An Environmentally Safe Production of Xylanases by *Fusarium* sp. EA 1.3.1 Using Agroindustrial Residues: Biochemical Characterization and Potential Applications

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Renewable energy-related biotechnologies such as biofuels produced from low-cost residual sources that represent clean technologies have become a partial solution to environmental problems. We sought to optimize the cultivation parameters of the fungus *Fusarium* sp. EA1.3.1 and biochemically characterize the naturally produced xylanases from the fungus. The development of the fungus was analyzed considering the variations in the resources available and by biochemical analysis of the crude extract. The composition and duration of the cultivation, nitrogen source, carbon source, salt solution, and initial pH of the medium were evaluated. The maximum xylanolytic production was obtained in Khanna medium enriched with a CP salts solution during four days of culture using yeast extract, wheat bran, and an initial pH of 8.5 for the culture medium. The optimum temperature and pH were 65°C and 6.5, respectively, for the xylanase activity from *Fusarium* sp. EA 1.3.1. The enzymatic extract presented general stability at 50°C, keeping 75% of its activity after 90 minutes of incubation, and its activity decreased to 20-40% with exposure to higher temperatures (60-70°C). The enzyme also presented high stability at pH 5.0 after 90 minutes, maintaining 85% of its relative activity. Thus, the isolated fungus presents high potential for xylanase production with desired biochemical and biophysical properties for industrial application.

Graphical Abstract
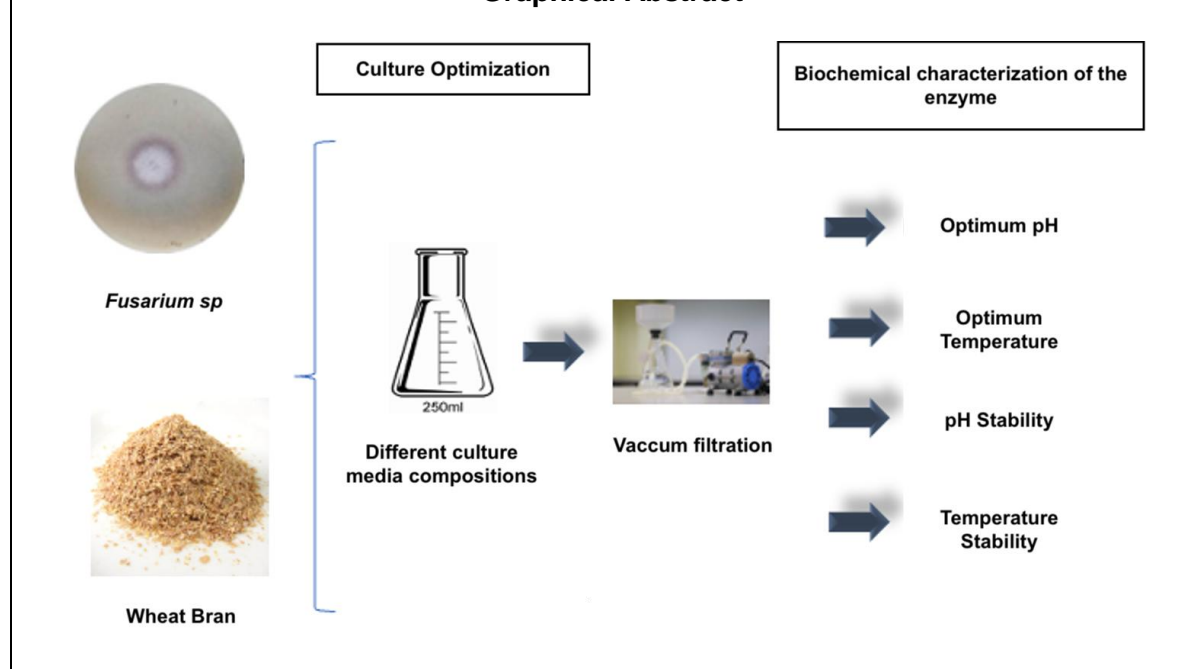
1. INTRODUCTION

Biotechnological processes are important for the world's technological development. These processes have economic and operational characteristics that confer advantages over conventional processes. In addition, concern with environmental issues drives research for new technologies that do not degrade but rather conserve the environment. The increase in the price of oil, the political and economic instability in the world and the growing environmental concerns have led to the search for alternatives to the use of petroleum, such as renewable energy sources. This search has mobilized international academic, industrial, social, and government sectors with an emphasis on the development of biotechnological processes [1].

Fuels obtained from low-cost sources that represent clean technologies have become a worldwide necessity. In addition to an economic need, there was a growing interest in the diversified use of agro-industrial waste to

minimize the environmental problems resulting from its disposal [2]. As an example, second-generation ethanol is obtained from the degradation of lignocellulosic materials (LCMs) [3].

The use of LCMs for the production of ethanol fuel has become a priority within the context of renewable energy [4]. However, the use of these residues involves a process that is still under development because the hydrolysis of these materials to fermentable sugars requires efficient, specific, and low-cost methods to make the process economically viable.

Several bioprocesses have been developed to use such materials as a substrate to produce biomolecules. Among these methods, microbial enzymes, which are biologically active molecules, are emphasized. Microorganisms provide a better, faster, and more easily controlled production of their metabolites, and, for this reason, these enzymes are preferably used in industry. In addition to the ease of obtaining them, products of microbial origin are independent of geographic or seasonal conditions and are less costly because cheap substrates such as agricultural residues can be used in their preparation [5].

Among the microorganisms studied, filamentous fungi are particularly interesting because they are fundamental pieces for biotechnological development [6]. In addition to the controlled application of enzymes, these microorganisms are also used to produce many other metabolites of interest for different areas of the biotechnology industry. Fungi are capable of producing different extracellular enzymes, and they are more active biocatalysts at low pH [7]. Generally, the enzymatic extracts obtained from these cultures have greater activity than those obtained from the cultivation of bacteria.

Among the enzymes produced by fungi, xylanases are very important. Hemicellulolytic enzymes act on the degradation of hemicellulose, a constituent of lignocellulosic biomass, to release pentoses, mainly D-xylose. The commercial xylanases available are mainly of fungal origin, and they have an optimum activity above 50°C, a temperature higher than that required for saccharification [8,9].

The importance of finding microbial enzymes that have an optimum activity compatible with the degradation of hemicelluloses would make the production of second-generation ethanol

feasible. In addition, these microorganisms can produce different complexes of lignocellulosic enzymes, allowing them to hydrolyze, not only the main chains that form these residues but also their side chains [10]. However, the enzymatic hydrolysis of lignocellulosic materials, even with high efficiency and specificity, has a very high cost to produce biofuels and requires a production system that results in a low cost of the final enzyme preparation. Therefore, this work sought to develop a bioprocess to obtain a concentrate rich in xylanases of fungal origin and determine the physicochemical parameters of cultivation of the microorganism for greater xylanolytic activity, in addition to biochemically characterizing the crude extract.

2. METHODOLOGY

This work was performed at the Laboratory of Mycology, Enzymology and Product Development (LMEDP), Universidade dos Vales do Jequitinhonha e Mucuri (UFVJM), JK campus, Diamantina, Minas Gerais, Brazil. The microorganisms were registered in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SisGen) under number A64AD93.

2.1 Microorganism under Study and Maintenance of Fungal Isolate

The microorganism was isolated in Diamantina, Minas Gerais, Brazil according to previously established protocols[11], and it was maintained in a solid medium composed of 4% Quaker® oatmeal, 2% bacteriological agar [12] in tilted test tubes. Transplanting was carried out periodically; the cultures were maintained at 30°C in a bacteriological oven for seven days and subsequently stored at 4°C. Fungi samples were also kept at 4 ºC on silica gel, according to previously publication [13].

The isolate was identified using molecular tools. Genomic DNA was extracted by macerating the mycelium in TES lysis buffer (100 mM Tris; 10 mM EDTA; 2% SDS) with the use of a pistil and then incubated at 65°C for 15 min. After that period, 140 µL of 5 M NaCl was added, and the mixture was incubated on ice for 30 min. To this mixture, 600 µL of chloroform: isoamyl alcohol (24:1) was added and centrifuged at 10,000g and 4°C for 10 min. The supernatant was collected and 300 µL of isopropyl alcohol and 50 µL of 3 M sodium acetate (pH 5.2) were added. The material was centrifuged under the conditions

mentioned above, and a new wash was performed with 600 µL of 70% ethanol, followed by another centrifugation step. The supernatant was discarded, and the pellet was dissolved in 50 µL TE (10 mM Tris; 1 mM EDTA) with 5 µL RNAse (10 mg/mL). The ITS region was amplified with the ITS4 and ITS5 primers using the PCR Master Mix kit (Promega) according to the manufacturer's instructions. The reaction product was developed on a 1% agarose gel, which was stained with Nancy (Sigma-Aldrich) and visualized on a UV transilluminator. The purification of the amplification reaction was performed with the Wizard® SV Gel and PCR Clean-up System kit (Promega) and quantified in a NanoDrop® (Thermo Scientific). The sequencing reaction was performed using the BigDye® Terminator Cycle Sequencing kit (Life Technologies) according to the manufacturer's instructions, and the material was applied to an ABI 3500XL sequencer (Life Technologies). The sequence was compared with homologous sequences available at NCBI-GenBank (www.ncbi.nlm.nih.gov) using the BLASTn tool.

2.2 Fungi Inoculum in Submerged Culture Medium

The inoculum process consisted of scraping the fungal tubes, diluting the spores in 5 mL of sterile distilled water, and transferring 1 mL of this suspension (2.51×10^8 conidia/culture) to a 125-mL Erlenmeyer flask containing 25 mL of the submerged medium.

2.3 Determination of Submerged Media for Xylanase Production by *Fusarium* sp. EA 1.3.1 and Time-course Growth Analysis

Initially, four different submerged culture media were analyzed: 1 – SR [14] ; 2 – Khanna [15]; 3 – CP [16], and 4 – Adams [17]. Wheat bran was used as a carbon source in all culture media. The media were kept at 30 ºC in a bacteriological incubator for seven days, and the media were removed every 24 hours. After the incubation, the cultures were filtered, and the xylanolytic activity was determined.

2.4 Different Nitrogen Sources Effect on the Cultivation of the EA 1.3.1 Isolate and xylanase Production

The filamentous fungus EA 1.3.1 was inoculated in a previously established submerged culture medium, and the composition of the nitrogen source of the culture was varied. The compositions were 0.1 g of peptone, 0.1 g of yeast extract, 0.1 g of urea, and combinations of these components; 0.5 g peptone with 0.5 g yeast extract; 0.5 g peptone with 0.5 urea; 0.5 g yeast extract with 0.5 g urea; and 0.33 g of peptone with 0.33 g of yeast extract and 0.33 g of urea. The media were kept at 30 ºC in a bacteriological incubator. After the incubation, the cultures were filtered and the xylanolytic activity was determined.

2.5 Different salt Solutions on the Cultivation of EA 1.3.1 Isolate and Xylanase Production

Different salt solutions of the previously determined submerged culture medium were analyzed with the objective of increasing the xylanolytic activity. The tested solutions were (1) Khanna salts [2.0% (w/v) of NH_4NO_3; 1.3% (m/v) KH_2PO_4, 0.362% (w/v) $MgSO_4$; 0.098% (w/v) KCl; 0.007% (w/v) $ZnSO_4$; 0.0138% (w/v) $MnSO_4$; 0,0066% (w/v) of $Fe_2(SO_4)_3$ and 0.0062% (w/v) of $CuSO_4$]; (2) CP salts [0.3% (w/v) of KH_2PO_4 and 0.05% (w/v) $MgSO_4$]; (3) 0.05% (w/v) of Wesson salts and their combinations of Khanna with CP, Khanna with Wesson, CP with Wesson, and Khanna with CP and Wesson. The control medium was a culture without salt solution, being kept at 30 ºC, stationary, in a bacteriological incubator. After the incubation time, the cultures were filtered, and the xylanolytic activity was determined.

2.6 Different Carbon Sources Effect on the Production of Xylanase by the Isolated Fungus EA 1.3.1

Different carbon sources were analyzed in the proportion of 1% (m/v): orange peels, tangerine peels, avocado peels, pumpkin shells, pineapple peels, banana peels, pumpkin seed, cane sugar straw, and wheat bran. The submerged media after the inoculum was kept stationary at 30°C in a bacteriological oven. After the incubation, the cultures were filtered, and the xylanolytic activity was determined.

2.7 Determination of the Initial pH for the Growth and the Production of Xylanases

The initial pH of the submerged culture medium was varied at the following values: 4.5, 5.0, 5.5,

6.0, 6.5, 7.0, 7.5, and 8.0. The submerged media after the inoculum were kept stationary in a bacteriological oven at 30°C. The cultures were filtered, and the xylanolytic activity was determined.

2.8 Production of Mycelial Mass and the Extracellular Extract Containing the Xylanase

The mycelial mass of the filamentous fungus was obtained through vacuum filtration with the aid of a Büchner funnel and Unifil® filter paper, 12.5 cm in diameter. After drying, the mycelial mass was weighed on an analytical balance. The filtrates containing the extracellular enzymes were subjected to the measurement of pH, volume, and measurement of xylanolytic activity.

2.9 Determination of Xylanolytic Activity

The activity was determined using 1% (w/v) birchwood xylan (Sigma®) in sodium acetate buffer 100 mol.L^{-1}, pH 5.5, as a substrate. The determination occurred through the formation of reducing sugars during the incubation of the enzyme with its substrate. The concentration of reducing sugars formed was measured using the 3', 5'-dinitro salicylic acid (DNS) reagent [18].

The enzymatic reaction consisted of the incubation of 500 µL of the xylan substrate and 500 µL of the soluble crude enzymatic extract at 55 °C for 5 minutes. After the reaction, 200 µL aliquots were removed and added to tubes containing 200 µL of DNS. The tubes were boiled for 5 minutes, and 2 mL of distilled water was added after cooling. The blank consisted of the immediate addition of a 200 µL aliquot of the reaction mixture to 200 µL of DNS. The readings were performed at 540 nm in a RayLeigh UV-2601® spectrophotometer against the zero-time reaction, in which spontaneous hydrolysis of the substrate was minimal. The method was previously standardized with a xylose standard curve (0 to 2.0 mg.mL^{-1}). The activity unit (U) was defined as the amount of enzyme that hydrolyzed one µmol of substrate per minute under the conditions of the assay.

$$U = [Enzyme] \times \mu mol \text{ of reducing sugars} \div 1 \text{ minute (Equation 1)}$$

2.10 Effect of Temperature and pH on Xylanase Activity

The reaction temperature and pH were analyzed using a Central Rotational Composite Design with two independent variables (DCCR2), X_1 (pH) and X_2 (temperature) of the reaction, totaling 12 tests, 4 central points and 4 rotationally distributed tests (axial points) at a distance α from the central point. The central pH value was 5.0 with an interval of 1.6, and the temperature was 50°C with an interval of 16 °C (Table 1). The DCCR performed by the Protimiza Experimental Design software with α of 5% provided the regression table, which showed that the experimental mean was statistically significant, since the p-value was less than 5%, as well as the pH, temperature, the pH square, the temperature square, and the interaction between pH and temperature were also significant.

$$Y_1 = 12,78 + 4,10 \, x_1 - 3,42 \, x_1^2 + 1,36 \, x_2 - 2,08 \, x_2^2 + 1,58 \, x_1 x_2 \text{ (Equation 2)}$$

Table 1. Temperature and pH values of the xylanolytic reaction were analyzed using the Central Rotational Composite Design statistical tool

Assay	X_1	X_2	pH	Temperature (°C)
1	-1	-1	3.6	36
2	+1	-1	6.4	36
3	-1	+1	3.6	64
4	+1	+1	6.4	64
5	-1.41	0	3.0	50
6	+1.41	0	7.0	50
7	0	-1.41	5.0	30
8	0	+1.41	5.0	70
9	0	0	5.0	50
10	0	0	5.0	50
11	0	0	5.0	50
12	0	0	5.0	50

Table 2. Analysis of variance for xylanase activity

Source of variation	Sum of squares	Degree of freedom	Mean square	F calculated	p-valor
Regression	246,9	5	49,4	71,7	0,00003
Residues	4,1	6	0,7		
Lack of fit	2,2	3	0,7	1,2	0,45517
Pure Error	1,9	3	0,6		
Total	**251,0**	**11**			

Table 3. Determination of the submerged culture medium and growth duration of the isolated filamentous fungus *Fusarium* sp. EA 1.3.1 for the production of a cocktail rich in xylanase

Days	Xylanolytic activity (U.mL^{-1}) in different culture media			
	Khanna	CP	Adams	SR
1	0	0.59 ± 0.29	0.59 ± 0.82	0.57 ± 0.30
2	3.62 ± 1.42	4.55 ± 0.54	3.60 ± 0.79	2.62 ± 1.34
3	25.99 ± 0.78	19.65 ± 1.32	8.32 ± 1.77	22.73 ± 1.04
4	30.77 ± 0.04	20.11 ± 4.17	20.25 ± 1.24	24.80 ± 0.09
5	29.53 ± 0.56	26.26 ± 0.97	22.36 ± 1.67	22.30 ± 0.16
6	26.34 ± 0.62	25.75 ± 5.18	21.64 ± 0.06	21.25 ± 4.19
7	27.32 ± 0.93	21.34 ± 2.02	20.08 ± 1.07	19.88 ± 0.18

Equation 2 was obtained, which is a quadratic model (second order), with concavity facing downwards. It was noted that the quadruplicate of the central point was close, demonstrating good reproducibility of the experiment. The DCCR performed with α of 5% provided the ANOVA table (Table 2) and the R² square of the model was 98.35%.

2.11 Temperature and pH Stability of the Xylanase Produced by the Isolated Filamentous Fungus E.A.1.3.1

Thermostability was evaluated by incubating the enzyme for 0, 5, 8, 14, and 24 h in the absence of substrate at temperatures of 50 ºC, 60 ºC, and 70°C. The samples were removed and placed in an ice bath, and the enzymatic activity of each sample was determined under the ideal pH and temperature conditions.

The stability of the enzyme to pH was evaluated by incubating the enzyme extract in 100 mM sodium acetate buffer (pH 4.5; 5.0 and 5.5) and 100 mM sodium phosphate (pH 6.0; 6.5 and 7.0) for 30, 60, 90, and 120 minutes in an ice bath in the absence of substrate. The enzymatic activities of the samples were determined in 200 mM sodium phosphate buffer under the ideal pH and temperature conditions.

2.12 Reproducibility of the Experiments

The experiments were performed in triplicate, and the mean activities and standard deviations were determined.

3. RESULTS AND DISCUSSION

3.1 Identification of the Isolated Filamentous Fungus EA 1.3.1

The fungus selected for this study was characterized as a significant producer of xylanolytic enzymes. According to the molecular analysis of the ITS region, a high degree of identity was verified with species of the genus Fusarium, *F. foetens* CBS 110286 (99.40%), and *F. circinatum* CBS 405.97 (98.80%). However, the ITS region was not efficient for the delimitation of some species of this genus, and the use of multiple molecular markers was required. Thus, isolate EA 1.3.1 was identified as *Fusarium* sp.

Several fungi species have been reported to produce xylanases that are highly efficient in degrading the xylan molecule [19]. Among the main producers of xylanases, which are frequently used in industrial processes, the genera *Trichoderma* and *Aspergillus* stand out. However, *Fusarium sp*ecies have also been reported as promising organisms for the production of these enzymes [20].

3.2 Effect of Submerged Culture Medium Composition and the Time-course of Cultivation on Xylanase Production

Fusarium sp. EA 1.3.1 presented a greater enzymatic activity (30.77 U.mL^{-1}) in the Khanna medium after 4 days, at 30ºC (Table 3). This

medium has a complex salt solution and smaller amounts of carbon and nitrogen sources. The second-best media composition was obtained in the CP culture medium, (26.26 U.mL^{-1}) after five days, and the third-best composition was in the SR medium (24.80 U.mL^{-1}) after four days of culture, followed by the Adams medium (22.36 U.mL^{-1}) after five days of culture (Table 3). According to the literature, several authors have used highly complex salt solutions as media for obtainment high xylanolytic production by different filamentous fungi [5,8,10,16,19,21]. Also, 4 days of cultivation represents a short time for the production of the complex xylanolytic enzymes, which leads to a decrease in the cost of large-scale production [22]. Curiously, after the 4-day period, the enzyme activity decreased, which is an expected behavior because of possible biochemical changes in the environment, such as depletion of nutrients or the accumulation of inhibitory products of enzymatic synthesis [23]. The results obtained are in accordance with data reported previously by *Aspergillus fumigatus* [24], *A. japonicus* [25], and *A. clavatus* [8], which

reached maximum xylanase production after 4 days of incubation using complex media compositions, whereas the filamentous fungi *Mycothermus thermophilus* and *Trichoderma reesei RP698* required 7 days of cultivation in a simple medium [26].

3.3 Different Nitrogen Sources for the Growth and Xylanase Production by *Fusarium* sp. EA 1.3.1

The microorganism under study requires a nitrogen source in the submerged culture medium for greater production of enzymes; that is, the activity of the enzyme in the control medium, without a nitrogen source, was only 8.5% of that obtained in the culture medium containing yeast extract (Fig. 1), the original source in the composition of the Khanna medium. The greatest extracellular activity was obtained in the Khanna medium containing yeast extract as a nitrogen source (31.40 U.mL^{-1}), followed by that obtained with the use of urea (18.42 U.mL^{-1}) (Fig. 1).
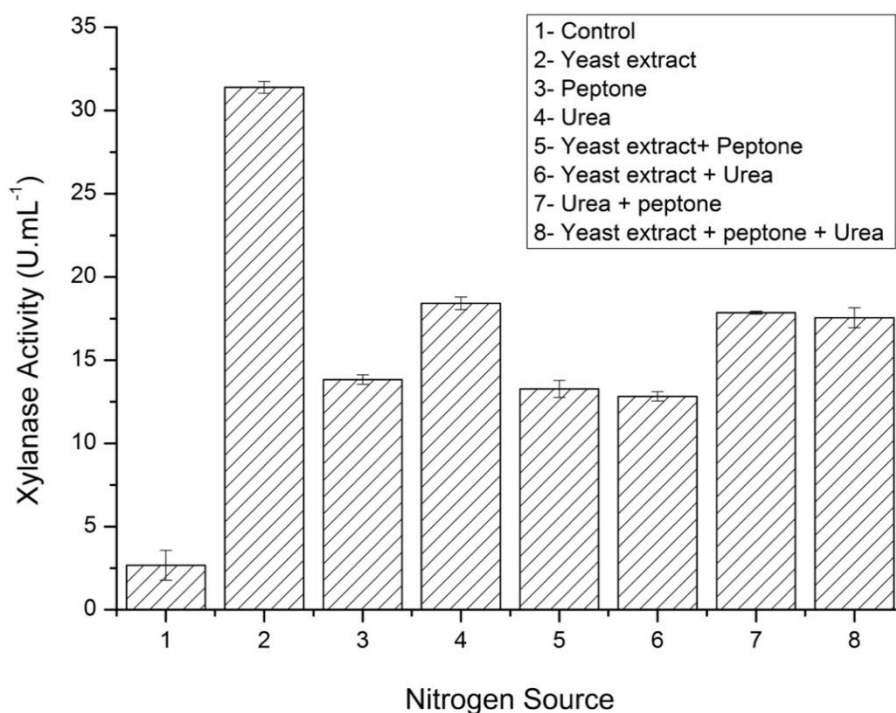

Fig. 1. Determination of the nitrogen source of the Khanna submerged culture medium for the growth of the fungus *Fusarium* sp. EA 1.3.1 and the production of a cocktail rich in xylanase

The combination of nitrogen sources was not favorable for the production of xylanases by the fungus under study. It is known that overuse can inhibit the production of xylanolytic enzymes and the growth of the microorganism. The same result was observed by Pasin et al. [12], who observed greater production of amylase when organic nitrogen sources (peptone and yeast extract) were used. Similar results were obtained by Kumar et al. [27], whose enzyme production was higher when the fungus *Thermomyces lanuginosus* was grown in a medium containing yeast extract, followed by a medium containing peptone and soybean meal. On the other hand, Gaffney et al. [28] observed that soybean meal induced a greater xylanolytic potential than the use of yeast extract.

3.4 Effect of Different Salt Solutions for the Growth and Xylanases Production by *Fusarium* sp. EA 1.3.1

The filamentous fungus under study required saline solution in adequate concentration to obtain a satisfactory xylanolytic activity because the activity of the Khanna culture medium without the salt solution was 25.4% of that obtained when the medium containing a solution of CP salts was employed (Fig. 2). Xylanolytic activities of 29.58 and 35.55 U.mL^{-1}, respectively, were observed when only the complex salt solution of Khanna salts or the simple solution of CP salts was employed (Fig. 2). Therefore, the Khanna submerged culture medium containing yeast extract and the CP salt solution for the cultivation of the microorganism, at 30 $^{\circ}$C, under stationary conditions was established as the best condition for the production of the xylanases.

The elements carbon, hydrogen, oxygen, nitrogen, sulfur, phosphorus, magnesium, and potassium are generally required in large quantities because they participate in almost all cellular components. The first three (C, H, and O) are supplied in the form of organic compounds, carbon dioxide, molecular oxygen, water, and nitrogen are supplied in the appropriate form (organic or inorganic). The elements S, P, Mg, and K are supplied to the fungus in the form of salts, and they are called macronutrients. Some fungi require growth factors, mainly vitamins, and others such as iron, copper, manganese, zinc, and molybdenum, which, because they are micronutrients, are required in minimal amounts.
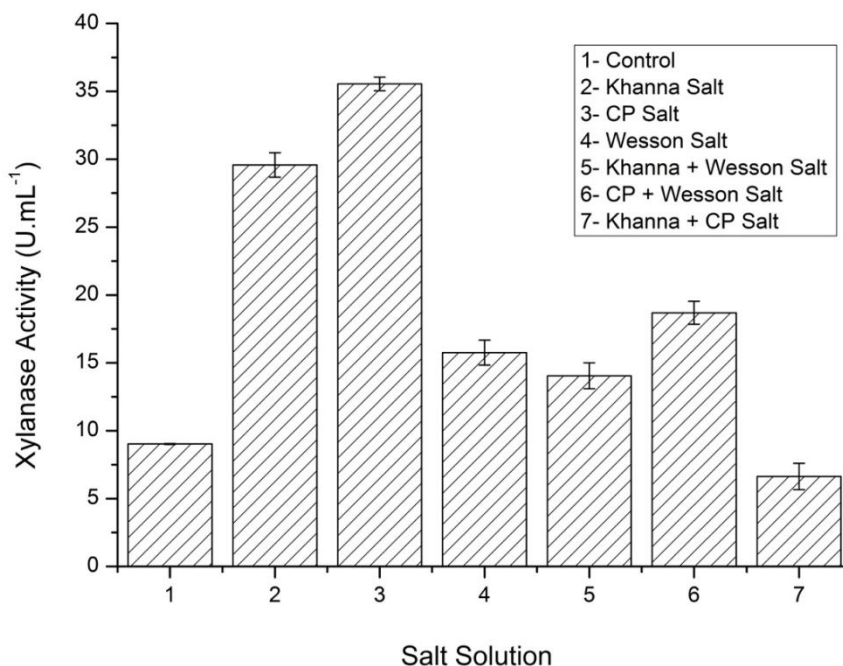

Fig. 2. Determination of the salt solution of the Khanna submerged culture medium containing yeast extract as a carbon source for cultivation of the filamentous fungus *Fusarium* sp. EA 1.3.1 and the production of xylanases at 30ºC without shaking

3.5 Different Carbon Sources Effect on the Production of Xylanase by *Fusarium* sp. EA 1.3.1

The use of agro-industrial residues is reinforced by studies that conclude that the production of xylanolytic enzymes needs to be induced, preferably, by complex molecules. Easily metabolized sugars, such as glucose, are repressors of the production of hemicellulases, even though the monosaccharides promote the growth of fungi [29]. Agro-industrial residues have been used for the production of xylanases by several fungi [3,5,12], mainly *Aspergillus* [10,30], to reduce the cost of production and to obtain a less expensive commodity.

As shown in Fig. 3, all the residues used in this study induced the production of xylanases. The greatest production was obtained in the presence of wheat bran (34.63 U.mL^{-1}). This result can be justified by its high hemicellulose content. Sugarcane straw (17.12 U.mL^{-1}) and pumpkin seeds (12.63 U.mL^{-1}) also favored a large production of enzymes (Fig. 3), and they can also be used as alternative sources for the production of these enzymes.

Bajaj et al. [31] and Pasin et al. [30] observed that wheat bran and eucalyptus chips were more effective for the production of xylanases by *Penicillium* sp. ZH-30 and *Aspergillus* sp, respectively, than pure xylan. On the other hand, Liao et al. [32] did not obtain good xylanolytic activity when they induced *Penicillium oxalicum* GZ-2O with wheat bran. This observation might be due to the large amount of wheat bran protein content, which can reduce xylanase production [33]. Sun et al. [33] found that the increase in starch content was directly correlated with a decrease in xylanase levels. Likewise, Rogoski et al. [34] reported a low xylanase activity when its production was induced with cassava peel, although maximum cell growth was obtained. Also, Terrasan et al. [35] observed that beer and wheat bran residues are good xylanase inducers, but pure xylan was more suitable for inducing the production of xylanases by *Penicillium janczewskii* than other agricultural residues. However, the use of commercial xylans as a carbon source is not economically viable for large-scale enzyme production [5]. The residues analyzed in this study are complex substances, and they can induce the production of several proteins, including cellulases when they are used as a carbon source. This production would favor the application of this extract for the degradation of lignocellulosic material for the generation of biofuels such as bioethanol.
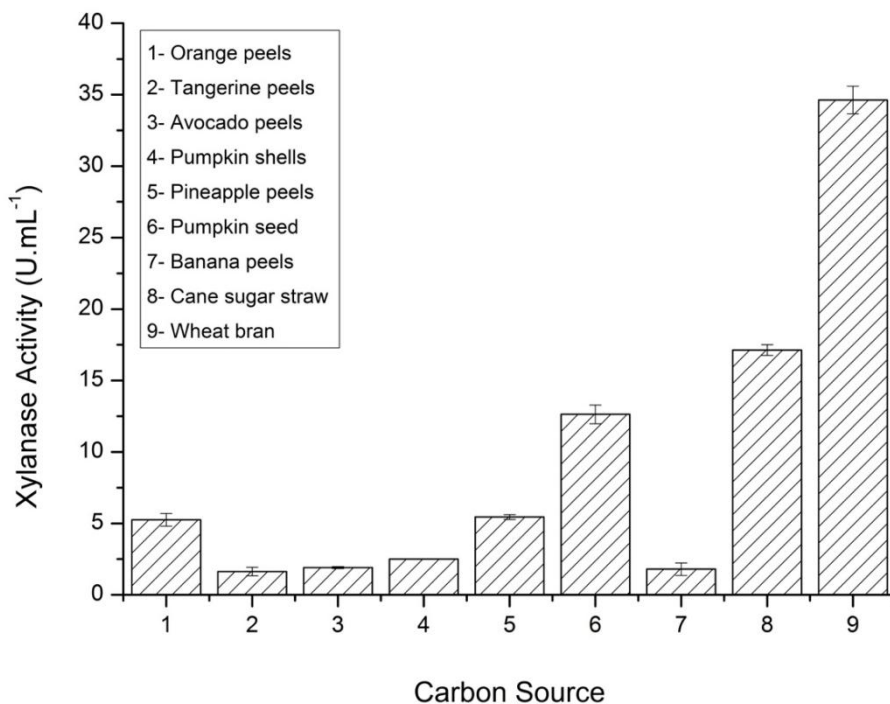

Fig. 3. Analysis of the potential of agro-industrial residues for xylanolytic production by the fungus *Fusarium* sp. EA 1.3.1

3.6 Effect of Initial pH on the Production of Xylanases by the *Fusarium* sp. EA 1.3.1

Large production of xylanase was obtained by the fungus at all the initial pH tested. According to Shah et al. [36] most fungi are able to grow in a wide pH range, and this fact was observed with the data obtained in our study. An increase in enzymatic activity with increasing pH of the culture medium was observed. Thus, pH 8.5 was chosen as the initial pH for the cultivation of *Fusarium* sp. EA 1.3.1 (Fig. 4). This result is different from that observed for *Aspergillus fumigatus* cultivated on corn cobs that yielded a greater production of xylanase at pH 5.4 [37], whereas a greater production by *P. oxalicum* was obtained at an initial pH of 5.0, and the production decreased when it was grown in more basic media [38]. Reports by Tai et al. [39] showed that several fungi produced more xylanase at pH 7.0, which is lower than that observed in our study.

3.7 Effect of Temperature and pH on the Activity of the Xylanases Produced by the Isolated Filamentous Fungus

After optimizing the composition of the culture medium to produce xylanases by the fungus *Fusarium* sp. EA 1.3.1, the effect of temperature and pH on the activity of these enzymes was studied. By analyzing the response surface and the contour curves, the existence of an optimum region for enzymatic activity was determined, where a range of combinations of pH and temperatures was found. This fact demonstrates the existence of various details regarding the catalysis by the enzyme under different conditions. Among them, 65°C and pH 6.5 were determined to be the optimum temperature and pH conditions, respectively, for the catalysis by the xylanases under study (Fig. 5). According to the literature, the pH 5.0 has been observed as the most common optimum pH for xylanases activity from diffent fungi species [21,40]. However, some studies have reported different results, such as the xylanase produced by *Aspergillus versicolor* (pH – 6.0) [41], *Aspergillus giganteus* (pH – 6.5) [42] and *Aspergillus nidulans* (pH – 5.8) [43].

Studies with filamentous fungi have described optimum temperatures for xylanolytic activity around 40 and 60°C [8]. Some commercial xylanases such as Sumizyme, Multifect XL, Pulpzyme and Cartazyme, also produced by fungi, exhibit excellent activity in this temperature range [44]. However, other studies observed the optimum temperature at 55 °C for xylanases from *Aspergillus niveus* [45], *Aspergillus* niger [45] and *Bacillus* subtilis [46] which are different from the result obtained in the present work.
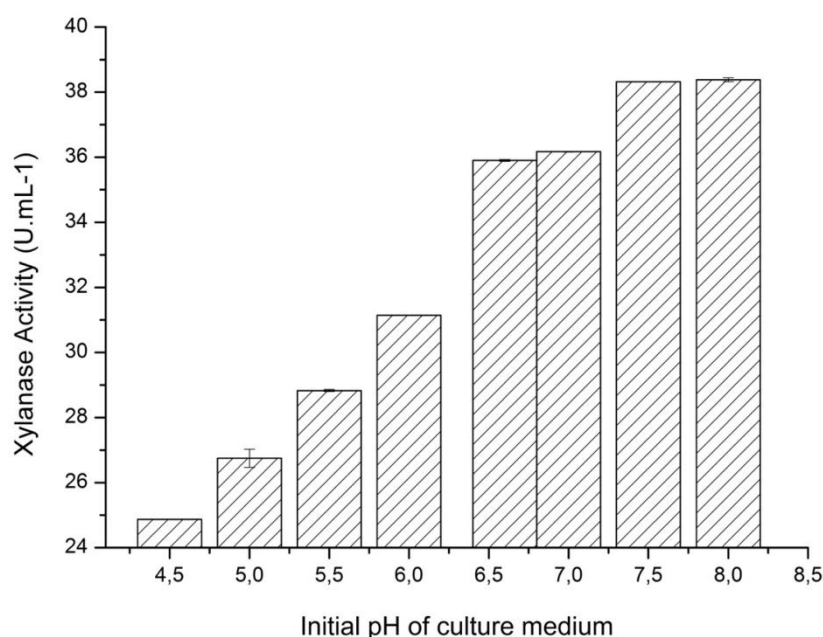

Fig. 4. Analysis of the initial pH of the Khanna culture medium for the production of a cocktail rich in xylanases by the fungus *Fusarium* sp. EA 1.3.1
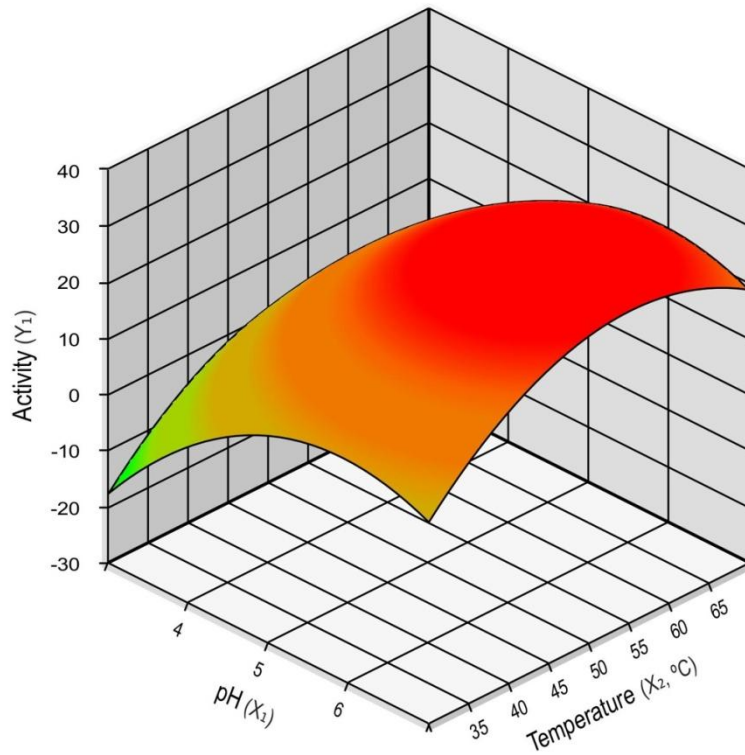
Fig. 5. Response surface and contour curve for the pH (X_1) and temperature (X_2) variables of the xylanolytic activity of the fungus *Fusarium* sp. EA 1.3.1
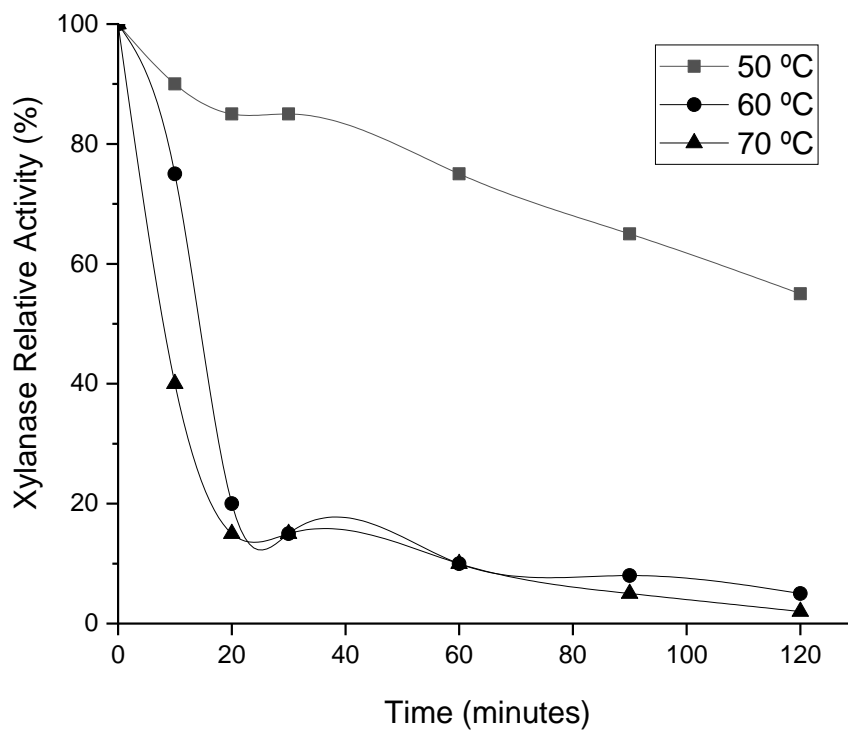

Fig. 6. Temperature stability of xylanases produced by the isolated filamentous fungus *Fusarium* sp. EA 1.3.1
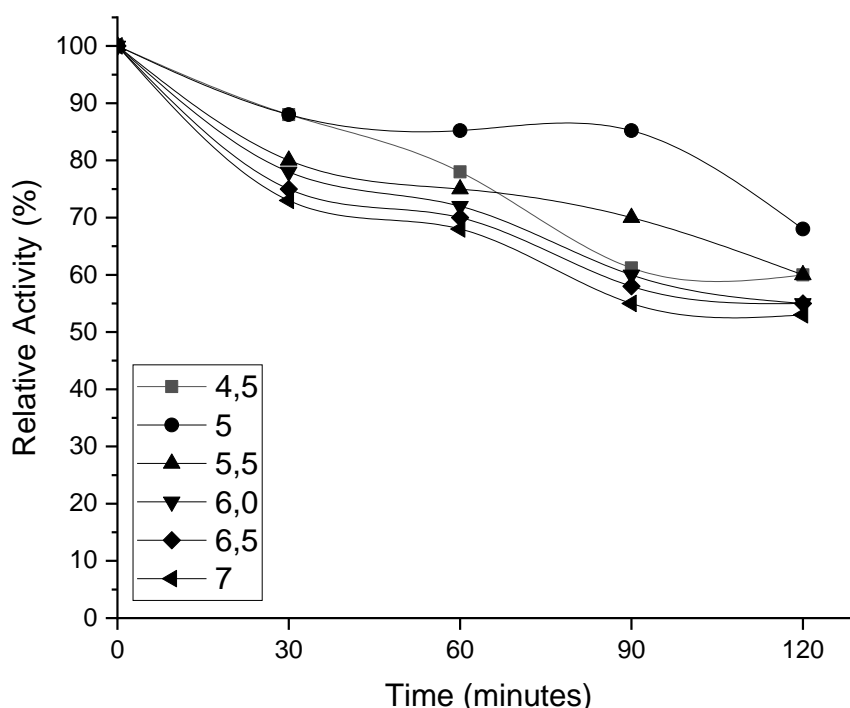
Fig. 7. Stability of xylanases produced by the isolated filamentous fungus *Fusarium* sp. EA 1.3.1 at different pH

3.8 Effect of Temperature and pH on Xylanase Stability

For possible applications of xylanolytic enzymes in industrial processes, the stability of the enzymes with respect to changes in temperature and pH must be elucidated. When incubating the xylanase at 50°C, it maintained 75% of its activity after 90 minutes, and the xylanolytic activity decreased to 55% of the initial activity after 2 hours. At 60 ºC, the activity decreased to 75% after 10 min, and to 20% of the initial activity after 20 min. Finally, the activity decreased to 40% within 10 minutes at 70 ºC (Fig. 6).

The enzyme remained stable with an activity greater than 50% of the initial activity after 120 minutes at all the pH range tested. Also, the enzyme maintained an activity greater than 85% of the initial activity after 90 minutes at pH 5.0 (Fig. 7). These results are interesting from an industrial point of view; the activity at different pH levels is of paramount importance for a future biotechnological application of the enzyme, regardless of the industrial sector [8].

4. CONCLUSION

The filamentous fungus *Fusarium* sp. EA 1.3.1 produced a highly active xylanase at 30°C in

Khanna submerged culture medium containing yeast extract as a nitrogen source, CP salts and wheat bran as a carbon source, at an initial pH of 8.5. This high xylanase production system established in this work was carried out with a cheaper cultivation, simple conditions (temperature and pH), a short cultivation time and containing agro-industrial residues (wheat bran) as a carbon source. By themselves, these conditions represent an important new way of producing enzymes with diverse industrial interests. In addition, the xylanase under study presented promising characteristics for industrial applications, mainly in relation to the optimal temperature and pH of the reaction catalyzed by xylanase.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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