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Spartina argentinensis valorization and process optimization for enhanced production of hydrolytic enzymes by filamentous fungus

Carlos Martín Pellieri [a], Antonela Taddia [a], Dana Belén Loureiro [a], Santiago Andrés Bortolato [b], Gisela Tubio [a,*]

[a] Instituto de Procesos Biotecnológicos y Químicos (IPROByQ) CONICET, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Argentina
[b] Instituto de Química de Rosario (IQUIR) CONICET, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Argentina

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ABSTRACT

Hemicellulose is a major component of plant cell walls and xylan is the most predominant polysaccharide. Xylan degrading enzymes integrate the xylanolytic system. Xylanolytic enzymes were produced by fermentation by *Aspergillus niger* and *Thermomyces lanuginosus* grown on grass considered unsuitable for farming called *Spartina argentinensis*. Significant parameters: type of fermentation used (SmF or SSF), type of leaves of *S. argentinensis* (green or senescent) and conidia final concentration for xylanase production were screened and optimized. The main results showed that the highest levels of xylanolytic enzyme production were obtained by 1×10^5 conidia/mL of *A. niger* in SmF at 96 h, 30°C, with a mixture of 20.20% of senescent and 79.80% of green leaves. The xylanase specific activity obtained was 62 U/mg, higher than the activity obtained (23 U/mg) in previous work (Taddia et al., 2019), and the concentration of xylanolytic production over that of glucanase activity was maximized five times. The optimized enzymatic extract obtained was characterized by LC-MS and HPLC of carbohydrates. Six enzymes were identified as constituents of the xylanolytic complex and seven carbohydrates. Moreover, the xylanolytic enzyme extract was stable for 30 days at 20 °C. Thus, *S. argentinensis* can be used within the framework of a circular economy, rendering a synergistic combination of the xylanolytic enzymes with industrial applications.

1. Introduction

Hemicellulose is a hetero-polysaccharide composed of pentoses (xylose and arabinose), hexoses (mannose, glucose and galactose) and sugar acids (methyl-glucuronic acid). Hemicelluloses are usually grouped into classes based on the main sugar residues present in the backbone, and these include xyloglucans, xylans, mannans and glucomannans, and β-glucans. Particularly, xylan consists of a d-xylose backbone linked with β-1,4- glycosidic bonds with side chains of α-arabinofuranose and/or α-glucuronic acids that in some cases contribute to cross-linking of cellulose microfibrils and

* Correspondence to: Universidad Nacional de Rosario, IPROByQ-Facultad de Ciencias Bioquímicas y Farmacéuticas, Suipacha 531, (S2002 LRK) Rosario, Argentina.
E-mail address: tubio@iprobyq-conicet.gob.ar (G. Tubio).

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lignin. Xylan degrading enzymes integrate the xylanolytic system (Bajpai, 2014). These enzymes have diverse specificity and modes of action, requiring a synergistic action where the xylan backbone is randomly cleaved by the action of endo-1,4-β-d-xylanases Xylose polymer is broken down to its monomeric form by action of β-d-xylosidases, while acetyl and phenolic side branches are removed by the action of α-glucuronidases and acetylxylan esterases. Additionally, α-l-Arabinofuranosidases catalyze the removal of the side groups. The ester bonds present on the xylan are cleaved by the action of p-coumaric esterases and ferulic acid esterases (Malgas et al., 2019).

The endo-1,4-β-xylanase (EC 3.2.1.8) is considered as the main enzyme responsible for xylanase activity (Amore et al., 2015). They are important biocatalysts and have a leading role in industrial processes such as food industry, sugar production, paper production, animal feed and the hydrolysis of biomass for the production of biofuel processes, however, the high cost of enzyme production has hindered its industrial application (Saleem et al., 2020; Sreena, 2020; Yang et al., 2006).

Various microorganisms such as filamentous fungi, bacteria, yeasts and actinomycetes are xylanase producers. Fungi are powerful producers of xylanase, and the genus *Aspergillus* and *Thermomyces* are among the most prolific. *Thermomyces lanuginosus,* a thermophilic fungus from *Ascomycota phylum*, has created interest because of its operationally stable enzymes, especially xylanase and *Aspergillus niger* was commonly classified as strong xylanase producer (Ang et al., 2013; Kumar and Shukla, 2018).

Large quantities of lignocellulosic wastes are produced annually around the world. They include agricultural residues, food farming wastes, tree pruning residues and urban solid wastes. A wide range of high added-value products, such as biofuels, organic acids, biopolymers, and molecules for food and pharmaceutical industries, can be obtained using solid wastes by biotechnological processes (Amore et al., 2015). Within lignocellulosic materials, perennial C4 grasses appear as a promising untapped resource. *Spartina argentinensis* is a dominant species that occupies an area of 33,500 km ^{2} in Argentina (Larran et al., 2015), making the soils in this area unsuitable for farming. Besides, cattle have low digestibility for this species. The low annual production of beef per hectare is usually due to these pastures. Farmers resort to the frequent burning of these pastures generating important environmental damage, in order to improve this rate. In addition, in these ecosystems, which are a great source of biomass, no sustainable productive activities have been carried out (Larran et al., 2015). Finding sustainable consolidated bioprocessing for enzyme production is an essential step towards attaining efficient and economical biological treatment methods.

Several previous works are focused on reducing the production costs using low-cost residues or substrates. Bajar and coauthors optimized the enzyme production through *Aspergillus heteromorphus* by SmF using anaerobically treated distillery spent wash and rice straw as substrates with the highest xylanase activity (11.6 U/mL) at the fifth day (Bajar et al., 2020). Recently, Namuch and coauthors evaluated lignocellulosic wastes (rice straw, sugarcane bagasse, rice bran and sawdust) and obtained maximum xylanase activity (376.81 U/mL) in 14 days using sugarcane bagasse as substrate by *Aspergillus flavus* (Namnuch et al., 2021). In our laboratory, we have evaluated eleven different carbon sources (Taddia et al., 2019), and optimized by *A. niger* the production of enzymes with wheat bran (Taddia et al., 2020) and brewer's spent grain (Podestá et al., 2019). However, studies carried out with *S. argentinensis* are not found in the current literature. The results confined that the agro-industrial waste substrate could be a cost-effective and eco-friendly alternative to produce value-added products like enzymes.

Research on optimizing the production of xylanase free of cellulase activity for further application in the pulp and paper industry is not available in previous work and some of these reports do not cite the use of factorial designs. Furthermore, the use of a resource without added value such as *S. argentinensis* would avoid environmental problems, reduce the costs of the process and would be able to obtain a value-added product for its application in the industry. In this work, the optimization of xylanase production using fermentation by *A. niger* and *T. lanuginosus* employing a response surface methodology was performed using *S. argentinensis* leaves as substrate, (i) Fractional Factorial Design was used to screen significant parameters affecting the production of xylanase, (ii) Central Composite Design was employed in order to form the response surface, fit the model and predict the optimum value and (iii) protein and monosaccharide composition of the optimized enzymatic extract was analyzed by nano LC-MS/MS and HPLC respectively.

2. Materials and methods

2.1. Chemicals and substrates

Carboxymethyl cellulose, beechwood xylan, azocasein, 3,5-dinitrosalicylic acid, D-glucose, D-xylose, bovine serum albumin and bicinchoninic acid were purchased from Sigma (St. Louis, MO, USA). All other chemicals and solvents used were of analytical grade.

S. argentinensis Parodi plants were originally transplanted from a watershed halophyte grassland community of the depressed area surrounding the Ludueña stream (33°S; 60°; 500 W) which belongs to the Experimental Campus of the Faculty of Agricultural Sciences from the National University of Rosario, Zavalla (33°10S; 60° 530 W). Green and senescent leaves were collected and treated separately. Both types of leaves were harvested, oven-dried to constant weight (60 °C - 48 h), ground to 3 *x* 2 *x* 1 mm pieces, sieved to separate the powder, and stored in plastic bags at −20 °C.

2.2. Microorganism

The strain of *Aspergillus niger* NRRL3 was provided by the culture collection of Agricultural Research Service, USDA. The strain of *Thermomyces lanuginosus* CBS 288.54 was provided by CBS-KNAW Fungal Biodiversity Center (An Institute of the Royal Netherlands Academy of Arts and Sciences).

2.3. Inoculum preparation

Spores of *A. niger* NRRL3 and *T. lanuginosus* from a five-day-old culture incubated at 30 °C and at 40 °C respectively, in potato-glucose agar medium in Erlenmeyer flask, were harvested after addition of 15 mL distilled water. After, they were appropriately diluted to the required density of conidia and stored in 10% w/v glycerol at −20 °C until used as the master suspension. The number of spores was counted using a Neubauer's counting chamber.

2.4. Culture medium

Culture medium for the *A. niger* NRRL3 strain containing Mandel's culture medium salts (g/L) was prepared using: KH_2PO_4: 3.00, $NaNO_3$: 3.00, $MgSO_4.7H_2O$: 0.50, $CaCl_2$: 0.50, $FeSO_4.7H_2O$: 0.0075, $MnSO_4.H_2O$: 0.0025, $CoCl_2$: 0.003, $ZnSO_4$: 0.002 and distilled water (Yuan et al., 2005). On the other hand, the culture medium used for *T. lanuginosus* strain was prepared as follows (g/L): yeast extract: 10, tryptone: 10, $(NH_4)_2SO_4$: 2, $MgSO_4.7H_2O$: 0.3, $CaCl_2$: 0.3, $FeSO_4.7H_2O$: 0.3 and distilled water. The salts were dissolved in water and pH was adjusted to 6 for both culture media (Jiang et al., 2005).

2.5. Fermentation process

In all cases, submerged (SmF) and solid-state (SSF) fermentation were performed by triplicate in flaks and blood culture glass jars, respectively. The systems of 250 mL considered as bioreactors were carried out by adding four grams of green and senescent leaves from *S. argentinensis*, with 100 mL and 10 mL of culture medium, respectively. The humidity in SSF was maintained at 65%. Then, the media were autoclaved at 121 °C for 20 min and after cooling (Microclave SL 9000 Ficoinox, Argentina), conidia suspensions to a final concentration were aseptically added to the bioreactors. Sterilized distilled water was used instead of the inoculums in controls systems. SmF and SSF were carried out and maintained at 30 °C for nine days for *A. niger* and *T. lanuginosus*. Also, some fermentations were performed at 50 °C for *T. lanuginosus*. In the oven (SLDB San Jor, Argentina), the temperature and humidity were controlled by a digital hygro-thermometer sensor (Clock & Hygro-Thermometer Boeco, Germany). The temperature was automatically maintained by the oven and the humidity was maintained using a glass filled with water. In SmF, an agitation speed of 150 rpm was maintained using a digital orbital shaker (OS-20 Boeco, Germany). After the incubation, the fermented broth was filtered and the supernatant was used for enzymatic assay. In SSF, the enzymatic extract was obtained by adding 15 mL of distilled water to each bioreactor. The resulting suspension was centrifuged at 3200 g for 5 min (DCS-16-RV PRESVAC, Argentina) and it was then filtered through a 0.45 μm membrane. Aliquots of 0.5 mL were removed every 24 h for the enzymatic activity and total protein determinations.

2.6. Analytical methods

The enzymatic activities of endo-1,4-β-xylanase (EX) and endo-1,4-β-D-glucanase (EG) in the enzymatic extracts from both fermentations were analyzed.

Beechwood xylan (1% w/v) and carboxymethyl cellulose (1% w/v) solutions were incubated with the enzymatic extract in 50 mM citrate buffer (pH 5.30) at 50 °C for 10 min for EX and EG activity determinations, respectively. The reducing groups released after the incubation period were determined by the dinitrosalicylic method (Miller, 1959). The absorbance of each sample was measured at 560 nm (UV–Vis Multiskan Go Thermo Fisher Scientific, USA). Blanks without enzymatic extract and without substrates were assayed as controls. Calibration curves with D-xylose and D-glucose were obtained under the same experimental conditions as the samples. One unit of enzymatic activity is defined as the amount of enzyme required to release 1 μmol of reducing sugar equivalent per minute (μmol sugar/min).

Total extracellular proteins excreted were quantified using the bicinchoninic acid method where bovine serum albumin was used as the standard protein (Walker, 2009).

Proteolytic activity was assayed according to Castro and Sato with some modifications using azocasein as substrate (Sores De Castro and Sato, 2014). The reaction mixture contained azocasein (1% w/v), 20 mM Tris–HCl buffer (pH 8.00) and optimized enzymatic extract, which were then incubated for 30 min at 37 °C. The reaction was stopped by adding trichloroacetic acid (10% w/v) and the sample was centrifuged at 5600 g for 15 min. The supernatant was neutralized with 1.8 *N* NaOH and the absorbance was measured at 420 nm.

2.7. Experimental design for EX production

Experimental design, a collection of mathematical and statistical techniques, is usually used for modeling and analyzing problems in which the responses are influenced by several parameters using the statistical program Minitab 17. In order to define the experimental variables and the experimental domain in the experimental design and optimization for EX production, previous experiments were performed.

In the planning phase, the production of EX and EG enzymes was evaluated as a function of its fermentation time by *A. niger* and *T. lanuginosus* (at 1.00×10^6 conidia/mL final concentration) in SmF and SSF with green and senescent leaves of *S. argentinensis* at 30 °C. Likewise, the same analysis was performed for *T. lanuginosus* at 50 °C.

Afterwards, fractional factorial design was used to select the significant parameters (screening phase). The obtained parameters were used on a central composite design, in order to form and optimize the response surface methodology.

2.7.1. Screening of significant parameters affecting EX production

Fractional factorial design was used to analyze three parameters at two levels (2^{3-1}) and in blocks of size two. Block design was performed in order to analyze the response of each filamentous fungus used (*A. niger* or *T. lanuginosus*). The parameters studied were: type of leaves (TL) of *S. argentinensis* (green (G) or senescent (S)) (X_1), type of fermentation used (SmF or SSF) (X_2) and conidia final concentration (CC) (1.00×10^5 or 1.00×10^6 conidia/mL) (X_3). The screening was performed in 10 experimental runs of fermentation process. The EX and EG activity were considered as the responses.

2.7.2. Optimization of significant parameters for EX production

The two significant parameters obtained from the screening process, achieved with fractional factorial design for the substrate, were examined using a central composite design. Response surface methodology was performed through a central composite design in a single block with a total of 13 experiments. The central composite design contained a 2^3 factorial matrix with four axial experiments and five replicates at the center points. The species *A. niger* was used, analyzing the factors: carbon source, which is a combination of green and senescent leaves in different proportions and defined as the percentage of senescent leaves (%SL), and conidia final concentration (CC). The levels of each factor to be optimized were chosen considering the minimum and maximum range of the screening stage; these were used as extreme values and three more numerical values were calculated based on the parameter α of the equation. The ratio between EX and EG activity (EX/EG) and the specific activity in the xylanolytic production (EX_{sa}) can be considered as a measure of selective enzymatic production, which allows the selection of the most adequate enzymatic extract according to its industrial application. Consequently, EX/EG and EX_{sa} were considered as the responses.

The quadratic regression models are one of the most widely used in practice. They allow a description of the object in a comparatively wide area of the input variable change. They are expressed as follows (Eq. (1)):

$$Y = \beta_0 + \sum_{i=1}^{m} \beta_i X_i + \sum_{i=1 j=i+1}^{m} \beta_{ij} X_i X_j + \sum_{i=1}^{m} \beta_{ii} X_i^2 \tag{1}$$

where Y is the predicted response, X are the coded levels of the independent parameters and β the regression coefficients of the model. The optimal levels of each parameter to obtain the maximal value of the response were obtained by solving the regression equation and analyzing the 3D surface and contour plots.

2.7.3. Experimental validation of the models

The fermentations were carried out in five replicates of each treatment under optimal conditions for the validation of the optimized parameters.

2.8. Mass spectrometry analysis

Proteins of the optimized enzymatic extract were analyzed using mass spectrometry. In order to obtain a single electrophoretic band containing all the proteins, the enzymatic solutions were run on SDS-PAGE electrophoresis gel up to 1 cm after entering the resolution gel, revealing it with Coomassie brilliant Blue. The bands were extracted and digested with trypsin. The resulting peptides were extracted and dried in a Speed-Vac. The samples were analyzed by nano-LC–MS/MS using an EASY-nLC 1000 system (Thermo Scientific) coupled to an LTQ Orbitrap XL mass spectrometer (Thermo Scientific, Germany). A C18 precolumn (Acclaim PepMap, 75 μm × 20 mm, 3 μm particle size, 100 Åpore size, Thermo Scientific) was used and a Reversed-phase chromatography was performed on an EASY-Spray Accucore column (C18, 50 μm × 150 mm, 2 μm particles, 100 Åpore size, Thermo Scientific) operated at 300 nL/min at 35 °C. A 120 min long multistep gradient was used for peptide separation. Peptides were analyzed using the Xcalibur 3.0.63 (Thermo Scientific) software. The acquired raw MS/MS data files were searched by Proteome Discoverer software (version 1.4, Thermo Scientific) and *Homo sapiens* Uniprot database, with trypsin specificity and a maximum of one missed cleavage per peptide. A high confidence filter for peptide identification was used.

2.9. Enzymatic properties of xylanolytic enzymes

The stability of xylanolytic enzymes from the optimized enzymatic extract was assayed at 20 °C for a period of 30 days. The enzymatic extract was withdrawn at different times, cooled down and residual activity was assayed. The stability of the enzyme was expressed as a percentage of the residual activity. The saccharides present in the optimized enzymatic extract were extracted using a Vivaspin 20 (Sartorius) centrifugal concentrator with a molecular mass limit of 5 kDa. The samples were centrifuged at 4500 g for 15 min. The reducing groups of saccharides in both fractions were determined by the dinitrosalicylic method (Miller, 1959). Then, the volumes of the concentrate in proteins and free of saccharide solutions were adjusted with distilled water to the same total protein concentration and xylanolytic enzymes activity of the enzymatic extract. The xylanase activity stability from the optimized enzymatic extract (control) and optimized enzymatic extract free of saccharides were analyzed at 20 °C for a period of 30 days. Aliquots at different times were cooled and residual activity was assayed. The stability of the enzyme was expressed as a percentage of residual activity.

2.10. High-performance liquid chromatography of carbohydrates

Samples were sent to the Carbohydrates Research Center (CIHIDECAR- CONICET-UBA). Initially, dissolved in distilled water and passed through a reverse phase column Solid-Phase Extraction in order to extract the pigments, proteins and particles (Strata® C18-E (55 μm, 70 Å). For this, 200 mg were dissolved in 1 mL of water and the column was washed with 1 mL of water and 1 mL of methanol, in case any remaining materials were retained. After that, the filtrate was lyophilized.

Afterwards, the sugar analysis was performed by high performance anion exchange chromatography (HPAEC-PAD) using a Dionex ICS-5000 HPLC system equipped with a pulse amperometric detector (PAD). A Dionex CarboPac MA1 exchange analytical column (4 × 250 mm) equipped with a guard column Dionex CarboPac MA1 (4 × 50 mm) was equilibrated with 450 mM NaOH at a flow rate of 0.4 mL/min at room temperature. The chromatographic analysis was registered using the program Chromeleon (c) Dionex, Version 7.2.4.8179 (Thermo Scientific).

The identification and quantification of carbohydrates was performed by comparing the retention times with internal standards of glucose, galactose, arabinose, xylose, mannose, rhamnose, fructose, mannitol, inositol, sorbitol, lactose, saccharose, maltose, arabitol, cellobiose, xylitol, galactitol and fucose (Sigma). Dilutions of known concentrations of carbohydrates were used as control for all assays.

3. Results and discussion

3.1. Planning

3.1.1. Evaluation of fungal growth and xylanase production

The incorporation of new natural carbon sources in fungal culture media requires a broad analysis because it is necessary to obtain basic information about the substrate to determine if it is feasible its use. Based on considerations mentioned above, it was tested whether *S. argentinensis* is an adequate substrate for the growth and production of EX by the fungal species *A. niger* and *T. lanuginosus*. Fig. 1 shows the effect of the leaf type (green or senescent) of *S. argentinensis* on the enzymatic and protein production during SmF at 30 °C. For both cases, the EX production is viable, using green or senescent leaves of *S. argentinensis*. Nevertheless, the production profile of EX indicates that the adaptation period for *A. niger* was shorter than two days, when the activity increased reaching its maximum (3.2 U/mL), while the *T. lanuginosus* species did so on the third day (2.7 U/mL). This behavior can be explained based on the colonization strategies of the microorganisms in a particular environment. *A. niger* appears to have adopted a strategy in which much of its metabolic energy is used in reproduction to occupy the environment as quickly as possible, regardless of the high nutrient content. The fungi that follow this behavior are characterized by an easy adaptation to environmental stress and the low availability of nutrients (Rojas Barreto and Hormaza Anaguano, 2014).

The proteins produced increase when the fermentation time increases. The enzymatic activities decreased after the maximum fermentation day. In some cases, this may be attributed to the production of other enzymes, such as proteases, which may generate enzyme degradation. However, no protease activity was detected in the enzymatic extracts. Products from xylanase and other hydrolase enzyme activities, for example, xylose, glucose, xylitol and ethanol are predicted to inhibit hydrolase (Maulana Hidayatullah et al., 2020). Otherwise, the decrease in enzymatic activity is also related to the regulation of the metabolic pathways involved in the production of hydrolytic enzymes.

The chemical composition of *S. argentinensis* biomass is different for both types of leaves. The green leaves contain hemicellulose 32.3%, cellulose 37.9% and lignin 7.22%. The senescent leaves contain hemicellulose 23.1%, cellulose 47.5% and lignin 7.89% (Larran et al., 2015). The lignin content between green and senescent *S. argentinensis* leaves is similar, so the difference in lignin content would not be affecting the enzymatic production when using either type of leaf, although the presence of lignin could interfere with the accessibility of cellulose and hemicellulose. So, it was of interest of this work, to determine how both factors (composition and structure) affect fungal enzyme production. For both, *A. niger* and *T. lanuginosus,* better yields were obtained when using green leaves as the sole carbon source, probably because the green

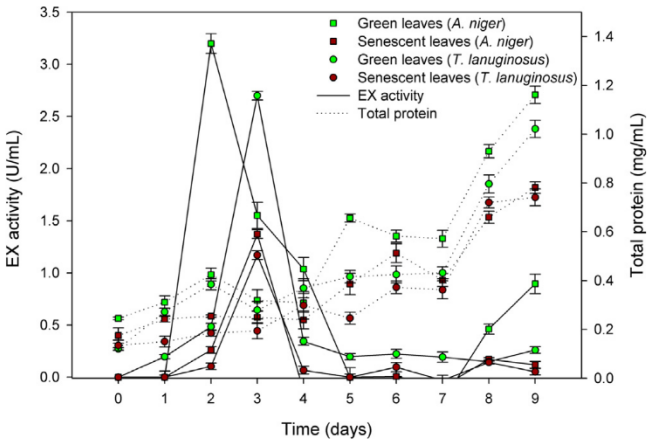

Fig. 1. Production of EX by *A. niger* and *T. lanuginosus* from green and senescent leaves of *S. argentinensis* during SmF at 30 °C and 1.00 × 10^6 conidia/mL final concentration.. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

leaves of *S. argentinensis* contain a higher concentration of hemicelluloses than the senescent leaves, and consequently a higher concentration of xylan (Amore et al., 2015).

The optimal growth temperature for *A. niger* is 30 ºC and for *T. lanuginosus* is 50ºC. However, the microorganisms *A. niger* and *T. lanuginosus* are capable of producing biomass and metabolites when the temperature is set in the ranges of 25-45 °C and 25-60 °C, respectively (Jiang et al., 2005). Although fungal growth and the production of a specific enzyme are related, they are two different processes, so the temperature that allows efficient fungal growth does not guarantee an optimal enzyme production. Therefore, for both strains, SSF was tested at two different temperatures (30 and 50 °C) where green leaves of *S. argentinensis* were used as a substrate.

Fig. 2 A shows that for *T. lanuginosus*, maximal EX activity was achieved on the third day of fermentation for both temperatures, however the activity at 30 °C is higher as that reached at 50ºC under the conditions assayed. On the other hand (Fig. 2 B), the species of *A. niger* also presented the maximal EX activity at 30 °C. Fernández Islas (2012) has already reported a similar pattern of EX production by *A. niger* in SSF using oat and wheat bran as substrates at 28, 36 and 45 °C, and the maximum EX activity was reached at the lowest temperature analyzed (Fernández Islas, 2012). The maximal production profiles at 30 °C with both species represents a practical advantage to operate at laboratory scale and a decrease in the energy cost of the fermentation, therefore in this work, this temperature was standardized for the growth of both strains.

As indicated above, the use of the xylanolytic complex can have important applications not only as bleaching of cellulose pulp, allowing a process with lower cost and lower environmental impact, but also in the food or animal industry. However, an efficient application of this enzyme needs that the enzymatic extract must be free of EG activity. For this reason, the joint production of EX and EG in both types of fermentation was evaluated. Figure 1S shows EX/EG activity ratio obtained at 30 °C in a SmF culture by *A. niger* using green leaves of *S. argentinensis* as a substitute for a carbon source. The highest EX/EG activity ratio (more than three times) was obtained in the second day of fermentation. In previous work, the highest ratio of EX to EG was obtained when *A. niger* was grown in the presence of a by-product obtained from the processing of wheat flour in SmF on the seventh day (Taddia et al., 2019).

3.1.2. Screening

From preliminary experiments, defined factors were included in the experimental statistical design. The goal from this step was to decide which of these had a significant effect on the EX or EG activity from fungal extracts of *A. niger* and *T. lanuginosus*. Three factors were analyzed using a fractional factorial design in two blocks, each given by a fungal species. The factors evaluated were the type of leaves (TL), conidia final concentration conidia/mL (CC) and type of fermentation (SmF or SSF), while the responses analyzed were EX and EG activities. Table S 1 shows the treatments matrix of fractional factorial design and the responses analyzed, while Table S 2 presents the significance of each factor in each response model, estimated by the analysis of the variance test (ANOVA).

For the EX activity response, the *F*-value of the model is 32.58, indicating that it was significant and there is only a 3% probability that the variations in the responses obtained were due to instrumental noise. At first glance, run #3 indicates that the highest activity value (1636.5 U) was obtained using a solid medium with G leaves as a carbon source, and a CC of 10^5 conidia/mL of *A. niger*. In contrast, run #7 shows that the same medium but inoculated with *T. lanuginosus* produced a response forty times minor. The type of leaves and the interaction between the type of leaves and conidial concentration were significant factors for this model, since the estimated *p*-values, at a confidence interval of 95%, were

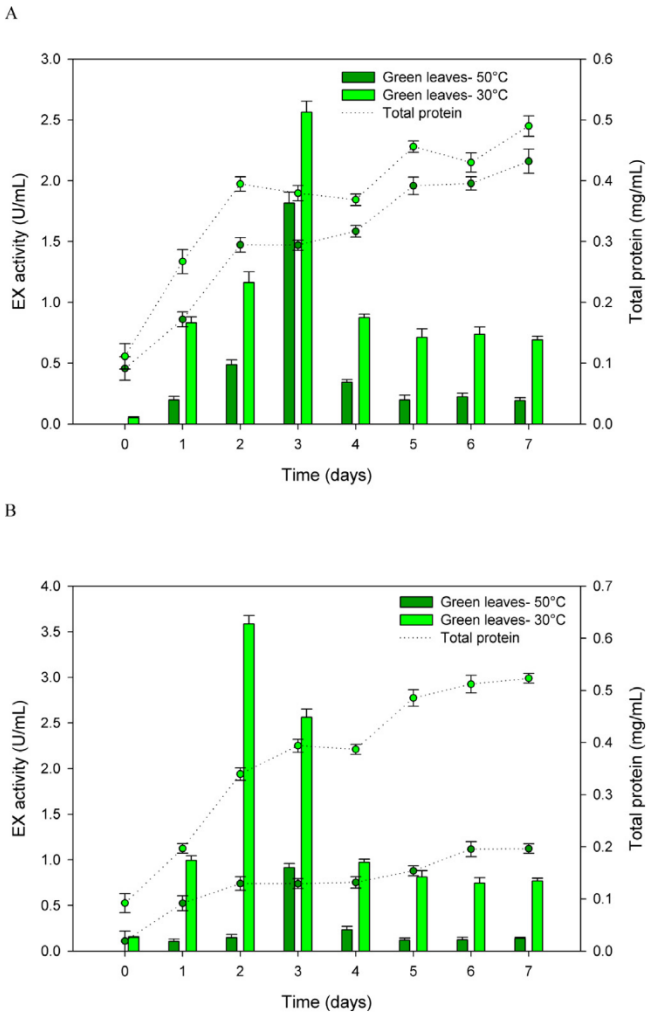

Fig. 2. Production of EX from green leaves of *S. argentinensis* during SSF at 1.00 × 10^6 conidia/mL and 30° and 50 °C by A - *T. lanuginosus* and- B- *A. niger*.. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

minor than 0.05 (in bold in Table S 2). The rest of the double interactions of the factors were not significant (p-values > 0.1).

When analyzing the effect of the type of fermentation on the response, it was observed that it was not significant. However, for practical reasons, it is convenient to choose one type of fermentation over the other. Consistent with previously reported results (Taddia et al., 2019), a morphological difference of growth was evidenced for the two fermentative systems. In SmF both species grew as 'pellet' while developing as dispersed mycelium in the SSF. These dissimilar morphologies suggest that the growth physiology and metabolism in each fermentation system can be very different. Furthermore, as mentioned above, the excretion of hydrolases occurs at the end of the hypha, therefore it is here where the nutrients present in the culture medium are assimilated. So, the presence of oxygen and an aerial vegetative body in the SSF, or the dilution of the hydrolases in SmF, can define the efficiency of the enzymatic production when scaling the process. Based on the description above, it was decided to continue the experimental design using only SmF as the type of fermentation.

It is interesting to note that the blocking effect was significant (p- value = 0.008), which suggests that the use of one microorganism or the other influences significantly when it is desired to maximize the enzymatic production. In this case, *A. niger* is the blocking factor that has a positive effect on the response.

Finally, it was observed that the different substrates used as a carbon source (green and senescent leaves) represent one of the factors that most influence the production of the lignocellulolytic enzymes analyzed, based in the composition and morphological structure of green and senescent leaves (Larran et al., 2015). The use of green and senescent leaves of S. argentinensis in the preliminary evaluation of this work, indicated that both microorganisms could grow using it as a carbon source, detecting differences in the values of enzymatic activities obtained when using different types of

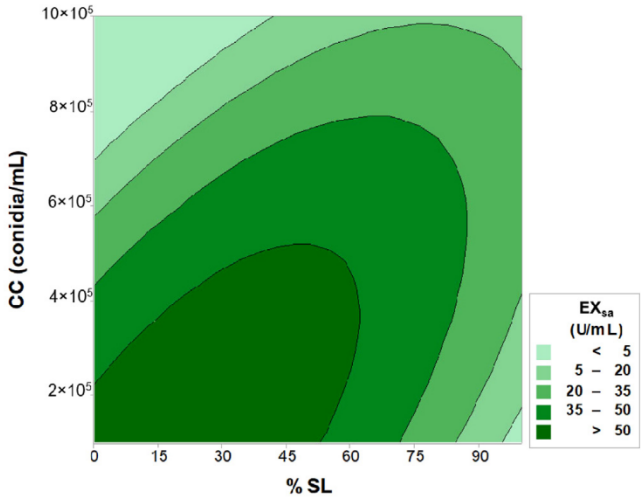

Fig. 3. Contour plot of the effects of the %SL and CC on the specific activity response in the xylanolytic production (EX_{sa}) of *A. niger*.

leaves. As was indicated before. The content of cellulose and lignin is higher in senescent leaves, while the hemicellulose content is higher in green leaves. Larran et al. observed a significant change of cell wall composition at advanced stages. Lignin content may vary because of multiple factors such as cell tissue, cell stage, environmental conditions and plant age (Larran et al., 2015). The results observed in the enzyme production systems using green and senescent leaves support the hypothesis that not only the contents of lignin, hemicellulose and cellulose, but also the differential cross-linking between them, may influence the enzymatic degradation of the biomass. Lignocellulosic materials have a complex chemical structure that hinders their degradation. Thus, the TL factor intended to cover not only the accessibility to the hemicellulose but also its differences in amount. In this sense, the state of foliar senescence and the bioavailability combined with the amount of conidia inoculated presented significant effects for the model on the xylanolytic production. Therefore, in the next step of optimization, it was decided to experiment with mixtures of green and senescent leaves as a carbon source.

On the other hand, for the EG activity response, the model was statistically not significant (p-values > 0.05), as it can be observed in the ANOVA test in Table S 2. Therefore, endoglucanase activity was not included as a response to the optimization strategy.

In conclusion, based on the analyzed results, *A. niger* was selected as the best enzyme producer, and the TL and CC factors were used to design an optimization strategy for the fungal production of EX.

3.1.3. Optimization

To determine the optimal levels of the significant factors that produce a better EX activity, a response surface methodology through a central composite design in a single block was proposed. Table S 3 summarizes the factors and levels used in each one of them, and the matrix of the designed treatments with the responses obtained. According to the discussion in the previous step, the TL factor was transformed into a new factor, which is a combination of green and senescent leaves in different proportions, and defined as the percentage of S leaves (%SL). The other factor, CC (conidia/mL), was not modified. Fig. 3 shows the effect of the interaction of %SL and CC factors, according to the specific activity response in the xylanolytic production (EX_{sa}) of *A. niger*. The maximum EX_{sa} levels (greater than 50) are represented in the lower left margin of the plot (darkest color region), where it occurs the interaction between low values of CC (less than 4×10^5 conidia/mL) and %SL minor that 45%. This means that the highest response values are achieved in a relatively wide experimental domain, suggesting that the process is robust, i.e., the control factors can be adjusted easily according to the potential scaling requirements to obtain acceptable responses.

Table 1A shows the ANOVA test for the EX_{sa} response. The F-value of the model is 6.13, indicating that the model is statistically significant and that there is only a probability of less than 0.05 that the value of F is due to experimental noise. In addition, the terms CC, %SL×%SL, and %SL×CC are significant terms of the model. According to the response surface regression procedure, the response can be modeled by a polynomial Eq. (2), according to:

$$EXsa = 57.4 + 0.31\ \%SL - 1.4 \times 10^{-5}CC - 1 \times 10^{-2}\%SL \times \%SL - 1 \times 10^{-7}CC \times CC + 1 \times 10^{-6}\%SL \times CC \quad (2)$$

Table 1B shows the ANOVA test for the EX/EG response. In this case, the model was significant (F-value = 13.96); there is only a probability of less than 0.001 that the value of F is due to experimental noise. The terms linear and square corresponding to the CC factor, the square term related to the %SL factor and the CC×%SL term were significant.

Table 1A
ANOVA test for the factors and their interactions that affect the xylanolytic specific activity according to the RSM.

Source of Variation	DF	SS	SM	*F*	*P*
Model	**5**	**4023.19**	**804.64**	**6.13**	**0.017**
Linear	2	1729.39	864.70	6.59	0.025
%SL	1	0.48	0.48	0.00	0.953
CC	**1**	**1728.91**	**1728.91**	**13.17**	**0.008**
Square	2	1464.99	732.49	5.58	0.036
% SL × % SL	**1**	**1098.32**	**1098.32**	**8.37**	**0.023**
CC × CC	1	543.25	543.25	4.14	0.081
Double interactions	1	828.81	828.81	6.31	0.040
% SL × CC	**1**	**828.81**	**828.81**	**6.31**	**0.040**
Error	7	918.73	131.25		
Lack of fit	3	137.19	45.73	0.23	0.869
Pure error	4	781.54	195.38		
Total	12	4941.92			

DF: degrees of freedom; SS: sum of squares; MS: mean squares.
%SL: percentage of senescent leaves.
CC: conidia concentration.

Table 1B
ANOVA for the factors and interactions that affect the response variable EX/EG in SmF of the experimental design.

Source of Variation	DF	SS	SSM	*F*	*P*
Model	**5**	**24.2486**	**4.8497**	**13.96**	**0.002**
Linear	2	12.4509	6.2254	17.91	0.002
%SL	1	0.0478	0.0478	0.14	0.722
CC	**1**	**12.4031**	**12.4031**	**35.69**	**0.001**
Square	2	6.7276	3.3638	9.68	0.010
% SL × % SL	**1**	**5.1558**	**5.1558**	**14.84**	**0.006**
CC × CC	**1**	**2.3691**	**2.3691**	**6.82**	**0.035**
Double interactions	1	5.0702	5.0702	14.59	0.007
% SL × CC	**1**	**5.0702**	**5.0702**	**14.59**	**0.007**
Error	7	2.4326	0.3475		
Lack of fit	3	1.0793	0.3598	1.06	0.458
Pure error	4	1.3533	0.3383		
Total	12	26.681			

DF: degrees of freedom; SS: sum of squares; MS: mean squares.
%SL: percentage of senescent leaves.
CC: conidia concentration.

According to the values presented in the ANOVA test, the following fitting expression to the EX/EG response (Eq. (3)) can be established:

$$\frac{EX}{EG} = 5.1 + 1.6 \times 10^{-2}\%SL - 3 \times 10^{-6}CC - 6.9 \times 10^{-4}\%SL \times \%SL - 1 \times 10^{-7}CC \times CC + 1 \times 10^{-7}\%SL \times CC \quad (3)$$

This equation can be used as a starting point for scaling, as well as a tool to test the robustness of the process by making predictions by interpolation. The response surface that models the interaction between %SL and CC as a function of the EX/EG response is shown in Fig. 4a. Fig. 4b shows that the effect of the interaction of the %SL and CC factors on the EX/EG response was similar to the one observed in Fig. 3 where the same interaction was studied on the EX specific activity response. The highest levels of the EX/EG responses (greater than 4) are also in the region delimited by the low values of CC (less than 4×10^5 conidia/mL) and %SL minor that 45%. Once more, the relatively wide experimental domain will make it possible to set the control factors according to the potential scaling requirements to achieve adequately EX/EG responses (see Table 2. Finally, Fig. 5 shows the three-dimensional plot of the Desirability Function.

3.1.4. Experimental validation of the models

The validation of the statistical model was achieved by performing five independent experiments. The validation of the model allowed to obtain a fungal extract with an EX_{sa} of 61.09 ± 4.17 U/mg and a EX/EG of 4.93 ± 0.27, maximizing the concentration of EX over that of EG five times as expected. Finally, the values of the individual responses optimized according to the desirability criterion ($EX_{sa} = 59.87$ and $EX/EG = 4.99$) were included in the validation ranges reported.

The specific enzymatic activity value achieved in the optimized system corresponds to an activity value of 372.5 U/mL recovered after 4 days in SmF which represents a substantial increment in comparison to the previously reported xylanase activity values, which were in general between 11.6 U/ mL (recovered after 5 days) to 376.81 U/ mL (recovered after 14 days) using spent wash and rice straw by *Aspergillus heteromorphus* (Bajar et al., 2020) or when using sugarcane bagasse

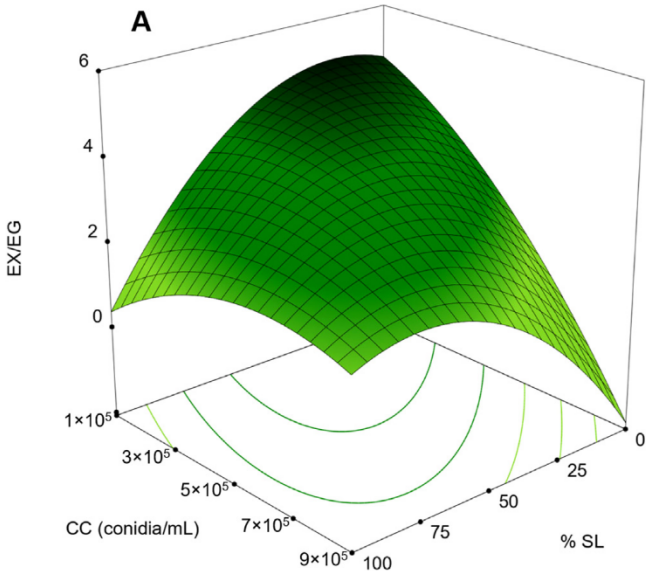

Fig. 4a. Plot of the response surface of EX/EG vs. CC and %SL factors.

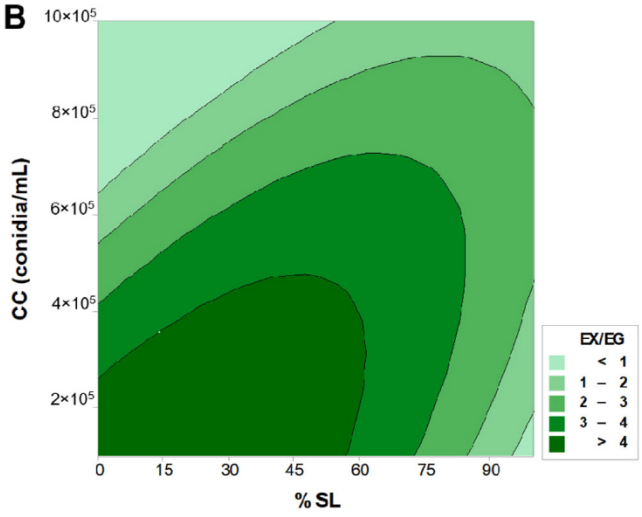

Fig. 4b. Contour plot of the effects of the %SL and CC factors on the EX/EG ratio in the xylanolytic production of *A. niger*.

Table 2
Responses predicted for the optimized system, with the corresponding levels of the factors, according to the desirability function employed by the optimal condition in 100 mL at 150 rpm, 30°C by *A. niger*.

Factors		Responses		Desirability function
%SL	CC (conidia/mL)	EX_{SA} (U/mL)	EX/EG	0.91
20.20	10^5	59.87	4.99	

%SL: percentage of senescent leaves.
CC: conidia concentration.
EX_{SA}: xylanase specific activity.
EX/EG: EX and EG activity ratio.

by *Aspergillus flavus* KUB2 (Namnuch et al., 2021) respectively. Others authors optimized xylanase production by FSS using wheat bran 2919 ± 174 U/gds (IDW) (Khanahmadi et al., 2018) or oil-palm-empty-bunches 3246 U/gds (Ajijolakewu et al., 2017).

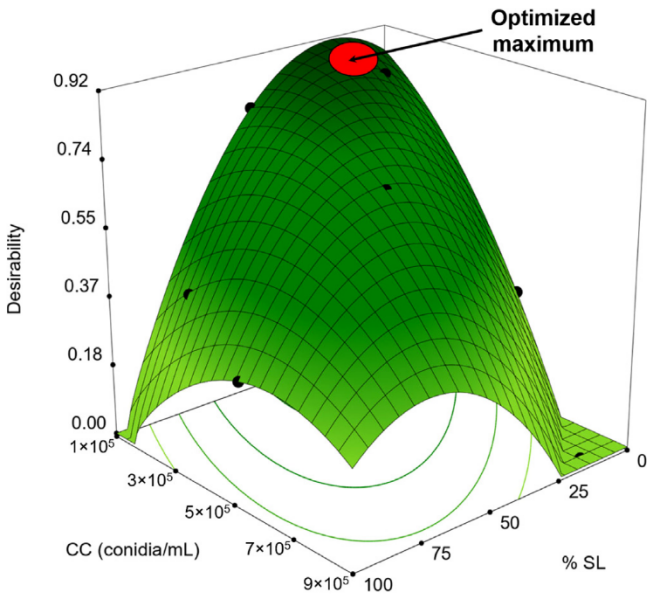

Fig. 5. Three-dimensional plot of the Desirability Function. The black circles indicate the desirability values corresponding to the experiments performed according to the response surface methodology design.

3.2. Mass spectrometry analysis

Optimized enzymatic extracts were analyzed by mass spectrometry. A total of 16 enzymes were detected and successfully identified. The majority of this enzymes have hydrolytic functions, which are involved in the degradation of wall polymers, starch or proteins.

Table 3 summarizes the extracellular and intracellular proteins identified as hydrolytic enzymes involved in the degradation of lignocellulosic substrates. Six enzymes of the xylanolytic complex were found. The endo-1,4-β-xylanase C and B, the main enzymes of the complex, presented a molecular weight of 35.5 and 24 kDa respectively with isoelectric points of 6.65 and 5.45 respectively. Similar results were reported by other authors (Guo et al., 2017; Mangan et al., 2018; Taddia et al., 2020). And the rest of the enzymes of the xylanolytic complex were identified as α-L-arabinofuranosidase axhA and B, α-galactosidase D and exo-1,4-β-xylosidase xlnD. Other enzymes, that do not belong to the cellulolytic complex, were also identified: β-glucosidase A and 1,4-β-D-glucan cellobiohydrolase B.

In this study, two proteins were identified as starch hydrolytic related enzymes: glucoamylase and acid α-amylase. Glucoamylase is the most efficiently secreted protein of *A. niger*, the glucoamylase (glaA) promoter as well as the signal sequence are widely used for heterologous protein production (Lu et al., 2010). Also, the aspartic proteinase that cleaves peptide bounds within the polypeptide chain at acidic pH, aspergillopepsin 1, was identified which indicated *A. niger* has the ability to degrade proteins into small molecular weight peptides by proteases during fermentation (Lu et al., 2010; Shi et al., 2016).

Also, other enzymes were identified as arabinan endo-1,5-α-L-arabinosidase Cis involved in the degradation of pectin and glycan, mannosyl-oligosaccharide-α-1,2-mannosidase 1B is involved in the protein glycosylation pathway, carboxylic ester hydrolase, glucan exo-1,3-β-glucosidase and transmembrane transport. This information indicated that the enzymatic extract of *A. niger* obtained from *S. argentinensis* has a great capacity to hydrolyze hemicellulose and cellulose, which is an advantage when the fungal extract is used for industrial applications, mainly in human and animal food industries.

3.3. Analysis of carbohydrates and enzymatic stability

The presence of small molecules such as sugars or vitamins, also present in the optimized enzymatic extracts, could potentially impact the protein stability and affect its quality (Wang et al., 2014). Reducing sugars are known to form glycation products with proteins via the Maillard reaction during the storage stage, thus changing their stability/activity characteristics and the stability through time is a key factor so as to determine the commercial success of an enzyme product. The sustainability of the stability for a longer period of time is a major challenge with such products since enzymes in solutions can change their conformation resulting in a loss of biological activity. Because of this, the presence of sugars in optimized enzymatic extracts was also studied.

Table 3
Composition of proteins secreted by *Aspergillus niger* NRRL3 determined by nano LC-MS/MS from a mixture of 20.20% senescent and 79.80% green leaves of *S. argentinensis* at 1 x 10^5 conidia/mL.

Accession Code	Protein	% Sequence coverage	Number of peptides	Number of amino acids	Molecular Weight (kDa)	pI	Relative abundance (%)
A2QFV7	endo-1,4-β-xylanase C (xlnC)	8.26	3	327	35.5	6.65	14.28
A2Q7I0	endo-1,4-β-xylanase B (xlnB)	7.56	1	225	24	5.45	3.57
A2R511	α-L-arabinofuranosidase B (abfB)	1.80	1	499	52.5	4.44	3.571
A2QFV9	α-L-arabinofuranosidase axhA (axhA)	5.42	1	332	35.8	4.86	7.14
A2QA27	exo-1,4-β-xylosidase xlnD (xlnD)	1.24	1	804	87.2	4.89	3.57
A2R2S6	α-galactosidase D (aglD)	1.67	1	660	71.2	4.83	3.57
A2RAL4	β-glucosidase A (bglA)	4.30	3	860	93.2	4.78	10.71
A2QAI7	1,4-β-D-glucancellobiohydrolase B (cbhB)	2.05	1	536	56.2	4.30	3.57
A2QHE1	glucoamylase (glaA)	3.75	2	640	68.3	4.45	7.14
A2QW02	acid α-amylase (An11g03340)	2.97	1	505	55.2	4.46	3.57
A2R3L3	aspergillopepsin-1 (pepA)	13.45	4	394	41.3	4.67	14.28
A5AAG2	arabinan endo-1,5-α-L-arabinosidase C (abnC)	5.03	1	318	34	4.87	3.57
A2QAS2	mannosyl-oligosaccharide-α-1,2-mannosidase 1B (mns1B)	6.24	3	513	55.9	5.05	10.71
A2R1N7	carboxylicesterhydrolase (An13g01880)	1.78	1	562	61.2	5.20	3.57
A2QV29	Aspergillus niger contig An10c0050 (An10g00800)	2.46	1	407	43.5	5.03	3.57
A2QAR3	Aspergillus niger contig An01c0370 (n01g12450)	1.17	1	941	98.7	4.70	3.57

Table 4
Carbohydrate analysis (neutral monosaccharides only) of optimized enzymatic extract employing sulfuric acid hydrolysis–gas chromatography.

Monosaccharides	Peak n°	µg/mg of sample
Galactose	10	1.566
Xylose	9	0.425
Glucose	9	0.544
Arabinose	8	0.769
Mannitol	7	0.162
Sorbitol	6	0.160
Xylitol	3	0.631

A qualitative and quantitative screening study was performed to detect the presence/absence of different sugars in the optimized enzymatic extract. Figure 2S shows the chromatogram of the monosaccharides from the optimized enzymatic extract. The 18 particular carbohydrates analyzed were glucose, galactose, arabinose, xylose, mannose, rhamnose, fructose, mannitol, inositol, sorbitol, lactose, saccharose, maltose, arabitol, cellobiose, xylitol, galactitol and fucose.

HPLC was used to elucidate the different saccharides present in the sample and the chromatograms obtained were examined. Different peaks were observed in the optimized enzymatic extract analysis with significant amounts of monosaccharides. The seven peaks obtained were identified as xylitol, sorbitol, mannitol, arabinose, glucose, xylose and galactose respectively, ordered according to their retention time (Table 4). These results show that the optimized enzymatic extract is a source of several valuable monosaccharides.

Although the optimized enzymatic extract contains only aspergillopepsin 1, which cleaves peptide bounds at acidic pH, the attack by proteases will not damage enzymes due to the optimized enzymatic extract did not contain protease activity. The stability of the xylanase activity from the optimized enzymatic extract (control) and in the optimized enzymatic extract free of saccharides were analyzed for 30 days. In both cases, it was observed that the enzymatic extract maintained 100% its activity after 30 days at 20 °C like other *A. niger* extracts using lignocellulosic biomass (Taddia et al., 2019). This suggests that the presence of saccharides does not affect the enzymatic activity.

4. Future prospective

The interrelationship between economy and the environment leads to a change in the model of development and growth that allows to optimize the use of available resources, materials and products in a new paradigm of "circular economy". It is included in this concept the use of biomass materials of lignocellulosic base considered as renewable resources and optimal substrates for the growth of microorganisms as a carbon and energy source.

Microorganisms are the major sources of enzymes. The employment of microorganisms, especially filamentous fungi, presents advantages that are framed in a model of sustainable production based on a circular economy. The optimization of the fermentation process using biomass materials of lignocellulosic base, as *S. argeninesis*, and filamentous fungi, like *A. niger*, enables the obtention of a cocktail enriched in hydrolytic enzymes. Certainly, in the continuation of this research, certain steps of the scale-up processes could be optimized, to obtain a cocktail in which the synergic action of the enzymes make it suitable for diverse industrial applications such as animal feed production, clarification of beverages, enzymatic detergents or the elaboration of bakery goods.

5. Conclusions

The election of the carbon source for fungal growth, a natural substrate with no commercial value such as *S. argentinensis*, gives the proposed strategy an additional advantage over traditional substrates due to the fact that it allows the reduction of costs generated. The optimal conditions for the production of xylanolytic complex enzymes were determined from *A. niger* with very good specific activity (EX_{sa} 61.09 U/mg) and a five times higher ratio compared to the enzymes of the cellulolytic complex. This demonstrates that the great potential of the design that leads to the production of EX by *A. niger* is considered an economically attractive and notable contribution to the industry. The enzyme was characterized and identified by nano LC-MS/MS as endo-1,4-β-xylanase C and B.

CRediT authorship contribution statement

Carlos Martín Pellieri: Conceptualization, Methodology, Investigation, Validation. **Antonela Taddia:** Investigation, Methodology, Validation, Writing – original draft, Writing– review & editing. **Dana Belén Loureiro:** Investigation, Methodology. **Santiago Andrés Bortolato:** Conceptualization, Investigation, Supervision, Writing – original draft, Writing– review & editing. **Gisela Tubio:** Conceptualization, Investigation, Resources, Funding acquisition, Supervision, Writing – original draft, Writing– review & editing.

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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Appendix A. Supplementary data

Supplementary material related to this article can be found online at https://doi.org/10.1016/j.eti.2022.102298.

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