08 ± 0.61^{C}	0.204 ± 0.014^{D}
11		8	Corn cobs	3.36 ± 0.98^{D}	$0.070 \pm 0.021^{E,F}$	6.76 ± 0.53^{B}	0.087 ± 0.006^{F}
12			Wheat bran	$0.88 \pm 0.05^{E,F}$	$0.024 \pm 0.001^{G,H}$	3.59 ± 0.28^{E}	$0.288 \pm 7E\text{-}4^{A}$
13			Lemon peel	8.48 ± 0.41^{A}	0.353 ± 0.022^{B}	11.57 ± 0.14^{A}	0.241 ± 0.003^{B}
14		9	Corn cobs	$0.07 \pm 0.003^{F,G}$	$0.003 \pm 3E\text{-}5^{H}$	0 ± 0^{H}	0 ± 0^{H}
15			Wheat bran	$0.45 \pm 0.03^{E,F,G}$	$0.019 \pm 2E\text{-}5^{H}$	0 ± 0^{H}	0 ± 0^{H}
16			Lemon peel	$0.55 \pm 0.02^{E,F,G}$	$0.024 \pm 1E\text{-}4^{G,H}$	0 ± 0^{H}	0 ± 0^{H}
LSD				1.1694	0.0303	0.515	0.0105

*Results with different capital letters were significantly different compared with their corresponding LSD value.

In this last case lemon peel was the carbon source in which the highest pectinolytic activity was obtained, although it must be noted that productions higher than mean production were also obtained with corncobs and wheat straw (Figure 2b). The exopectinase activities obtained on xylan or xylan-rich agroindustrial residues, as wheat bran and corncobs, suggested the inducing ability of some components of these substrates, as arabinose, for pectinolytic system in this *Aspergillus* strain, as has been reported before for another species (de Vries and Visser, 2001; Arotupin, 2007).

As expected, among polysaccharides the highest xylanolytic activities were obtained on xylan; while for exopectinases and pectin lyases, the highest titers were observed on pectin. This is in accordance with previous reports, which signaled xylan and pectin as the natural inducer of the corresponding enzymatic activities (de Vries and Visser, 2001; Liu *et al*., 2017).

The high exopectinolytic productions obtained by using citric residues as substrates has been also reported for *A. niger*, *A. flavipes* and *A. terreus* (Mrudula and Anitharaj, 2011; Martínez-Trujillo *et al*., 2011). However, there are few reports in which lemon peel has been used as the only carbon source. Regarding this, *A. flavus* CECT-2687 showed similar exopectinolytic activities than those reported for *A. niger* in this substrate (Patil and Dayanand, 2006).

In regard to the evolution of enzyme production along the culture, the highest activities obtained on polysaccharides or agroindustrial residues are shown on Figure 3. It is worth noticing that the maximum xylanolytic activity was reached between 24 and 48 h of the culture (Figure 3a), which in fact happened in all substrates. Titers obtained on pectin or lemon peel were similar, reached the maximum activity during the first 24 h of the culture and diminished about 50 % after that (Figure 2b). However, exopectinolytic productivity obtained on lemon peel was slightly higher than that obtained when the fungus grew up on pectin, both at initial pH of 8 (Table 2).

In respect to pectin lyases produced on polysaccharides and agroindustrial residues, this activity was not observed on xylan, but good titers were obtained on wheat bran and corncobs, both xylan-rich substrates. Nevertheless, the highest pectin lyases production was obtained on lemon peel, mainly at pH 8.0 (Table 2); and it was higher than that obtained on pectin at the same pH value. This behavior has two possible explanations: 1) by the concentrations used in this particular experimental condition (10 g l^{-1}) pectin can be acting as a repressor; or 2) at pH 8.0 pectin is de-esterified (Laufenberg *et al*., 2003), which makes it a less suitable inductor of this activity (de Vries and Visser, 2001). However, specifically on lemon peel, the pectin contained in the husk is about 25 % (Laufenberg *et al*., 2003); which means that when using this agroindustrial residue as the only carbon source the fungus has about 2.5 g l^{-1} of pectin, and under this concentration the repressive

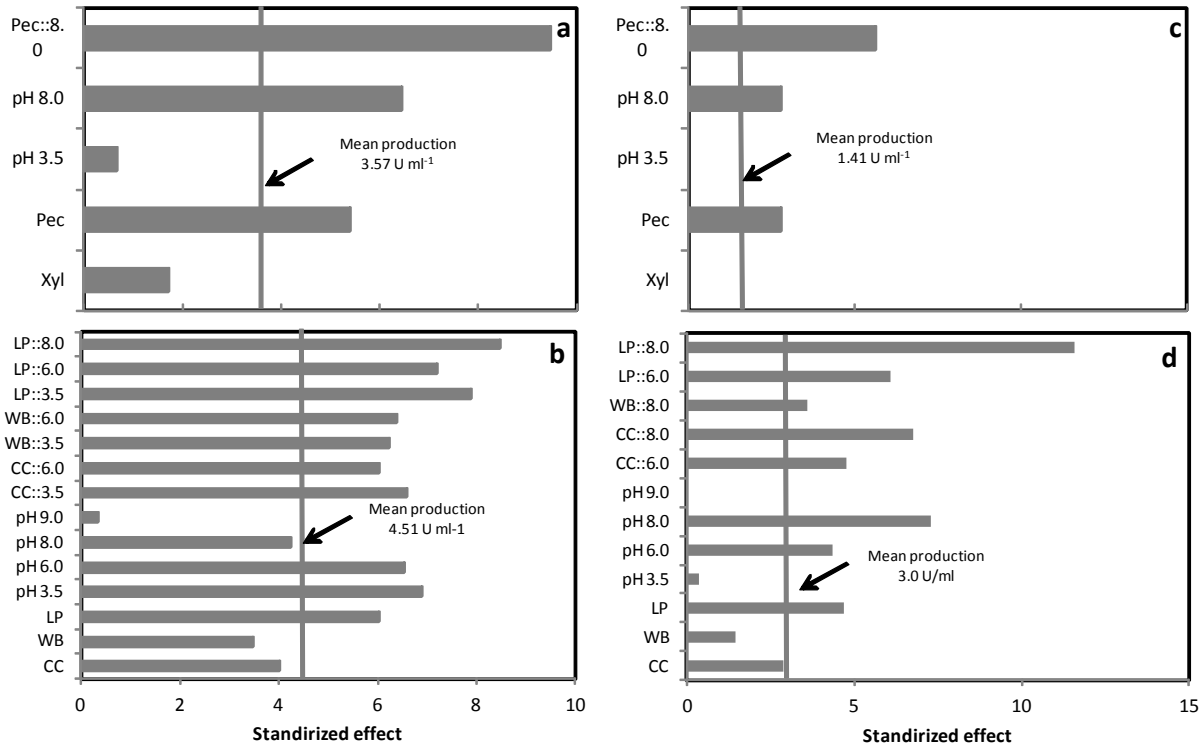

Fig. 2. Pareto chart of standardized effects of each factor and the significant interaction among carbon source:pH for production of exopectinases (a and b) and of pectin lyases (c and d) after 72 hours of fermentation on polysaccharides (a and c) and agroindustrial residues (b and c). All effects higher than those corresponding to mean production (shown by the vertical solid gray line) indicate that the corresponding factor is significant for pectinases production. As to the interaction effect, in the figure shows only those with values higher than mean production. Pec, pectin, Xyl, xylan, LP, lemon peel, CC, corn cobs, and WB, wheat bran. The two dots couple (::) depicts the interaction between two different factors.

effect of pectin could be reduced. Besides, pectin in lemon peel is associated with other polysaccharides, and because of this is less susceptible to de-esterification caused by the alkaline pH. Both facts can explain the highest pectin lyase activities obtained on lemon peel.

Pectin lyases produced by *A. flavus* species is not frequently reported. In fact, there are only a few reports for a non-toxigenic strain of *A. flavus* growing on pectin (Yadav *et al*., 2008; Yadav *et al*., 2013). However, the high values of pectin lyase activity obtained in this work when agroindustrial residues were used as substrates indicate the potential and versatility of our strain for growing and colonizing plant tissues. In fact, it was recently reported the role of pectinases and pectin lyases as a virulence factor for the infection of apple fruit by *A. niger* (Liu *et al*., 2017).

Regarding the initial pH, this parameter had an important effect on pectin lyases productions compared with those exerted by substrates, especially in those experiments developed at initial pH of 8.0 (Figure 2 d). However, in this case the joint effect of pectin and initial pH of 8, as well as lemon peel and initial pH of 8 had the main effect on pectin lyase production (Figures 2 c and d). Also, it should be noted that good lyase titers were obtained at acidic pH on some agroindustrial residues, mainly corn cobs (Table 2). However, this production was not significant in respect to mean production (Figure 2d). High pectin lyases production obtained at initial pH of 8.0 has also been reported for another *Aspergillus* lyases (Delgado *et al*., 1993).

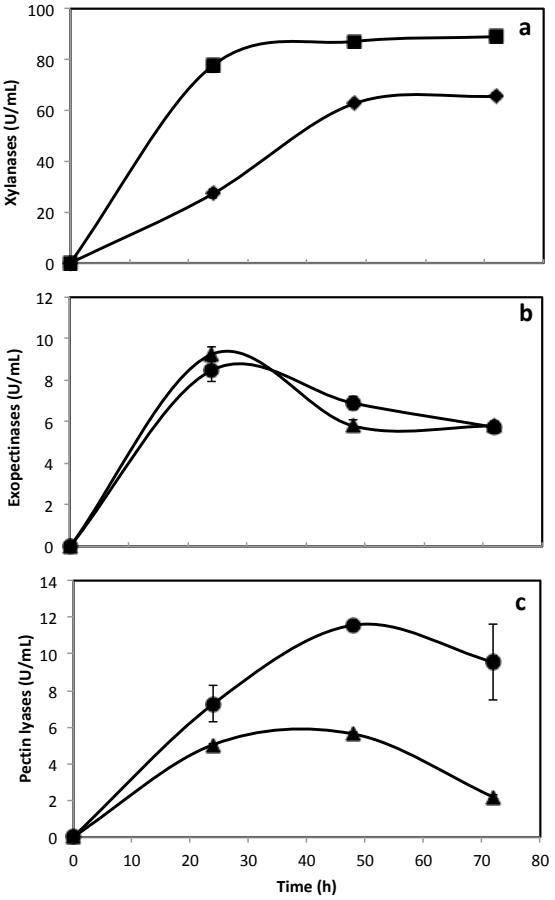

Fig. 3. Xylanases (A), Exopectinases (B) and Pectin lyases (C) production by *A. flavus* batch cultures developed on: xylan initial pH 8.0 (♦), corn cobs initial pH 8.0 (■), pectin initial pH 8.0 (▲) or lemon peel initial pH 8.0 (●).

On the other hand, even when pectin lyase production obtained on lemon peel was considerably higher than that obtained on pectin on pH 8.0, the productivity value was almost the same (Table 2). In this case the highest activity was observed at 48 h of the culture, and it diminished after that (Figure 3).

As the joint effect of carbon source and pH on the production of xylanases and pectinases were significant (Figures 1 and 2), a zymographic analysis of the enzymes produced on agroindustrial residues under different initial pH values were done; this suggested difference in protein patterns (data not shown). On a gel incubated at pH 8.8 with 73% esterification degree pectin, we found a clear band of around 27 KDa.

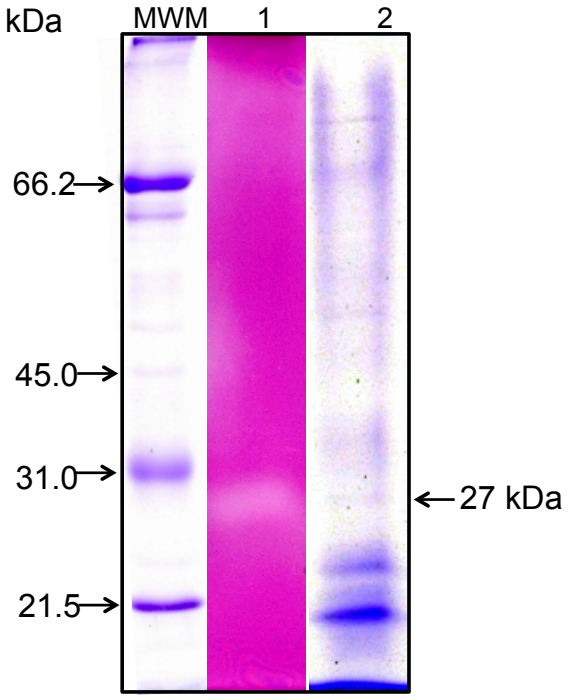

Fig. 4. Electrophoretic pattern of filtrates obtained with *A. flavus* CECT-2687 growing on lemon peel at initial pH value of 8.0. MWM, molecular weight markers; Lane 1, zymogram for pectin lyases and Lane 2, SDS-PAGE protein profile. The arrow on the right side of the figure depicts the protein band corresponding to a lyase activity.

As it is well known that an alkaline pH is necessary for the stimulation of lyases activities, which are viewed as clear zones in zymograms (Hadj-Tajeb *et al.*, 2011), we concluded that this band could correspond to a pectin lyase (Figure 4). This enzyme is different to those reported by Yadav *et al.* (2008 and 2013), which had different molecular mass and biochemical characteristics. Work is in progress in order to purify and characterize this pectin lyase.

The behavior on the production of these xylanases and pectinases by *A. flavus* CECT-2687 is consistent with the fact that the production of these enzymes by *A. flavus* could be regulated by the environmental pH found by fungi during the invasion process, as has been signaled by Aro *et al.* (2005) for several microbial enzymes. *A. flavus* could attack the cell wall of plants, by producing only those enzymes that exert its function in a specific condition. As the pH of some tissues that are attacked by this kind of fungi is around neutrality or slightly alkaline, the adaptability of *A. flavus* to different environments and its ability

to produce high levels of these enzymatic activities when using pH values similar to that condition could be obvious.

Conclusions

Xylanases and pectinases production by *A. flavus* CECT-2687 depends on carbon source and pH. This last factor has the main effect on the production, but the joint effect of both factors was important too. The greatest xylanolytic production was obtained on xylan, wheat bran and corn cobs, while pectinases were produced mainly on pectin or lemon peel; although good productions of exopectinases and pectin lyases were obtained on those xylan-rich substrates. In all the cases the greatest production was obtained with the initial pH of 8. Enzymatic activities produced by this *A. flavus* strain showed the potential and versatility of this species, and helped to explaining its ability to colonize both plant tissue and cereals.

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