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Facultad de Ciencias Agropecuarias

Universidad Nacional de Trujillo

RESEARCH ARTICLE

Xylanase production by Penicillium sp. Pn004 and its application for grass hydrolysis: High value subproduct from non-centrifugal sugarcane bagasse and wheat bran

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Received: 16 November 2023. Accepted: 24 March 2024. Published: 8 April 2024.

Abstract

Worldwide more than 7 billion tons of lignocellulosic biomass will be produced by 2025. In Colombia, these residues are mainly disposed of in rivers and open fields, and only 10% is reused and recycled. Non-centrifugal sugarcane bagasse (SCB) is a residue obtained after sugarcane juice extraction during the manufacturing process of *panela* and is mostly used as fuel for boilers at sugar mills. Wheat bran (WB) is the main waste from wheat flour production. Nonetheless, the chemical composition of these by-products makes them suitable for use as substrates for hemicellulolytic enzyme production from fungi. Here, a whole process for production in a solid-state fermentation system, recovery, and ultrafiltration process for concentration of xylanases by *Penicillium* sp. Pn004 is presented. The higher productivity (26.7 ± 1.59 U gds^{-1} day-1) was reached on the fifth day of fermentation with an enzyme activity of 130.0 ± 7.95 U gds^{-1}. The batch ultrafiltration process allowed increasing the xylanase activity up to 19-fold in the retentate, from 66.47 U mL^{-1} to 1486.83 U mL^{-1}, without reaching a steady state flux through the membrane. Finally, the enzymatic extract achieved a 43% release of sugar from kikuyu grass (*Cenchrus clandestinus*), showing its potential to be used as an additive for silage or for enzymatic saccharification of lignocellulosic materials for sugar production.

Keywords: Enzyme production; agro-industrial waste; enzymatic hydrolysis; ultrafiltration process; *Penicillium*.

DOI: https://doi.org/10.17268/sci.agropecu.2024.015

Cite this article:

Martínez, A. D., Blanco Paz, A., Chavarro-Anzola, V., Barrios Murcia, J. C., & Bautista, E. J. (2024). Xylanase production by Penicillium sp. Pn004 and its application for grass hydrolysis: high value subproduct from non-centrifugal sugarcane bagasse and wheat bran. *Scientia Agropecuaria*, *15*(2), 201-212.

1. Introduction

The high generation of agro-industrial waste is a major concern due to its environmental and economic impact. Today, one third of the food is estimated to be wasted worldwide, from the cultivation to the commercialization. This led to a growing interest in transforming available and low cost agro-industrial residues into high-value raw materials or substances through new technologies, as more efficient and less polluting processes are implemented (Blasi et al., 2023; Mishra et al., 2023; Peñaranda et al., 2018; Wagh et al., 2024). Worldwide more than 7 billion tons of lignocellulosic biomass will be produced by 2025, Asia would account for almost half of it (45%), follow by Africa (19%) and South America (13%) (Magalhães et al., 2019). In Colombia, more than 71 Mt of these residues are generated per year, mainly by the coffee, oil palm, sugarcane, corn, rice, banana, and plantain industries, which in most cases are incinerated or disposed of in rivers and open fields (50%), sanitary landfills (40%), and a small percentage is recycled or reused (lower than 10%) (Cuadrado-Osorio et al., 2022). In this scenario, *Corporación Colombiana de Investigación Agropecuaria* (AGROSAVIA) is committed to the exploitation of agro-industrial residues as raw materials for new processes to produce high added value products with application in the agricultural and livestock industry.

Due to their complex composition, agro-industrial waste can be exploited to manufacture high value-added products such as enzymes, organic acids, antibiotics, pigments, biofuels, to produce

renewable energy, as well as pulp and paper (Kuddus et al., 2023; Sundaram et al., 2024). In recent years, the use of sugarcane and cereal grains by-products as substrates to generate bioproducts through fermentation processes has been gaining relevance (Bakari et al., 2023; da Silva et al., 2020; Dutra et al., 2020; Kumar et al., 2023; Lee et al., 2023; Marques et al., 2018; Paschoa et al., 2024; Qadir et al., 2018; Visentin et al., 2024). For instance, *Aspergillus niger* was used for the production of cellulases and xylanases in a solid-state fermentation system using sugarcane bagasse and brewery spent grain (Moran-Aguilar et al., 2021). Also, wheat bran was the substrate chosen for the production of Beta-glucanase by *Aspergillus niger* CCUG3399 on a solid-state fermentation process (Heidary Vinche et al., 2021). Finally, *Rasamsonia composticola* a thermophilic fungus was used to produce an endoxylanase using wheat bran and pretreated sugarcane bagasse in solid-state fermentation (Franco et al., 2024).

Bagasse is the main by-product from the sugarcane juice extraction during the production of sugar, non-centrifugal cane sugar (NCS)-locally known as *panela*, and bioethanol. It has been estimated than 30.2-34% of the processed sugarcane corresponds to bagasse (Mendieta et al., 2020). World sugarcane production for 2024 is estimated to be 2.21 billion tons (Martinez-Hernandez et al., 2018), thus around 0.6 billion tons of bagasse will be available for use as a source of byproducts such as bioethanol (de Oliveira Rodrigues et al., 2020; Peñaranda et al., 2018), enzymes, metabolites, cellulose, or to be used as fuel at the sugar mills (Savou et al., 2019). Sugarcane bagasse (SCB) is a lignocellulosic material composed of cellulose (40-45 wt%), hemicellulose (30-35 wt%), and lignin (20-30 wt%) (Barciela et al., 2023; Silva et al., 2018). Wheat bran (WB) is also a lignocellulosic agro-industrial residue from the wheat flour processing, which makes up to 25% of wheat (Saini et al., 2023). WB contains hemicellulose (18 wt%), cellulose (20 wt%), and lignin (6 wt%) (Chen et al., 2023). Raw WB is mainly used for animal feeding thanks to its content of iron, magnesium, manganese, and vitamins B3 and B6 (Chen et al., 2023; Katileviciute et al., 2019).

Filamentous fungi are saprophytic microorganisms found in natural ecosystems (soil, agro-industrial residues, decomposing materials), which are known to produce and secrete many bioproducts of interest, e.g., organic acids, polysaccharides, alkaloids, mycotoxins, antibiotics, and a wide range of hydrolytic enzymes capable to decompose complex, and insoluble biopolymers from both plant and animal tissues(de Oliveira Rodrigues et al., 2020; Rosa et al., 2024). Regarding biomass exploitation, filamentous fungi have lignocellulosic systems consisting of extracellular enzymes, such as laccases, peroxidases, cellulases and hemicelluloses, which can completely decompose the structure of this material (de Oliveira Rodrigues et al., 2020). Xylanase enzymes from filamentous fungi are potentially the most interesting for industrial uses because they are produced in greater amounts than those from yeasts and bacteria (Dahiya et al., 2024). Xylanases have broad industrial applications such as: plant biomass hydrolysis, improvement of the nutritional value of animal feed, clarification of fruit juices, pulp bleaching, enhancing of immunogenic activity of plants, production of xylitol, removal of xylan-based stains in detergent industry (Ismail et al., 2022).

Several studies (Gupta et al., 2019; Mustafa et al., 2023) have been conducted with individual filamentous fungi from *Trichoderma*, *Aspergillus*, and *Penicillium* genera for production of xylanolytic enzymes by solid-state fermentation (SSF) of SCB and other lignocellulosic residues, reporting good growth behavior and high enzymatic activities. For instance, *Penicillium citrinum* reached a maximum xylanase activity of 28.9 U gds^{-1} on agave bagasse and 38.4 U gds^{-1} on SCB (Valle-Pérez et al., 2021). *P. echinulatum* produced a maximum titer of 37.87 U gds^{-1} on a SSF system with a mixture of SCB and wheat bran as substrate (Camassola et al., 2010). *P. ochrochloron* RLS11 produced 4 U/mg CMCase and 40.0 U/mg of xylanase on a SSF system using sugarcane straw as substrate (Ning et al., 2024).

This study aimed to develop a whole production, recovery, concentration, and application process of an enzymatic extract (ECE) with mainly xylanase activity. The ECE was produced through SSF fermentation of agro-industrial wastes (non-treated SCB and WB) using a strain of Penicillium sp., recovered by solid-liquid extraction, and concentrated with an ultrafiltration system in batch configuration. Finally, ECE was evaluated for hydrolysis of kikuyu grass to demonstrate its potential for use as silage additive or for enzymatic saccharification of lignocellulosic materials for sugar production.

2. Methodology

2.1 Substrates

Raw SCB was provided by AGROSAVIA's experimental center CIMPA located at Barbosa Municipality in Santander. Fiber size of the material was selected to be approximately 10 cm and then air-dried at 40°C for 24 hours reaching a humidity <10%. Water content was measured by electronic moisture analyzer (MLS50-3, Kern & Sohn GmbH, Germany).

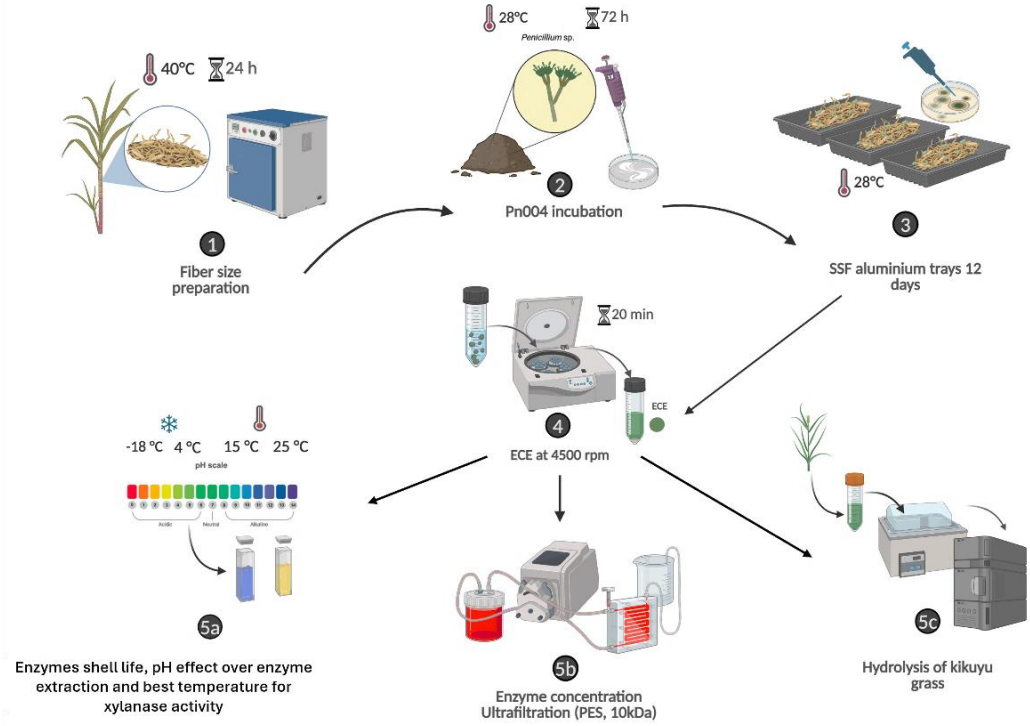

Figure 1. Schematic representation of the xylanase production process and its characterization, concentration, and application on the hydrolysis of Kikuyu grass.

Dried SCB was stored in sealed plastic bags at 4°C until use. Raw WB was purchased from a local supplier and stored at room temperature (20-22°C) until use. Regular table sugar (Ingenio Riopaila Castilla, Colombia), yeast extract (LP0021, Oxoid Ltd., United Kingdom), and hydrochloric acid 37% (Merck) were also used during experimentation.

2.2 Microorganisms and inoculum preparation

Penicillium sp. Pn004 was isolated from the soil of the Boyacá region of Colombia and deposited at the Germplasm Bank of Microorganism of AGROSAVIA. Their use was granted by Contract to Access Genetic Resources and its Derived Products No. 168 of 2017.

The fungus was cultured in potato dextrose agar (PDA) at 28°C for 3 days. The inoculum was prepared by aseptically scraping each Petri dish into a sterile 0.01% Tween 80 solution and shaking vigorously to obtain a conidia suspension. Concentrations were adjusted to 1×10^6 conidia mL^{-1} and confirmed by hemocytometer. Figure 1 shows a schematic representation of the whole production process and application of the enzymatic crude extract, for a better understanding.

2.3 SSF conditions and sampling

Aluminum trays (10×7×4 cm) containing solid substrate (non-treated SCB and WB; ratio 1:0.8) enriched with a nutritional solution (1 wt% sugar and 0.32 wt% yeast extract; pH 5.0 adjusted with hydrochloric acid) at 1:3 w/v ratio was sterilized at 121°C for 15 minutes. Inoculation was performed by adding the conidia suspension (ratio 1:1.05 w/v) following a zigzag pattern over the solid substrate. Immediately, trays were covered with linear low-density polyethylene (LLDPE) plastic (stretch film) and placed for up to 12 days in an incubation room at 28±2 °C and relative humidity maintained at 65%-70% during first 24 h. SSF assays were carried out in triplicate, keeping trays for each sampling day. Depending on the experiment, sampling was performed on days 1, 3, 5, 7, 10, and/or 12.

Water contents of solid substrates were estimated by loss on drying according to the Official Method (AOAC 925.10, 2012) with modifications. Briefly, 2-3 grams were weighed on a tared Petri dish and oven dried at 105°C to constant weight (around 4 hours). All measurements were conducted in triplicate.

2.4 Enzymatic crude extract separation

Enzymes were recovered by solid-liquid extraction mixing all the fermented substrate per tray with a 0.01% (v/v) Tween 80 solution at a ratio of 1:2 (w/v) and blended with a metallic spatula. Then, the mixture was kept under refrigeration for 1 h and filtered through a veil. The filtrate was centrifuged at 4500 rpm for 20 minutes and then the supernatant was vacuum filtered using Whatman

No. 1 filter paper. The filtrate fraction, corresponding to the enzymatic crude extract (ECE), was stored at 4°C until used for xylanase and cellulase activity assays.

2.5 Enzyme activity assays

Xylanase (endo-1,4-β-xylanase) activity was determined by incubating the ECE with 2% (w/v) birchwood xylan (Bailey et al., 1992) (Megazyme, P-XYLNBE-10G, Ireland; xylose content ≥85%), both in 0.05 M sodium citrate buffer pH 4.8 at 50 °C for 60 minutes. The reducing sugars released were estimated by the 3,5-dinitrosalicylic acid (DNS) assay (Miller, 1959), using xylose as standard, and measuring the absorbances at 540 nm. One unit of xylanase activity was defined as the amount of enzyme capable to release 1 µmol of reducing xylose per min at the corresponding assay conditions. The enzymatic activities reported in U gds^{-1} correspond to enzyme units per gram of dry substrate. All biochemical tests were conducted in triplicate.

2.6 Shelf life of enzyme extracts

The effect of the pH (5, 6.5, and 8) on the shelf life was studied with an enzymatic extract recovered by using McIlvaine buffer (0.05 M), which is referred to as ECE-buffer. As control, a treatment without buffer was used, where the recovery of the enzyme was conducted as before with a 0.01% (v/v) Tween 80 solution (ECE-Tween). All the treatments were stored at different temperatures (-18, 4, 15, and 25 °C) for seven days. The responses were expressed as relative activity [(U_t/U_o) x (100%)], where U_o and U_t correspond to initial and final activities, respectively.

2.7 Effect of pH during enzyme extraction and best temperature for xylanase activity

An additional set of experiments were run to understand the effect of the pH (3, 4, 5, 6, 7, 8) during the enzyme extraction from the solid substrate and the effect of the temperature on the xylanase activity assay. For this, ECE-buffer and ECE-Tween recovered at different pH values were incubated with 2% (w/v) birchwood xylan (Bailey et al., 1992) in 0.05 M citrate buffer at pH 4.8, and temperatures between 50 °C and 80 °C, with 10 °C increments. The total protein content was measured using the Bradford method by using Bovine serum albumin (BSA) as standard protein (Bradford, 1976). The units were calculated as specific activity (U mg^{-1}).

2.8 Hydrolysis of kikuyu grass

Kikuyu grass (*Cenchrus clandestinus*) was collected from AGROSAVIA´s research center Tibaitatá located in Mosquera, Cundinamarca (4°42'28"N 74°13'58"O). Then, it was washed five times with deionized water, dried overnight at 60 °C, and cut into pieces of about 2 cm. Enzymatic hydrolysis was carried out at 60°C for 48 hours without agitation in 0.05 M citrate buffer at pH 5, with a load of grass of 5.8 % (w/v) and addition of sodium azide (sodium azide for synthesis ≥ 99%, Merck) at 0.005% (w/v). The hydrolysis was initiated by adding the enzymatic crude extract at different xylanase activities of 1, 5, 10, 15, and 20 U mL^{-1}. Samples were withdrawn at 0, 2, 4, 6, 8, 24, and 48 h. The reaction was stopped by immediately immersing the tubes into boiling water for 10 minutes. Then, the samples were centrifuged at 8000 rpm for 10 minutes and the supernatants were analyzed for glucose content through an Ultra Performance LC system (UPLC).

On a second experiment, kikuyu grass was treated with 5 U mL^{-1} in 0.05 M citrate buffer at pH 5 at a loading of 5.8 % (w/v) for 72 hours at 60°C. Samples of grass, before and after the enzymatic treatment, were dried overnight at 70 °C. Total sugar contents were analyzed by UV-Vis spectrophotometric method (DuBois et al., 1956) . Samples from liquid phase were taken at 0, 16, 24, 48, and 72 hours and the reaction were stopped by immediately immersing the tubes into boiling water for 10 minutes. Then, the samples were centrifuged at 8000 rpm for 10 minutes and the supernatants were analyzed for glucose content by UPLC.

2.9 UPLC analysis

The glucose was quantified using a strong cation-exchange column (Bio-Rad Aminex HPX-87P, 300 x 7.8 mm) in a UPLC Waters Acquity system coupled with a refractive index detector (RI-2414). The column oven was set at 85°C, and the IR was set at 40°C. The mobile phase was ultrapure deionized water at 0.6 mL min^{-1} in a 30-minute run.

2.10 Enzyme concentration by ultrafiltration

Concentration of ECE was performed in a crossflow ultrafiltration system using a Vivaflow® 200 module (Sartorius Stedim Biotech GmbH, Germany), coupled to a Masterflex L/S pump system in a batch configuration. The crossflow cassette consisted of a polyethersulfone ultrafiltration membrane (Molecular Weight Cut Off (MWCO): 10,000 Da, active area: 200 cm^2) operated at 2.5 bars (200-400 mL min^{-1}). Purification factor (PF), rejection (R), concentration factor (X), yield, and permeate flux (J) were defined according to Equations 1-5 respectively (Berninger et al., 2018; Lutz, 2015; Yilmaz et al., 2019).

$$Purification\ factor\ (PF) = C_p/C_f \quad \text{Eq. 1}$$
$$R = 1 - (C_p/C_f) \quad \text{Eq. 2}$$
$$X = V_0/V_f \quad \text{Eq. 3}$$
$$Yield_{concentrate} = X^{R-1} \quad \text{Eq. 4}$$
$$J = V/(A \times t) \quad \text{Eq. 5}$$

Where C_p is xylanase activity in the concentrate (U mL^{-1}), C_f is xylanase activity in the ECE (U mL^{-1}), V_0 starting volume (mL), V_f final volume (mL), V is the volume of permeate (L), A is the effective filtration area (m^2), and t is time (h).

2.11 Statistical analysis

The results are reported as means of the replicates along with the standard deviation. Data were analyzed by one-way ANOVA with a 5% significance using Excel® for Microsoft 365 (Microsoft Corp., USA) and Minitab 16 (Minitab, LLC, USA) software. Plots were done using RStudio with ggplot2 (Villanueva et al., 2019) and plotly (Inc, 2015) packages.

3. Results and discussion

3.1 Effect of fermentation time on xylanase production

Several variables are important during the development of a SSF process to ensure a good productivity, such as aeration, moisture, pH, nutritional components, particle size, porosity, and fermentation time. During enzyme production, time is a key factor in order to control the enzymatic activity for situations such as enzyme degradation due to the activity of proteases, enzyme inactivation with toxic components produced during the fermentation, decrease in production rate due to catabolic repression (Chilakamarry et al., 2022). Thus, the kinetics for xylanase production was obtained within 12 days of fermentation. The highest xylanase activity and productivity were reached at the seventh day of fermentation, with values of 196.61 ± 5.29 U gds^{-1} and 28.08 ± 0.76 U gds^{-1} day-1, respectively (Figure 2). Nonetheless, the productivity at day five was similar with a value of 26.70 ± 1.59 U gds^{-1} day^{-1} with an enzyme activity of 130.00 ± 7.95 U gds^{-1}. Therefore, the following SSF tests were conducted for five days, since a good productivity was achieved, and the cost related to utilities and use of the space were reduced.

The enzyme concentrations reached in our study are higher than those published previously for a strain of *P. echinulatum* (Camassola et al., 2010), which reached a xylanase activity of 36.38 ± 5.38 U gds^{-1} and a productivity of 12.12 ± 1.79 U gds^{-1} day^{-1} on the third day of fermentation using non-treated SCB+WB (1:0.67) as substrate. Meanwhile, the xylanase activity is comparable to that reported for *P. citrinum* MTCC 9620 during SSF of non-treated SCB supplemented with Czapek Yeast Extract media Ghoshal *et al.* (2016), with an enzyme activity of 156.50 U gds^{-1} on the fifth day, which was the starting point for fermentation optimization After this, they achieved an enzyme activity of 1665.80 U gds^{-1} for xylanase, highlighting the importance of an optimization process to increase the production of enzymes.

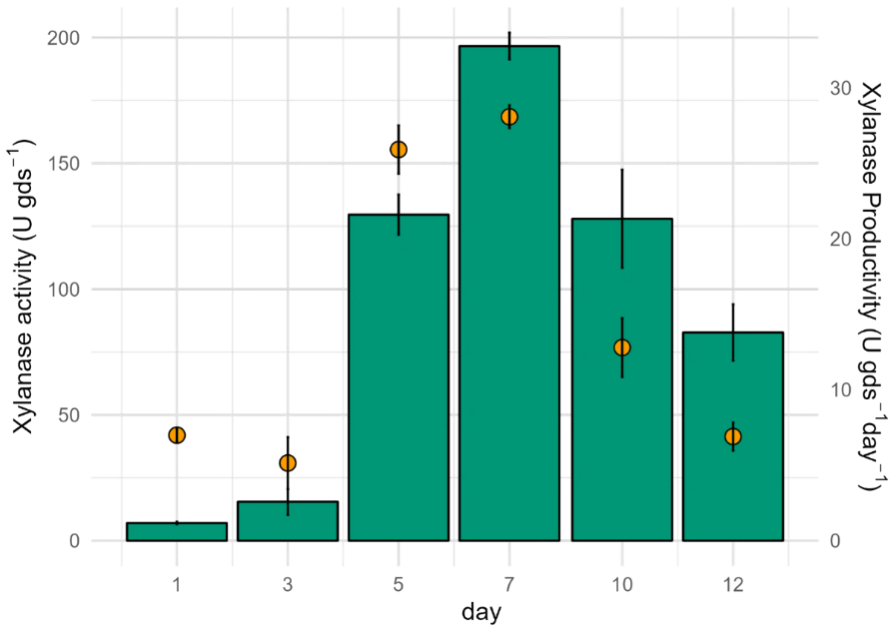

Figure 2. Xylanase activity and productivity of *Penicillium* sp. Pn004 in a SSF process over time. Columns corresponds to xylanase activity (U gds^{-1}) and dots corresponds to productivity (U gds^{-1} day^{-1}). The error bars are the standard deviation of the triplicate experiments.

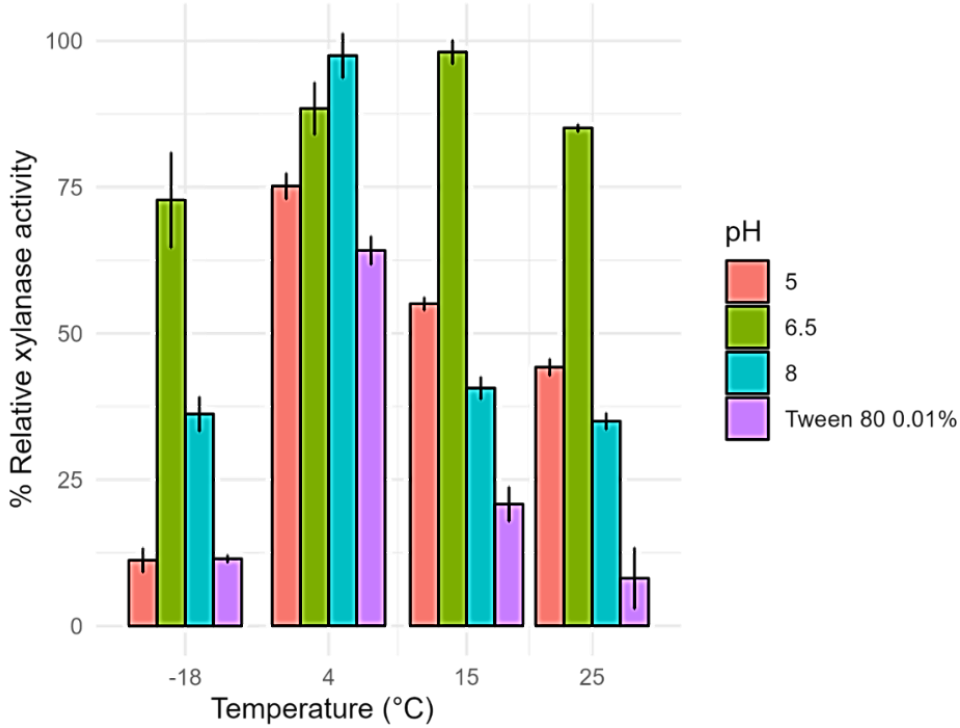

Figure 3. ECE shelf life after seven days of storage under different temperatures (-18 °C, 4 °C, 15 °C and 25 °C) and pH (5, 6.5, 8, and without buffer) estimated as relative activity. The pH of the Tween solution 0.01% was adjusted to 4.8.

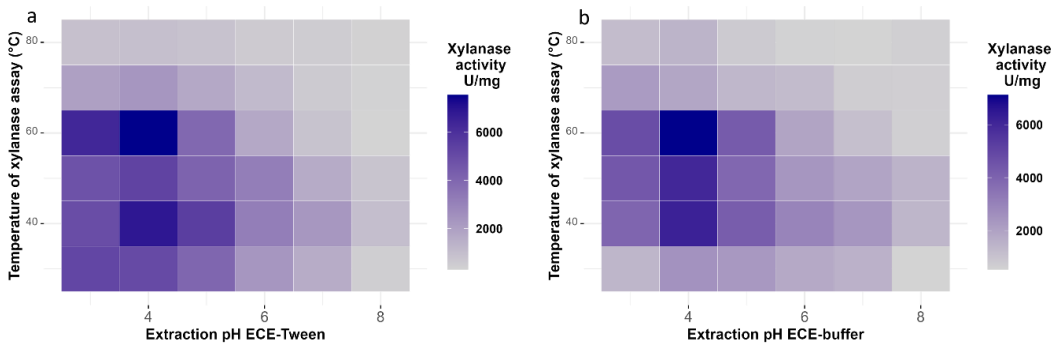

Figure 4. Influence of extraction solution pH and temperature of biochemical assay on the xylanase activity from an ECE produced by *Penicillium* sp. Pn004. a. ECE extracted with 0.01% Tween 80 solution (ECE-Tween) b. ECE extracted with McIlvaine buffer (ECE-buffer).

3.2 Shelf life of enzyme extracts

Temperature and pH during storage can alter the protein structure of enzymes, thus affecting its catalytic properties (Andrea et al., 2016). Therefore, the effect of pH, buffering, and temperature on enzyme activity during storage for seven days was evaluated. The results showed that shelf-life of xylanases depend on both variables. The best conditions for storage were pH 6.5 and 15°C with a relative xylanase activity of 98% ± 2%, and pH 8 and 4 °C with 97.5% ± 3.8% of relative xylanase activity, as shown in Figure 3. Freezing (-18 °C) was the most deleterious temperature at any pH (5, 6.5, 8, and without buffer) with reduction of relative xylanase activity between 27-89 %. Nonetheless, it has been reported that the relative xylanase activity was maintained after 120 days of freezing (-20 °C and 4 °C) with loss of only 6 and 4 % respectively on an ECE from *P. roqueforti* ATCC 10110 produced in SSF using yellow mombin residue (de Almeida Antunes Ferraz et al., 2019) as substrate. This difference could be due to the use of different buffer solutions during the extraction of the ECE. Here, the McIlvaine buffer was used, whereas de Almeida Antunes Ferraz et al. (2019) obtained the ECE with 0.05 M citrate buffer solution at pH 4.8 from the solid substrate.

The greatest decrease in xylanase activity was observed when a 0.01% Tween 80 solution was used for the extraction process, regardless of storage

temperature. The relative xylanase activity on the seventh day of storage was 64% at 4 °C and only 8% at 25 °C. These results highlight the importance of buffer solutions on enzyme shelf life to mitigate the loss of catabolic activity due to changes in pH and ionic strength of the solution over time, which shall affect the shape of the active site and its solubility (Chapman et al., 2019; Eun, 1996).

3.3 Effect of pH during enzyme extraction and best temperature for xylanase activity

Recovery of enzymes from fermented solid substrates is an important step. Several factors could affect this stage such as the type of an extractant, solid:liquid extraction ratio, extraction time, temperature, pH and extraction rate (Adhyaru et al., 2016; Alves et al., 2022; Jonathan et al., 2021). Thus, an experiment was set up to study the effect of the pH and the type of the extraction solution. Higher xylanase recovery was achieved when the pH of the extraction solution was acidic (pH 3-5) than neutral and alkaline pH, regardless the type of solution (McIlvaine buffer or Tween 0.01% solution) as shown in Figure 4. The highest enzymatic activity was obtained for the ECE-Tween at pH 4 and 60 °C (7547.15 U mg^{-1}), followed by the ECE-buffer at pH 4 and 60 °C (7120.45 U mg^{-1}). When the pH of the extraction solution decreased to 3 at 60°C, a 16% reduction in xylanase activity was observed for ECE-Tween, whereas for ECE-buffer it was 33%. An increase in pH to 5 at 60°C reduced the ECE-buffer xylanase activity by 40%, and in the ECE-Tween by 47%. Previously, it was reported than acidic conditions favor the recovery and activity of xylanase enzymes produced by SSF using Aspergillus *tubingensis* FDHN1. Acidic conditions could help to maintain the stability of the enzyme, and to break ionic bonds, hydrogen bonds, and van der Wall forces that link the enzymes to the fungal mycelia and solid substrate (Adhyaru et al., 2015). The effect of increasing the temperature above 60 °C on the xylanase activity was more deleterious than decreasing it below the same value, regardless of the pH value (Figure 4). At 70 °C and pH 4, the ECE-buffer lost 73% of its xylanase activity, whereas the ECE-Tween lost 69%. Meanwhile, a reduction in the temperature to 50 °C at pH 4 decreased the xylanase activity by 30% for ECE-Tween and by 15% for ECE-buffer. The optimal temperature (60°C) for xylanase activity found in this study matches those of previous studies, where no significant differences were detected in a pH range from 3.0 to 5.0 for an ECE produced by *P. roqueforti* ATCC 10110 (de Almeida Antunes Ferraz et al., 2019). Also, the optimal temperature found for *P. glabrum´s* purified xylanase was of 60°C and optimal pH of 3.0 (Knob et al., 2013).

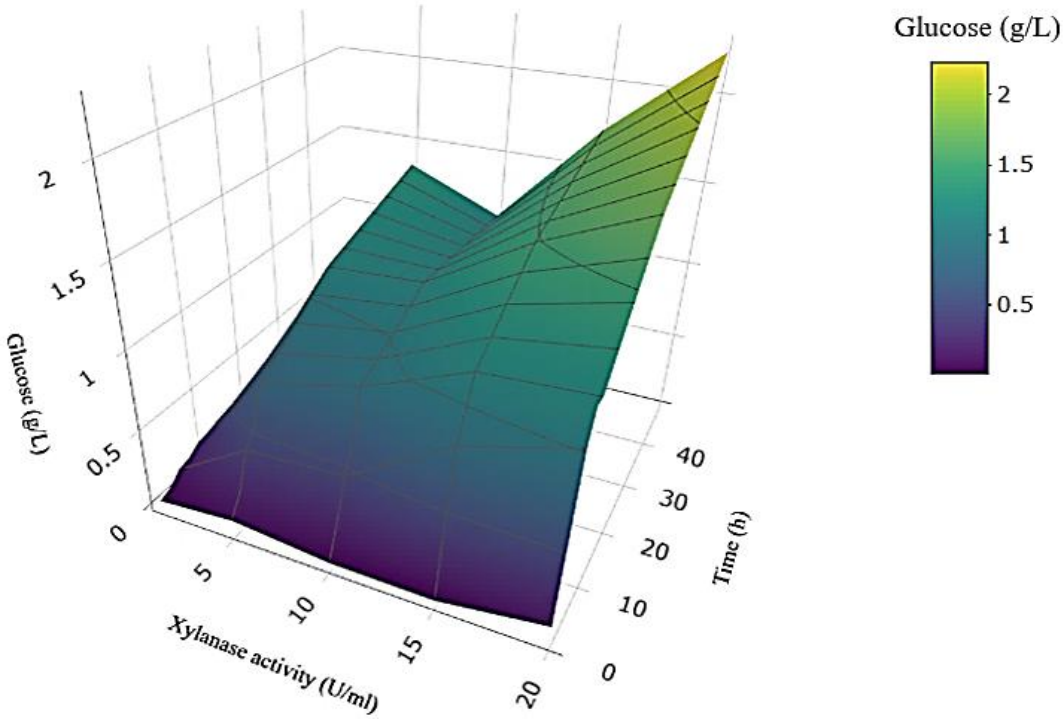

Figure 5. Glucose release over time from untreated kikuyu grass hydrolysis under different concentrations of xylanase in the ECE of *Penicillium* sp. Pn004.

Table 1
Glucose content in liquid supernatant and total sugars in kikuyu grass before and after hydrolysis with ECE with 5 U mL^{-1} xylanase activity

Time (h)	Glucose content in liquid (g L^{-1})	Glucose released (mg gdb^{-1})	Total sugar in kikuyu grass (mg gdb^{-1})
0	0.00	0	35
16	0.37	6.5	
24	0.52	9.05	
48	0.57	9.88	
72	0.61	10.56	15

3.4 Hydrolysis of kikuyu grass

Cellulolytic enzymes, such as cellulases, xylanases, and cellobioses, have drawn attention as silage additives because of their ability to degrade the plant cell wall (Muck et al., 2018). These enzymes, combined with lactic acid bacteria (LAB), could improve silage fermentation as the enzymes release carbohydrates from the cell wall, making them available for LAB to ferment into lactic acid and lower silage more rapidly (Mejía-Avellaneda et al., 2021). Usually, the pH of silage drops below 4, so enzymes must be able to work in these acidic conditions. The ECE produced in this work showed higher enzymatic activity in the pH range of 3-5, making it a good alternative as a silage additive. Therefore, an experiment to measure the release of sugars from kikuyu grass under different enzyme activity of ECE was designed. Figure 5 shows that the concentration of glucose released from the grass at a substrate loading of 5.8 % (w/v) increases with time and the concentration of xylanase.

The highest glucose concentration was 2.23 g/L or 38.78 mg g^{-1} at 48 h of kikuyu grass hydrolysis with an enzyme activity of 20 U mL^{-1}. It has been reported that a higher enzyme loading is required to achieve a higher release of reducing sugars and efficient saccharification, as well as a longer incubation time (Menegol et al., 2014).

Finally, to establish the potential of the ECE for the release of reducing sugars from grass to support the growth of the LAB during silage application, kikuyu grass was treated with 5 U mL^{-1} of xylanase activity at a loading of 5.8 % (w/v). A yield of 43% of total sugars was found after 72 hours of treatment (Table 1), equivalent to 20 mg of total sugar gdb^{-1}. During the 72 hours of treatment, the glucose concentration in the liquid media increased from 0.00 to 0.61 g L^{-1} (Table 1). Previously, a hydrolysis of kikuyu grass with commercial enzyme Celluclast produced only 0.032 g L^{-1} of reducing sugars (Vásquez et al., 2010). Cellulase from *Trichoderma reesei* ATCC 26921 was used with a load of 15 FPU g^{-1} of biomass for enzymatic hydrolysis of hybrid grass with a production of around 200 mg/g of reducing sugars within 144 h. Finally, Kans grass treated with an enzymatic crude extract (20 FPU gdb^{-1}) from *T. reesei* released 34.67 mg of reducing sugars gdb^{-1} for a 2% biomass loading after 72 hours of incubation (Kataria et al., 2011).

3.5 Enzyme concentration by ultrafiltration

One of the bottlenecks in the production process of enzymes is the concentration and purification stage. Since the cost of enzyme purification for a bioproduct intended for the agro-industrial sector could be high, we decided to work with an enzyme cocktail and apply only the concentration step. The ultrafiltration process allows the concentration of the ECE and it is also considered an initial purification method by using membranes with MWCO lower than that of the enzyme of interest. Therefore, the retentate shall have the concentrated enzyme (Jonathan et al., 2021). The membrane used in this study had a MWCO of 10 KDa smaller than the reported molecular weight of xylanases from *Penicillium:* 41 kDa for a GH10 xylanase produced by *P. funiculosum* (Lafond et al., 2011); 27.0 kDa for a xylanase from *P. expansum* (Murthy et al., 2012); 23.9 kDa and 33.1 kDa for xylanases I and II from *P. sclerotiorum* (Knob et al., 2010). The ultrafiltration process using the batch configuration allowed the concentration of the ECE in the retentate at a PF of 19, increasing the xylanase activity from 66.47 U mL^{-1} to 1486.83 U mL^{-1}, as shown in Figure 6. The permeate flux through the membrane did not reach an steady-state even at PF of 19, and thus, the fouling effects were more important than the cake layer formation (Yilmaz et al., 2019). The yield in the concentrate at PF of 19 was 86%. The values reported are greater than that reported by Guo et al. (2012), which is a two-step purification process (ultrafiltration-gel filtration chromatography) reaching a PF of 17 with a yield of 68.11% from *Bacillus subtilis* (Guo et al., 2012). Also, xylanase from *Bacillus subtilis* BE-91 was purified by a two-step process of ultrafiltration separation and gel chromatography reaching a yield above 69% and a PF of up to 18 (Liu et al., 2011).

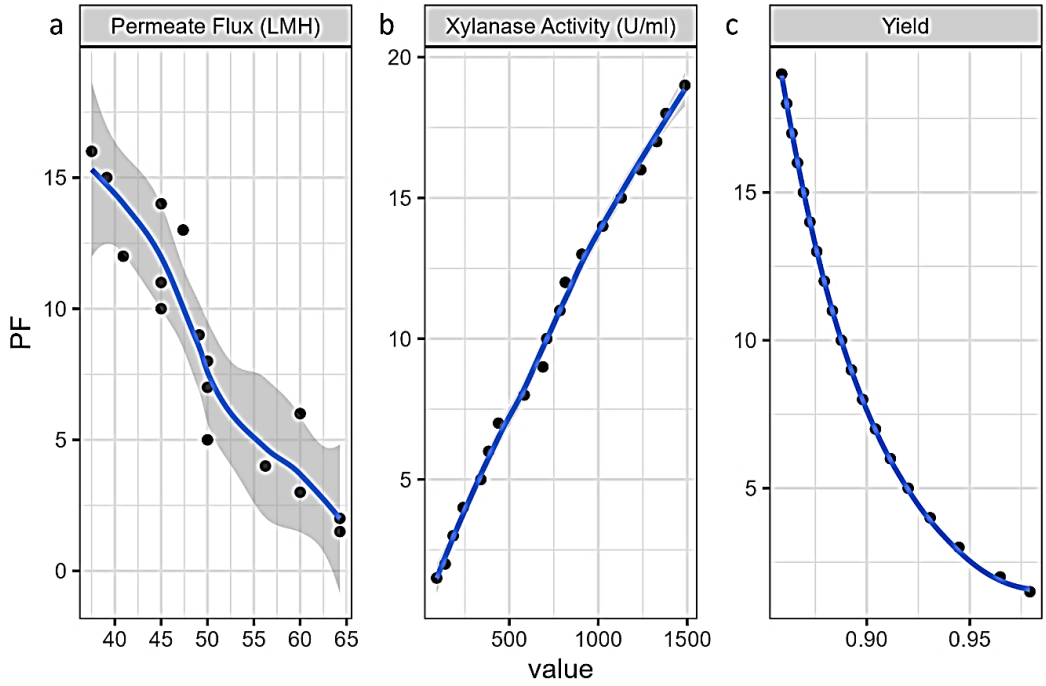

Figure 6. Concentration of ECE obtained from SSF by *Penicillium* sp. Pn004through an ultrafiltration process. a. Permeate Flux (LMH, L m^{-2} h^{-1}), b. Xylanase activity (U mL^{-1}), c. Yield (%). The solid line corresponds to the curve fitted to the data by local regression (Loess).

4. Conclusions

The results of this study show the potential to transform an agro-industrial waste, non-centrifugal sugarcane bagasse and wheat bran into a value-added product, such as xylanase by SSF fermentation using *Penicillium* sp. Pn004. It was found that, at neutral pH, the shelf life of the obtained enzymatic crude extract was longer, while acidic conditions are recommended for enzyme recovery from the solid substrate, and the optimum temperature for xylanase activity assay was 60 °C. The ultrafiltration process using the batch configuration was suitable for reaching a higher xylanase concentration without reducing the flux through the membrane and having a good yield. Furthermore, higher enzyme loading, and longer incubation time allow a higher release of reducing sugars from kikuyu grass using the enzymatic extract obtained in this study. This extract could be used as an additive with lactic acid bacteria (LAB) for silage as it allows the release of sugars from the grass, which could be used as a carbon source by the LAB and favor their action on the silage. Also, it could be used for enzymatic saccharification of lignocellulosic materials for sugar production. Nonetheless, more work should be done to increase the productivity of xylanases by enhancing the media composition and scaling up the process, to make a more profitable production process. Our study also gave the initial conditions for ultrafiltration process of xylanases in a crude enzymatic extract, which shows the potential of this technique to concentrate and purify enzymes obtaining high yields and product purity at the same time. Finally, a whole process at laboratory scale was developed for the production, recovery, and concentration of xylanases enzyme with high productivity and yield using a *Penincillum* sp. strain for enzymatic saccharification of grass silage.

Consent of publication

All authors have read and approved the manuscript for publication.

Conflict of interest

The authors hereby declare that they have not conflicts of interest in publishing this manuscript.

Acknowledgements

We would like to thank AGROSAVIA and the Ministry of Agriculture and Rural Development (MADR, by its Spanish acronym) for their funding. We are also grateful to the National University of Colombia - Medellin Campus for their support in the analysis of samples during the study. The microorganism used in this study are part of Framework Contract for Access to Genetic Resources and their Derived Products No. 168 of 2017.

Author contribution

Conceptualization, [EJB, ADM, ABP]; Formal analysis [ADM, ABP, VCA, JCBM], data collection [ADM, ABP, VCA, JCBM], writing original draft preparation [ADM, ABP, VCA, JCBM, EJB], writing, review and editing [ADM, ABP, EJB], Funding acquisition [EJB]; Supervision [ADM, ABP, EJB].

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