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Research Article

Fusarium graminearum as a producer of xylanases with low cellulases when grown on wheat bran

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ABSTRACT

The xylanolytic potential of endophytic fungi isolated from leaves of *Theobroma cacao* was explored for the first time. Four fungal strains showed significant amounts of xylanase activity and low cellulase levels when grown on wheat bran as the sole carbon source. Strain Ec220 of *Fusarium graminearum* had the highest xylanase production (1.79 U/ml), whereas its cellulase activity was minimal (0.24 U/ml). Optimal conditions for xylanase production were: 154 h of incubation time, pH 5.79 and 29.8 °C. Furthermore, two protein spots detected by two-dimensional gel electrophoresis showed molecular weights (26.05 and 27.70 kDa) and isoelectric points (6.18 and 9.20) corresponding to previously reported *F. graminearum* xylanases, Xyl A and Xyl B, respectively. Therefore, endophytic fungi of *T. cacao* can be an important source of xylanolytic activities when cultured on wheat bran, and xylanases with low cellulases found in strain Ec220 require further characterization as they show promise for possible industrial applications.

1. Introduction

Lignocellulosic materials are becoming increasingly relevant in industrial processes because they are the main source of renewable energy in the world, are widely available, and can be obtained at very low costs as agro-industrial wastes [1]. However, the greatest limitation to using lignocellulosic materials in the industry is its high resistance to degradation into simpler fermentable sugars, which is due to the polymeric complexity and strong covalent and non-covalent bonds formed by its three main components: cellulose (40-50%), hemicellulose (25-30%), and lignin (15-20%) [2–4]. Thus, there is a current industrial need for natural enzymes with high hydrolytic capacity to achieve a more efficient degradation of lignocellulosic biomass. Several authors have highlighted the use of plant cell wall degrading enzymes (PCWDE), such as cellulases and hemicellulases, for the efficient bioconversion of lignocellulose into lower molecular weight compounds that can be used in industry [5,6].

Xylanases (EC 3.2.1.8), one of the most common types of hemicellulases, have received growing attention due to their biotechnological potential for a wide range of industrial applications, such as pulp bleaching, paper deinking, biofuel production, and bread making, among others [7]. Furthermore, cellulase-free xylanases have proven to be effective in selectively removing hemicellulose and other non-cellulolytic polysaccharides that coat the cellulose fibers of biomass [8,9]. This is very useful in the biofuel industry since cellulose can be readily exposed to enzymatic attack, which allows a better conversion of lignocellulosic material to simpler compounds like ethanol [10]. In the paper industry, the use of these enzymes makes it possible to obtain superior quality paper because they hydrolyze the xylan-lignin linkages over pulp fibers with minimum disruption of the cellulose components. Additionally, they have become an eco-friendly and economic alternative in pulp bleaching as they help to greatly reduce the use of expensive, detrimental chlorinated chemicals [11,12]. However, it is still necessary to prospect new natural sources to find organisms capable of producing abundant amounts of xylanases with high hydrolytic power and specificity at low production costs [4,13].

Microorganisms, particularly filamentous fungi, are the main producers of xylanases at a commercial level due to their easy cultivation and high production of extracellular enzymes [14]. Endophytic fungi are among the most promising microbial sources to screen for enzymes with high xylanolytic activity. This is because these fungi live in symbiosis with plants, so they are capable of secreting a wide range of PCWDE to colonize leaves, roots and more complex plant structures [15,16].

Endophytic fungi of the cacao tree (*Theobroma cacao*) have not been

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prospected for their xylanolytic potential as far as we know. In a previous study with fungal isolates from cacao pod husks, strains of *Fusarium* and *Aspergillus* had the highest cellulase activity [17]. *F. graminearum* (*Gibberella zeae*) is well known for being an aggressive pathogen that cause severe damage to wheat and barley crops annually, but in *T. cacao* it acts as an endophyte without apparent harm to the host plant [18,19]. Various authors have highlighted the ability of *F. graminearum* to secrete a variety of PCWDE when using lignocellulosic biomass as substrate. Wheat bran, for instance, is considered as the most effective carbon source among agro-industrial wastes for enzyme production, especially for induction of xylanases, which is attributed to its high (51.8%) hemicellulose content [20–22]. Thus, the aim of this study was to explore and optimize the xylanase production of endophytic fungi isolated from *T. cacao* when cultured on wheat bran as the sole carbon source.

2. Materials and methods

2.1. Chemicals and reagents

All reagents were of analytical grade and used as received with no further purification. Carboxymethylcellulose (CMC), yeast extract, trichloroacetic acid (TCA), acetone, phenol, Tris base, sodium dodecyl sulfate (SDS), 2-mercaptoethanol, glycerol, bromophenol blue, Coomassie Brilliant blue G-250, methanol and acetic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Potato Dextrose Agar (PDA) was obtained from Becton Dickinson (Sparks, MD, USA), beechwood xylan was sourced from Biosynth (Berkshir, UK), hydrochloric acid fuming 37% (HCl) was purchased from Mallinckrodt (Staines-upon-Thames, UK), sodium hydroxide (NaOH) was obtained from Macron (Bologna, Italy). Wheat bran and soy flour were obtained from local markets.

2.2. Fungal strains

The five fungal strains used in this study were obtained from the collection of microorganisms of the Biotechnology Research Center of Ecuador (CIBE) at ESPOL Polytechnic University. These strains were endophytic fungi isolated from healthy leaves of Nacional type cacao (*Theobroma cacao*) in three different localities of Ecuador, which was endorsed by the framework contract MAE-DNB-CM-2018-0091. Isolates were identified taxonomically in a previous study by Villavicencio-Vásquez et al. [23] as: *Fusarium graminearum* strain Ec220, *F. equiseti* strain Ec023, *Lasiodiplodia theobromae* strain Ec157, *L. theobromae* strain Ec120, and *Xylaria sp.* strain Ec099. Fungal strains were activated on PDA at 28 °C for seven days. Cultures were then transferred to 2% (w/v) soy flour agar plates and grown at 28 °C for 12 days, stored at 4 °C and sub-cultured after every 30 days.

2.3. Enzyme production

Production of enzymes of interest was conducted by submerged fermentation (SmF) on 500 ml Erlenmeyer flasks, using 100 ml of a liquid minimal salt medium (MSM) [24] supplemented with 0.6% (w/v) yeast extract and 1.0% (w/v) wheat bran as the sole carbon source [25]. Additionally, 1 ml of a trace element solution [in (w/v): 0.64% $CuSO_4.2H_2O$, 0.11% $FeSO_4.7H_2O$, 0.79% $MnCl.4H_2O$ and 0.15% $ZnSO_4.7H_2O$] was added per 1 L of MSM medium. The pH of the medium was adjusted to 6.0 with 12 M HCl. Flasks with MSM content were autoclaved at 121 °C for 30 min, and then inoculated with five 5 mm-diameter agar plugs from a fresh culture and incubated for 10 days at 30 °C and 120 rpm on a rotatory shaker (New Brunswick Scientific, Edison, NJ, USA). Culture samples were obtained daily and then centrifuged for 10 min at 15,000 rpm. The resulting supernatants were collected and used as crude enzymatic extracts for determination of enzyme activities.

2.4. Quantification of enzyme activities

Xylanase and cellulase activities were measured according to the amount of reducing sugars released in the medium by means of the 3,5-dinitrosalicylic acid (DNS) assay modified by Bailey et al. [26]. Briefly, a 1.0% (w/v) substrate solution dissolved in 0.2 M sodium acetate buffer (pH 5.0) was prepared, using beechwood xylan for xylanase activity and CMC for cellulase activity as substrates. Next, 50 µl of enzymatic extract was added to 450 µl of substrate solution, and then the mixture was incubated at 50 °C for 30 min. The reaction was stopped by the addition of 750 µl DNS reagent, at 100 °C, for 10 min [27]. Tubes were then cooled in cold water for 10 min and the absorbance was measured at 540 nm against the blank. As standard curves, several concentrations of xylose and glucose were prepared for xylanase activity and cellulase activity, respectively. One enzyme unit (U) was defined as the amount of enzyme required to liberate 1 µmol of reducing sugar (xylose or glucose) per minute.

2.5. Optimization of xylanase production by response surface methodology (RSM)

Temperature, initial pH of the medium and incubation time are crucial factors that affect the production of xylanases [22,28]. Two central composite designs (CCD) were carried out through RSM, where xylanase activity was considered as the response variable and factors as independent variables. For the first CCD, factors temperature and incubation time were used at two different levels, followed by a second CCD with pH and incubation time as factors, at four different levels (Table 1). pH adjustment was carried using 12 M HCl and 5 M NaOH solutions. Quantitative data obtained from these experiments were fitted using the following second-order polynomial equation proposed by Soni et al. [29] for the optimization of the response variable:

$$Y = \beta_0 + \sum_i \beta_i X_i + \sum_{ij} \beta_{ij} X_i X_{ij} + \sum_{ii} \beta_{ii} X_i^2 \quad (1)$$

In this polynomial equation, Y corresponds to the response variable, X_i and X_j are independent variables, β_0 is the intercept term, β_i is the linear coefficient, β_{ij} is the interaction coefficient and β_{ii} is the quadratic coefficient.

2.6. Statistical analysis

Experimental data was analyzed using the RStudio (v 4.0.0) software package "rsm" for the response surface analysis and the analysis of variance (ANOVA) for the estimation of the regression coefficient (R^2), prediction of the statistical model, and fitness of data into the model. The statistical significance of the model was examined by Fisher distribution test (*F test*), whereas Tukey's multiple comparison test was used

Table 1
Levels and range values of the independent variables for the two central composite designs (CCD) used for the optimization of xylanase production

Variables		Level				
Factor code	Factor	Lower (-2/-3*)	Low (-1)	Central Point (0)	High (+1)	Higher (+2/+3*)
First CCD						
X_1	Temperature (°C)	-	25	30	35	-
X_2	Incubation time (h)	-	144	168	192	-
Second CCD						
X_2	Incubation time (h)	96	120	144	168	192
X_3	pH*	4.0	5.0	5.5	6.0	7.0

* For factor pH, "Lower" level was -3 and "Higher" level was +3, according to its central point.

for the DNS assay results. Data was presented as the average of three replicates ± standard deviation (SD).

2.7. Freeze-drying, precipitation and protein determination

Enzymatic extracts of *F. graminearum* Ec220, obtained under optimal conditions, were freeze-dried at - 40 °C for 72 h. Subsequent protein precipitation was performed following the TCA/acetone + phenol protocol established by Wang et al. [30]. The protein concentration was determined by the method of Bradford [31], using the Protein Assay Dye Reagent (Bio-Rad) with bovine serum albumin (BSA) as standard.

2.8. SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli [32]. Protein samples were diluted 2:1 (v/v) in sample buffer [1.28 M Tris base 4X, pH 6.8, 10% (w/v) SDS, 8.21% (w/v) 2-mercaptoethanol, 26% (v/v) glycerol and 0.02% (w/v) bromophenol blue] and boiled for 10 min. Electrophoresis was performed on a 12% (w/v) polyacrylamide gel in a Mini-PROTEAN 3 equipment (Bio-Rad, Hercules, CA, USA) at 120 V for 140 min. Protein bands were visualized by staining with 0.05% (w/v) Coomassie Blue G-250 [prepared in 50% (w/v) methanol, 10% (w/v) acetic acid and 40% (w/v) distilled water] and then analyzed in the Image Lab v 6.0.1 program (Bio-Rad).

2.9. 2-D PAGE

A quantity of 165 μg of precipitated sample dissolved in 81 μl of ReadyPrep-BioRad® rehydration buffer [10 ml of 8 M urea, 2% (w/v) 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 50 mM dithiothreitol (DTT), 0.2% (w/v) Bio-Lyte® and 0.005% (w/v) bromophenol blue] was applied to a 7-cm IPG strip with pH range 3-10 (ReadyStrip™ IPG Strips, BioRad). Isoelectric focusing (IEF) was performed on the IPG strip using a Protean i12 IEF Cell system (Bio-Rad). Subsequently, the strip was equilibrated applying two equilibration solutions for 30 min each: Buffer I [20 ml of 6M urea, 2% (w/v) SDS, 0.375 M Tris-HCl (pH 8.8), 20% (w/v) glycerol and 2% (w/v) DTT] and Buffer II (same components as Buffer I except DTT). The strip was washed with 1.875 M Tris-HCl (pH 8.8) to remove excess equilibrium solution. The second dimension was performed in a 12% SDS-PAGE gel at 35 mA for 2 h. Protein spots were detected using the PDQuest™ 2-D analysis software (v 8.0.1, Bio-Rad).

3. Results and discussion

3.1. Determination of xylanase and cellulase activities

Enzyme activities were measured by the DNS assay method, using the crude enzymatic extracts from MSM liquid cultures with wheat bran as the sole carbon source. Incubation conditions were 30 °C and pH 6.0, considered as standard for fungal growth [33].

Four out of the five fungal strains prospected in this study presented significant xylanase activity in the range of 0.878-1.779 U/ml. Furthermore, *F. graminearum* strain Ec220 had the highest xylanase activity (1.779 ± 0.042 U/ml) among all strains with statistical significance ($p < 0.001$) as shown in Table 2. This value was greater than the xylanase activity (0.678 U/ml) previously reported in a similar study by Dong et al. [34], where *F. graminearum* was grown on wheat bran hydrated with a modified synthetic medium, but under different culture conditions. Interestingly, a research study with birchwood xylan, a conventionally used substrate for xylanase production, found an even lower xylanase activity (0.380 U/ml) than those with wheat bran [35]. Thus, as many authors have also highlighted, wheat bran is an inexpensive lignocellulosic material that can induce xylanase production more effectively than any other agro-industrial waste or even costly substrates like xylans [20,22,34].

Table 2
Enzyme activities of the five fungal strains prospected in this study.

Strain	Enzyme Activity (U/ml) *		Time (h) of crude extract collection**	
	Xylanase	Cellulase	Xyl.	Cel.
F. graminearum Ec220	1.779 ± 0.042^{a}	0.243 ± 0.002^{a}	144	168
L. theobromae Ec157	1.392 ± 0.014^{b}	0.621 ± 0.001^{b}	168	168
F. equiseti Ec023	1.341 ± 0.024^{b}	0.176 ± 0.007^{d}	120	144
L. theobromae Ec120	0.878 ± 0.001^{c}	0.400 ± 0.004^{c}	168	120
Xylaria sp. Ec099	0.043 ± 0.001^{d}	0.171 ± 0.006^{d}	216	144

* Statistically significant differences ($p < 0.001$) between strains with regard to enzyme activity values were represented by a letter superscript (a, b, c and d) according to Tukey's test for multiple comparisons.

** Time (h) on which the highest enzyme activity (xylanase and cellulase, respectively) was recorded for each fungal strain under standard culture conditions: 30 °C and pH 6.0.

Cellulase activity was also determined, which was useful to evaluate how selective wheat bran was to induce xylanase activity in the MSM medium. The Ec220 strain had its highest cellulolytic activity (0.243 ± 0.002 U/ml) at 168 h of incubation time. This was significantly lower ($p < 0.001$) than that presented by the two strains of *L. theobromae* (Ec157 and Ec120), which obtained the highest cellulase activities in the DNS assay (Table 2). In addition, these results are similar to those reported by Kikot et al. [36], who also found lower cellulase activity (0.320-0.330 U/ml) compared to xylanase activity (0.840-0.880 U/ml) when *F. graminearum* isolates were grown on commercial oat bran as the carbon source, a lignocellulosic material with similar chemical composition to wheat bran. On the other hand, a study that utilized cellulose-rich agricultural by-products indicated a maximum cellulase production (1.01 U/ml) by *F. graminearum* with corn stover as substrate [37]. This can be explained by the chemical composition of these residual materials since the chemical composition of wheat bran has a higher hemicellose content (51.8%, mainly arabinoxylans) than cellulose (18.5%), whereas corn stover is made of 35-40% cellulose and only 18-22% hemicellose [21,38,39]. Based on this, it can be assumed that wheat bran selectively induced high xylanase activity and low cellulases in *F. graminearum* Ec220, which was the strain selected for the optimization of factors for xylanase production.

From a biotechnological point of view, this result has great application potential in the paper and detergent industries where there is a current need to find cellulase-free or low-cellulase microbial xylanases that can help improve the quality of finished products, reduce production costs and minimize environmental pollution. For instance, in the pulp and paper industry, cellulase-free xylanases have proven to be highly efficient in selectively removing hemicellulose components without altering or damaging cellulose fibers during pulp bleaching, resulting in a cleaner, high-quality paper with minimal use of expensive chlorinated compounds [40,41].

3.2. Optimal conditions for xylanase production

In this study, RSM was carried out to establish the optimal temperature, incubation time and pH, three crucial factors for xylanase production. In the first CCD with temperature (X_1) and incubation time (X_2) as factors, the adjusted R^2 was 0.875, which means that the response variable or xylanase activity was explained in 87.5% by these two factors. However, ANOVA (*F-test*) showed that only the linear terms X_1 and X_2, and not their interaction, were statistically significant ($p < 0.001$) in the statistical model predicted by the RSM [Eq. (A.1) in Supplementary material]. Also, the lack of fit (LOF) was significantly greater than the pure error ($p < 0.001$), so there was no good fitness of model, which can be due to a lack of variation in the experimental data [42]. As shown in Fig. 1, at temperature levels -1 (25 °C) and +1 (35 °C), yields were considerably lower and had little variation throughout the incubation

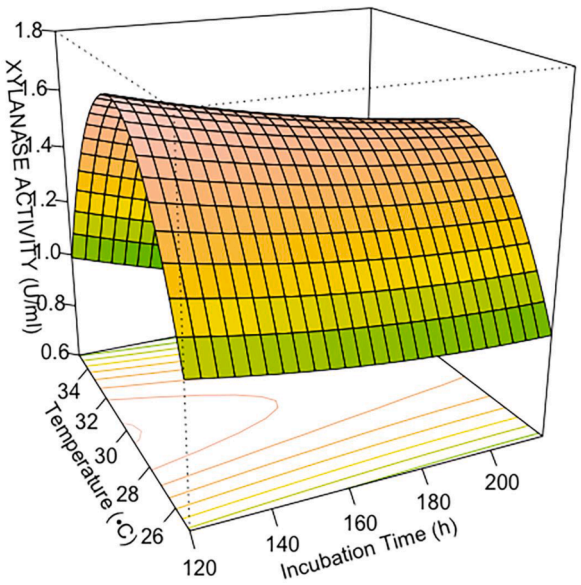

Fig. 1. 3D response surface plot of xylanase activity as a function of temperature (X_1) and incubation time (X_2).

time. Yet, the experimental values at the central point (30 °C) were twice higher than the yields obtained at the other two levels, and they were the only values that agreed with the outcome predicted by the model (Table A.1 in Supplementary material). Additionally, this temperature was very similar to the optimal temperature (29.8 °C) predicted by the RSM, which is also in good agreement with what has been reported by other authors as optimal for growth and enzyme production of *F. graminearum* [18,43].

Since xylanase production could not be optimized by the first CCD, a second CCD was carried out. However, this time temperature was not considered as a factor because it was notorious that at 30 °C strain Ec220 produced the highest yields of xylanase (Table A.1 in Supplementary material), which was confirmed by the predicted optimal temperature (29.8 °C). Thus, temperature was maintained constant in the second CCD. Instead, initial pH (X_3), alongside incubation time (X_2), were used as factors to evaluate their effect on xylanase activity. Four different levels with two central points and six axial points were conducted for each factor, for a total of 24 experimental treatments, as indicated in Table 3. First, the results of the RSM were fitted to Eq. (1) to obtain Eq. (2). Afterward, the statistical significance of Eq. (2) was analyzed by ANOVA (*F-test*). All terms: linear (X_2 and X_3), the interaction (X_2X_3), and quadratic (X_2^2 and X_3^2) were significant ($p < 0.001$). Also, $R^2 = 0.808$ was similar to the adjusted $R^2 = 0.773$, which suggests that the predicted values agreed with the experimental values [29]. In addition, the LOF was not significant ($p > 0.05$), indicating that the model was adequate for this experimental design and can be used to predict the response variable. Thus, the effect of incubation time (X_2) and initial pH (X_3) on the xylanolytic activity of strain Ec220 (Y) can be represented by the following equation:

$$\textbf{Xylanase Activity}\,(Y) = +\,1.728\; +0.182\;X_2 + 0.164\;X_3 - 0.064\;X_2\;X_3 - 0.165\;X_2^2 - 0.114\;X_3^2 \quad (2)$$

The initial pH of the medium is a very significant variable during the fermentation process, since it can influence numerous enzymatic systems, the transport of enzymes across the cell membrane and the speed at which nutrients are utilized, which ultimately has an effect on the growth and enzyme production of the microorganism [44,45]. Fig 2 shows the 3D RSM plot and its contour graph, where the effect of time and initial pH with respect to the response variable can be observed.

Table 3
Experimental arrangement in coded values of the central composite design (CCD) with incubation time and pH as factors along with the predicted and actual xylanase activities as response.

Treatment	Incubation time (X_2)	pH (X_3)	Predicted value (U/ml)	Experimental value (U/ml)*
1	-2	+3	0.554	0.761 ± 0.023
2	-1	+3	1.039	1.099 ± 0.454
3	0	+3	1.194	0.818 ± 0.276
4	+1	+3	1.019	0.720 ± 0.330
5	+2	+3	0.514	0.665 ± 0.029
6	-2	+1	0.882	0.419 ± 0.003
7	-1	+1	1.495	1.768 ± 0.123
8	0	+1	1.778	1.779 ± 0.042
9	+1	+1	1.731	1.534 ± 0.080
10	+2	+1	1.345	1.450 ± 0.078
11	-1	0	1.381	1.354 ± 0.023
12	0	0	1.728	1.550 ± 0.014
13	0	0	1.728	1.762 ± 0.013
14	+2	0	1.432	1.142 ± 0.045
15	-2	-1	0.298	0.043 ± 0.005
16	-1	-1	1.039	0.940 ± 0.021
17	0	-1	1.450	1.614 ± 0.019
18	+1	-1	1.531	1.655 ± 0.043
19	+2	-1	1.282	1.724 ± 0.012
20	-2	-3	-1.198	0.000 ± 0.000
21	-1	-3	-0.329	0.002 ± 0.000
22	0	-3	0.210	0.008 ± 0.001
23	+1	-3	0.419	0.101 ± 0.020
24	+2	-3	0.298	0.063 ± 0.022

* Values are expressed as mean ± SD, n=3

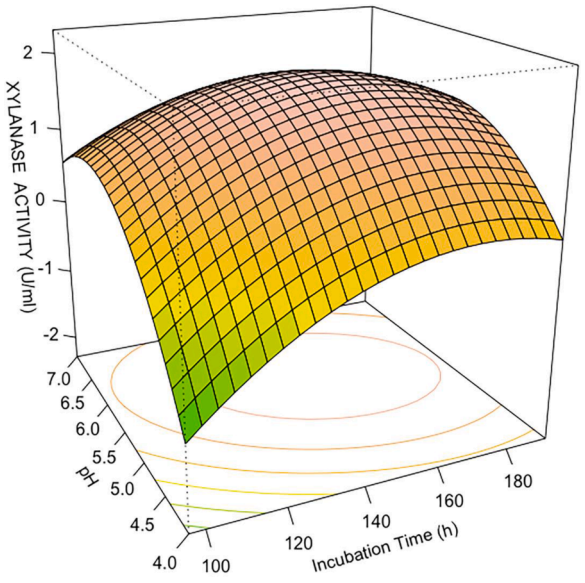

Fig. 2. 3D response surface plot of xylanase activity as a function of incubation time (X_2) and pH (X_3).

Xylanolytic activity increased exponentially from pH 4.0 to nearby pH 6.0, and then moderately decreased at pH 7.0. The stationary point predicted by the RSM, where Eq. (2) predicts the highest yield, was reached at an optimal pH value of 5.79. To our knowledge, optimal pH for xylanase production has not been reported in previous studies with *F. graminearum* isolates in SmF. However, Peter Mshelia et al. [43] reported a pH of 5.6 for optimal growth of *F. graminearum* and a study with *F. solani* found an optimal pH of 5.5 for xylanase production [46], which are in agreement with the results obtained in this study.

In the case of incubation time, the highest xylanase production occurred between 144 and 168 h, with the optimal value predicted at 154 h, which is concordant to previous studies with *F. solani* and

Aspergillus niger [8,47]. As shown in Table 3, the statistical model Eq. (2) was able to fairly predict most experimental values. Nevertheless, an RSM model that includes three or more factors is recommended for future studies.

Finally, the conditions established as optimal for xylanase production by strain Ec220 were: 154 h of incubation time, pH 5.79 and temperature 29.8 °C. The model was validated at optimal conditions, where the observed response (1.786 ± 0.076 U/ml yield of xylanase) was not statistically different ($p > 0.05$) from the predicted yield (1.817 U/ml) by the model Eq. (2). Furthermore, the production achieved after statistical optimization was the highest xylanase activity attained in this investigation.

3.3. Proteomic analysis

Crude enzymatic extracts, obtained under optimal conditions for xylanase production, were freeze-dried and precipitated for a better resolution of the proteins present in the secretome of strain Ec220. SDS-PAGE of the precipitated enzyme sample revealed three bands of great intensity at 36.7, 30.3 and 27.0 kDa, and one with lower intensity at 26.1 kDa (Fig. 3). Pollet et al. [48] found similar results with four purified xylanases of *F. graminearum*. They classified the ones with higher molecular weight (MW), Xyl D (39.7 kDa) and Xyl C (34.1 kDa), as

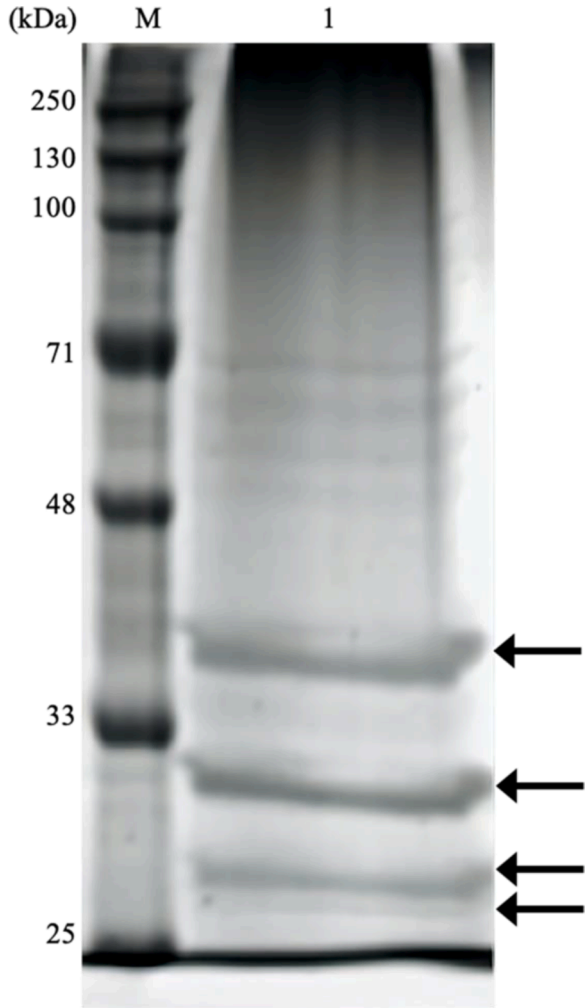

Fig. 3. SDS-PAGE of strain Ec220 secretome under optimal conditions with wheat bran as substrate. (1) Precipitated enzyme sample; arrows point at bands with MW (36.7, 30.3, 27.0 and 26.1 kDa, from top to bottom) similar to those previously reported for xylanases of *F. graminearum*. (M) Protein molecular weight marker (RunBlue Prestained).

glycosyl hydrolase family (GH)10, while the other two with lower MW, Xyl A (27.3 kDa) and Xyl B (26.0 kDa), were classified as GH11 xylanases. This suggests that the proteins expressed more predominantly in the secretome of Ec220, after being cultured on wheat bran, could be one or more of the xylanases formerly described in isolates of *F. graminearum*.

A two-dimensional gel electrophoresis (2-D PAGE) was carried out in order to achieve a better determination of the protein profile of strain Ec220. In the data analysis, 192 protein spots were identified. As shown in Fig. 4, the first protein spot (P1) had a MW of 26.05 kDa and an isoelectric point (pI) of 6.18, which is in good agreement with the MW (24-27.3 kDa) and pI (6.20) previously reported for Xyl A of *F. graminearum* [48]. The second protein spot (P2) had a pI of 9.13, which was similar to the pI (9.20) described for Xyl B of *F. graminearum*; however, its MW (27.70 kDa) was slightly outside the range (23-26 kDa) indicated in other studies [48,49]. In addition, the presence of GH11 xylanases has also been reported in other *Fusarium* species, such as *F. oxysporum* (MW: 23.5 kDa and pI: 8.70) and *F. verticillioides* (MW: 24 kDa and pI: 8.60) [34,50,51].

Moreover, none of the protein spots detected had coincidences with MW and pI values previously reported for GH10 xylanases. This differs from what was reported in a previous study where both GH11 and GH10 xylanases were present in the secretome of *F. graminearum*, using wheat bran as inducer of enzymatic activity [20]. Interestingly, a study that evaluated the effect of pH and temperature on two purified xylanases from *F. graminearum* cultures found that the xylanase with high molecular weight (~41 kDa) was more temperature-sensitive and slightly less stable at pH 5.5 in comparison to the low molecular weight xylanase (~20.9 kDa) [34]. Thus, it is likely that high molecular weight xylanases (GH10) degraded more rapidly as the enzyme sample underwent different experimental conditions before the proteomic analysis.

Furthermore, a study that characterized both types of xylanases found that GH10 and GH11 have different substrate binding structures and specificities. The TIM-barrel structure of GH10 xylanases provides them with a higher hydrolytic power to reduce soluble xylans more effectively than GH11. However, in a natural scenario, where xylan forms complex structures with celluloses and lignins inside plant cell walls, the wide shallow binding structure of GH11 xylanases allows them to be more efficient at catalyzing biomass degradation in comparison to GH10 [52]. Based on the different catalytic mechanisms these two types of xylanases possess to degrade xylan, this suggests that microorganisms may express both at different concentrations and times according to the conformation and availability of xylan. In this research studio, mostly biomass xylans were available for degradation as the substrate used for xylanase production was a complex lignocellulosic material such as wheat bran. Therefore, this supports the fact that the two protein spots identified in the secretome of strain Ec220 have high degree of similarity with the characteristics of the GH11 xylanases of *F. graminearum* reported in previous studies.

4. Conclusion

Xylanase activity of endophytic fungi strains isolated from cacao (*Theobroma cacao*) leaves was significant when grown on wheat bran as the sole carbon source. In *Fusarium graminearum* strain Ec220, particularly, wheat bran was effective to selectively induce high xylanase production with low cellulase activity. The statistical model obtained through RSM was suitable to predict the optimal conditions for xylanase production. The proteomic analysis of strain Ec220 secretome revealed two protein spots with characteristics very similar to *F. graminearum* xylanases reported previously. To the best of our knowledge, this is the first work that provides insight on the xylanolytic potential of endophytic fungi of *T. cacao* when cultured on low-cost agricultural residues like wheat bran, especially strain Ec220 that produced xylanases with low cellulases, which are currently sought-after biotechnological products for their great applicability in several industrial processes.

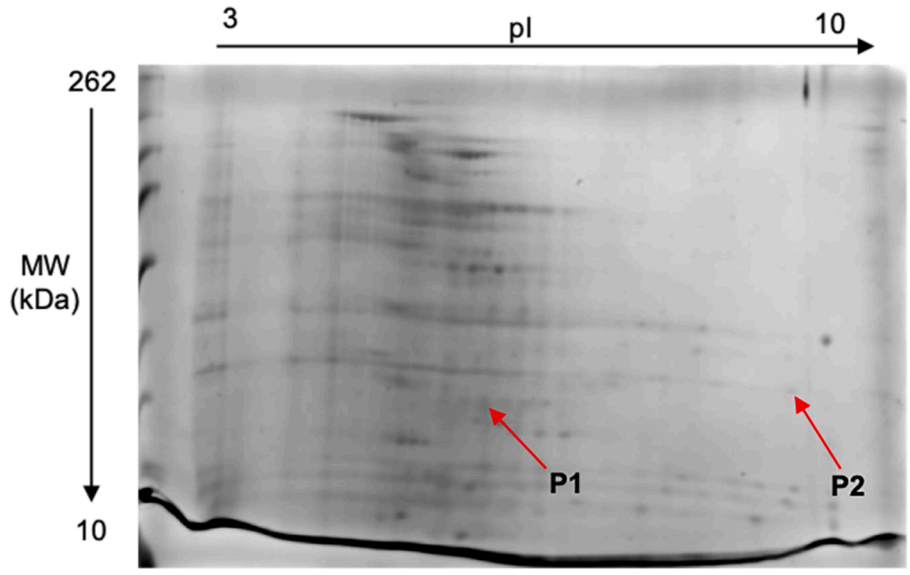

Fig. 4. Two-dimensional gel electrophoresis of strain Ec220 secretome under optimal conditions with wheat bran as substrate. Two protein spots (P1 and P2) suggestive of two xylanases (Xyl A and Xyl B) previously reported for *F. graminearum* are shown on the gel. The protein molecular weight marker was in the range of 262-10 kDa (Spectra Multicolor Broad Range).

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Author agreement

We the undersigned declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere. We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome. We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us. We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property. We understand that the Corresponding Author is the sole contact for the Editorial process (including Editorial Manager and direct communications with the office). She is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs. We confirm that we have provided a current, correct email address which is accessible by the Corresponding Author.

CRediT authorship contribution statement

Jhon Cruz-Davila: Investigation, Methodology, Formal analysis, Visualization, Writing – original draft, Writing – review & editing, Data curation, Software. **Jeffrey Vargas Perez:** Methodology, Software, Formal analysis, Supervision. **Daynet Sosa del Castillo:** Conceptualization, Methodology, Writing – review & editing. **Nardy Diez:** Conceptualization, Resources, Validation, Visualization, Writing – review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

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