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https://doi.org/10.17113/ftb.61.04.23.8073 original scientific paper

Xylanase Production by Solid-State Fermentation for the Extraction of Xylooligosaccharides from Soybean Hulls§

Running head: Xylanase Production Using SSF for the Extraction of XOS from Soybean Hulls

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Received: 2 January 2023
Accepted: 26 July 2023

SUMMARY

Research background. The development of a novel process for the production of xylooligosaccharides based on the 4R concept is made possible by the integration of numerous techniques, especially enzymatic modification, together with the physical pretreatment of renewable materials. This study aims to integrate the use of agricultural wastes for the production of xylanase by a new strain of *Penicillium* sp. and value-added products, xylooligosaccharides.

Experimental approach. To produce xylanase, a solid-state fermentation was performed using wheat bran as substrate. In order to obtain the most active xylanase crude extract, the time frame of the cultivation process was first adjusted. Then, the downstream process for xylanase purification was

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§Paper was presented at the 10th International Congress of Food Technologists, Biotechnologists and Nutritionists, 30 November-2 December 2022, Zagreb, Croatia

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developed by combining different membrane separation units with size exclusion chromatography. Further characterization included determination of the optimal pH and temperature, determination of the molecular mass of the purified xylanase and analysis of kinetic parameters. Subsequently, the hydrolytic ability of the partially purified xylanase in the hydrolysis of alkali-extracted hemicellulose from soybean hulls was investigated.

Results and conclusions. Our results show that *Penicillium rubens* produced extracellular xylanase at a yield of 21 U/g during solid-state fermentation. Using two ultrafiltration membranes of 10 and 3 kDa in combination with size exclusion chromatography, a 49 % yield and 13-fold xylanase purification was achieved. The purified xylanase (35 kDa) cleaved linear bonds β-(1→4) in beechwood xylan at a maximum rate of 0.64 μmol/(min·mg) and a Michaelis constant of 44 mg/mL. At pH=6 and 45 °C, the purified xylanase showed its maximum activity. The xylanase produced showed a high ability to hydrolyze the hemicellulose fraction isolated from soybean hulls, as confirmed by thin-layer chromatography. In the hydrothermally pretreated hemicellulose hydrolysate, the content of XOS with different degrees of polymerization was detected, while in the non-pretreated hemicellulose hydrolysate, the content of xylotriose and glucose was confirmed.

Novelty and scientific contribution. Future research focused on creating new enzymatic pathways for use in processes to convert renewable materials into value-added products can draw on our findings.

Keywords: soybean hulls; xylanase; *Penicillium rubens*; xylooligosaccharides; renewable materials; solid-state fermentation

INTRODUCTION

The plant tissue contains a high concentration of biologically active components, which are often included in pharmaceutical, cosmetic, and food products. Biologically active components are stored in plant tissues in intracellular membranes and the extracellular matrix (*1*). Although the global industry offers a plethora of biobased products and ingredients derived from plant biomass, oligosaccharides stand out because of all the beneficial effects that have been noted with them. Since oligosaccharides have all the properties of functional food supplements and are considered functional foods with therapeutic potential, the food industry is particularly interested in them (*2*). The fact that prebiotic oligosaccharides will reach a market value of $7.37 billion by 2023 shows that oligosaccharides have made the greatest scientific progress, which has promoted their commercial production (*2*,*3*). Nowadays, xylooligosaccharides (XOS), sugar oligomers composed of xylose

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monomer units linked by β-(1→4) glycosidic bonds, are in particular focus (*3*). Due to their technological and nutritional properties, they are used in the food, pharmaceutical and agricultural industries*, e.g.* to improve dietary fiber content, viscosity, and color intensification to give food a firmer and stickier texture; as antioxidants, cholesterol-lowering, antihyperglycemic, in obesity, to balance the intestinal microbiota, and in severe constipation in pharmaceutical applications; and in agriculture for dietary supplementation (*4*). Extraction of XOS from plant tissue is an extremely challenging and complicated multistep process because the structure of the plant cell is quite complex and consists of cellulose, hemicellulose, and lignin fractions that are intertwined. Hemicellulose is the second most abundant polysaccharide whose basic building block is xylan, which consists of β-1,4-linked D-xylopyranose units in a linear framework, with branched substituents such as arabinose, acetic acid, uronic acid, ferulic acid, and coumaric acid observed at certain points in the structure (*5*). The conversion of xylan from agroindustrial residues to value-added xylooligosaccharides is considered a sustainable process. These processes for obtaining xylooligosaccharides are mainly two-step and consist of physical and chemical pretreatments of the lignocellulosic fraction aimed at reducing the complexity of the structure and partial solubilization of xylan, and xylanase catalyzed hydrolysis of resulting products to XOS with different degrees of polymerization. The xylanase preparations can be added directly to the reaction, either immobilized or generated *in situ* by microorganisms (*4*,*6*).

The presence of various substituents and branches in the hemicellulose structure indicates that its hydrolysis and thus recovery of the XOS requires the simultaneous action of various enzymes such as endo-xylanases and xylosidases together with arabinofuranosidases, ferulic acid esterases, uronidases, and others (*5*). Xylanases are a class of hemicellulolytic enzymes that catalyze the degradation of xylan and convert plant biomass into products with commercial yield (*7*). They have gained popularity in industry as they are used in a variety of applications including saccharification of plant biomass, improvement of animal feed quality, production of food, juices and wine, and the natural sweetener xylitol (*5*). The use of xylanases in saccharification and isolation of XOS from plant tissues requires the use of highly selective enzymes whose prices are prohibitive for industrial use. In this context, several studies have recently been conducted to investigate the lucrative production of xylanases using microorganisms, especially fungi, and waste biomass as substrates. For example, Javed *et al.* investigated the possibility of xylanase production in agricultural waste such as wheat bran using the fungus *A. niger* KIBGE-IB36 (*8)*. The xylanolytic potential of endophytic fungi *F. graminearum* isolated from leaves of *Theobroma cacao* was explored for the first time for the production of xylanase (low cellulase levels) when grown on wheat bran as the sole carbon source (*9*). *A. awamori* AFE1 and cassava peel were used for the production of thermally stable, acidophilic,

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and surfactant-tolerant xylanase (*10*). Furthermore, xylanases were produced on solid-state fermentation by *A. niger* CCUG33991 using low-cost agro-industrial residues such as wheat bran, sorghum stover, corn cob, and soybean meal in tray bioreactor (*11*).

Fungi cultivated on solid substrate is a widely accepted approach for the production of a variety of industrially important enzymes, including xylanase, as this cultivation method is very similar to the conditions found in the natural habitats of fungi. For this reason, this type of cultivation provides a high yield of xylanase activity units when the composition of the nutrient substrate and the growth conditions are adequately controlled. Several fungal producers have been controlled to produce commercial xylanases, including *Aspergillus niger*, *Humicola insolens*, *Termonospora fusca*, *Trichoderma reesei*, *Trichoderma longibrachiatum*, and *Trichoderma koningii*. *Penicillium purpurogenum*, *Penicillium janthinellum*, *Penicillium funiculosum*, *Penicillium herquei*, *Penicillium capsulatum*, and *Penicillium crysogenum* were also used for the production of xylanolytic enzymes (*6*).

The objective of this work is to: 1) develop a method for using wheat bran as the sole carbon source for the production of xylanase using *Penicillium rubens*; 2) develop a method for purifying the xylanase produced; 3) thoroughly biochemically characterize the partially purified xylanase; and 4) to verify the activity of the partially purifed xylanase in the biorefinery concept for valorization of agricultural wastes. For this purpose, the performance of the obtained enzyme is tested for the enzymatic extraction of XOS from the alkali-isolated hemicellulose from soybean hulls.

MATERIALS AND METHODS

Substrates and chemicals

Winter wheat of the Serbian variety Simonida was processed at a nearby mill in Čenta (Serbia) to provide wheat bran (protein (*N* x 6.25), (18.03±0.04) % dry mass, ash (3.93±0.05) % dry mass, and cellulose crude fiber (6.87±0.021) % dry mass) for cultivation of *P. rubens* in this study. The company Sojaprotein Bečej, provided soybean hulls for the production of XOS. Standard oligosaccharides for quantitative evaluation of soybean hull hydrolysates (xylose, xylobiose, xylotriose, xylotetraose, and xylopentaose) were purchased from Megazyme (Wicklow, Ireland). Orcinol, agarose, arabinose, fructose, glucose, sucrose, Bradford reagent, bovine serum albumine, n-butanol, Sephadex G-75, gel filtration markers, glycerol, DTT were purchased from Sigma-Aldrich (St. Louis, USA). Beechwood xylan from Carl Roth (Karlsruhe, Germany) was used to test the xylanase activity. Bromophenol blue and Commasie Brilliant Blue R-250 were purchased from Fisher Scientific (Waltham, USA). Sulfuric

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and acetic acid were purchased from Zorka Pharma (Šabac, Serbia) while aceton was purchased from Lach-Ner (Bratislava, Czech Republic).

Screening and identification of xylanase producers

A competent producer of xylanases was selected from an extensive collection of fungal strains belonging to the Department of Biochemical Engineering and Biotechnology of the Faculty of Technology and Metallurgy. The “Quick-DNA Fungal/Bacterial Miniprep Kit” from Zymo Research (Irvine, USA) was used to isolate genomic DNA from fungi. The isolated genomic DNA was then used for gene amplification by polymerase chain reaction (PCR). The internal transcribed spacer region (ITS) of the rRNA gene was amplified with a pair of universal primers (Ecogen, Barcelona, Spain), ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC -3'). PCR amplification was performed in 50 µL of the reaction mixture containing 1 µL of DNA, 25 µL of DreamTaq PCR Master Mix (Thermo Scientific, Waltham, USA), 0.5 µM of each primer, and nuclease-free PCR water supplemented to 50 µL. On the 'T100 Thermal cycler' Biorad™ (BIO-RAD, Hercules, California) PCR reactions were performed according to the following protocol: initial denaturation at 95 °C for 5 min, 30 cycles consisting of denaturation at 95 °C for 30 s, primer hybridization at 55 °C (ITS) and 57 °C (28s rRNA) for 30 s, 72 °C extensions for 60 s, and final extension at 72 °C for 7.5 min. Horizontal electrophoresis (20 min at 100 V) with 2 % agarose was used to determine the PCR product length. The PCR products were purified using the „DNA Clean & Concentrator ™“ kit (Zymo Research, Irvine, USA) and sent to the sequencing service Macrogen (The Netherlands) for sequencing. The sequences of the PCR products were analyzed using the BLAST program on the National Center for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov/).

Production of xylanase from P. rubens growning on wheat bran

Xylanase was prepared by solid-state fermentation (SSF). Fermentation was carried out in 100 mL Erlenmeyer jars containing 5 g of wheat bran. The Erlenmeyer jars filled with wheat bran were tightly sealed with cotton lids before autoclaving at 121 °C and 0.12 MPa for 20 min. The growth medium*, e.g.* wheat bran, was then moistened with 2 mL sterile citrate buffer (0.1 M; pH=5). The fungal isolate was grown on slanted malt agar (malt extract 20 g/L; agar 18 g/L) in an incubator at a temperature of 30 °C. After 72 h of incubation, the fungal isolate was scraped with 10 mL of sterile distilled water containing a few drops of glycerol to prepare a spore suspension. Then, 1 mL of the spore suspension (10^6 spores/mL) obtained by scraping the culture plate as described previously was aseptically added to the growth medium to perform inoculation. Xylanase was recovered from the fermented medium after a 192 h fermentation period at 30 °C. A simple distilled water procedure was

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used to obtain xylanase from fermented wheat bran. Specifically, the fermented medium was mixed with 25 mL of distilled water and incubated for 30 min at 30 °C and 150 rpm on a rotary shaker (KS 4000 I control, IKA, Staufen, Germany). The Thermo Scientific, Waltham, USA, Heraeus™ Fresco™ 17 microcentrifuge was used to separate the crude extract containing xylanase (10 min, 16800 x *g*, 4 °C), which was then stored at - 20 °C until use. The obtained enzyme apparently maintained full activity after storage for 4 weeks.

Analysis of enzymatic activities

DNS used to evaluate xylanase activity was described by Miller (*13*). The amount of reducing sugars released from xylan was measured spectrophotometrically at 540 nm using xylose as a standard. The Unit of enzymatic activity (U) was defined as the amount of enzyme required to release 1 μmol of xylose equivalent per minute under the assay conditions. Enzymatic activity was expressed as enzyme units per gram of substrate (U/g). Specific activities were expressed as enzyme units per milligram of protein (U/mg).

Protein content determination

Protein concentration was determined by the Bradford method *(14*). Commercially available Bradford reagent was mixed with the sample according to the reagent manufacturer's recommendations, and the protein content was calculated based on a standard curve prepared with bovine serum albumin (BSA).

Purification of P. rubens xylanase

Ultrafiltration membrane fractionation

Ultrafiltration was used to initiate the purification process of xylanase from *P. rubens*. Standard polyethylene sulfone membranes (Sartorius AG, Vivaflow® 50, Göttingen, Germany; molecular cut-off of 10 kDa) with a membrane area of 50 cm^2 were loaded with crude xylanase extract using a peristaltic pump (Heidolph Pumpdrive 5206, Schwabach, Germany) until a twentyfold volume reduction of the crude extract was achieved. After fractionation, protein content and xylanase activity were determined in permeate and retentate fractions. The xylanase-rich retentate was additionally concentrated using Amicon® Ultracentrifugal filter units with a molecular weight cut-off of 3 kDa (Merck, Burlington, Massachusetts, USA).

Gel filtration chromatography

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The xylanase-rich sample obtained after concentration using centrifugal filter units was chromatographed on a Sephadex G-75 (15 x 250 mm, Omnifit® Labware column, Sigma Aldrich, St. Louis, USA) pre-equilibrated with 100 mM citrate buffer, pH=6. The column was operated at a flow rate of 0.75 mL/min, and the eluted fractions were collected using a fraction collector (Buchi C-660 Labortechnik AG, Flawil, Switzerland). The xylanase activity and protein conten were determined in each fraction, as described. While the other fractions in which xylanase activity was detected were pooled and freeze-dried (Beta 1-8 freeze-dryer, Martin Christ, GmbH, Osteroide am Harz, Germany), the fraction with the highest xylanase activity was kept for electrophoretic determination of xylanase purity and molecular mass. Gel filtration was used for molecular mass determination of purified xylanase. A mixture of gel filtration protein molecular mass markers, namely lysozyme from egg white (14.0 kDa), carbonic anhydrase from bovine erythrocytes (29.0 kDa), ovalbumin (44.0 kDa), and bovine serum albumin (66.0 kDa) were also separated on the same column under identical conditions, and their elution volumes were determined. A standard plot was then made between V_e/V_o on the x-axis and log MW on the y-axis to calculate the molecular weight of the purified xylanase.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis

Electrophoresis was used to evaluate the efficiency of each step of purification of xylanase. Sample buffers with the following composition: 2.5 mL of 2X Tris-HCl buffer, 2.0 mL of glycerol, 2.0 mL of 1M DTT solution, 4.0 mL of 10 % (*m*/*V*) SDS, and 0.4 mL of 5 % (*m*/*V*) bromophenol blue were mixed with the samples collected at each stage of the xylanase isolation process at a ratio of 1:1 (*V*/*V*) and heated at 95 °C for 5 min. Samples were loaded onto a polyacrilamide gel (stacking: 4 % and resolving: 20 %) along with pre-stained protein ladder standard markers (10-260 kDa), and electrophoresis was performed at 120 V for 75 min in a vertical mini-Hoefer system equipped with an electrophoresis bath and power supply. The gels were impregnated with Coomassie brilliant blue R-250.

Purification parameters

The effectiveness of the purification process was evaluated by analyzing: 1) activity yield - Y, % and 2) purification factor - PF. The following mathematical formulas were used to determine these parameters:

$$\boldsymbol{Activity\ Yield}\ (\boldsymbol{Y}), \% = \frac{Total\ activity\ in\ the\ purification\ phase}{Total\ activity\ in\ the\ crude\ xylanase\ extract} \times 100 \qquad /1/$$

$$\boldsymbol{Purification\ Factor}, \boldsymbol{PF} = \frac{Specific\ activity\ in\ the\ purification\ phase}{Specific\ activity\ in\ the\ crude\ xylanase\ extract} \qquad /2/$$

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Characterization of partially pure P. rubens xylanase

Effect of pH and temperature on the *P. rubens* xylanase activity

The optimal pH of partially pure xylanase was determined by measuring its activity in buffers with different pH values (3-10 in steps of 1 pH unit) such as sodium citrate buffer (100 mM, pH 3-6), sodium phosphate buffer (100 mM, pH 6-8), Tris-HCl (100 mM, pH 8-9), and glycine-NaOH buffer (100 mM, pH 10) at 37 °C. Enzyme activity was also measured at different temperatures (25; 37; 45; 50 and 60 °C) under standard assay conditions to determine the optimum temperature at pH=6.0.

Enzyme kinetics

In evaluating the kinetics of hydrolysis of beechwood xylan with *P. rubens* xylanase, the initial concentration of the substrate was varied (0.25; 0.5; 1.0; 2.0; 3.0; 4.0; and 5.0 % (*m*/*V*)), and the hydrolysis reaction was carried out under optimal conditions for the indicated xylanase at [pH]=6 and [T] = 45 °C. The conventional DNS method as described previously was used to follow the kinetics for each set of experimental conditions. The mathematical model of Michaelis-Menten kinetics included in OriginLab® software package (Origin(Pro), Version 2010, OriginLab Corporation, Northampton, MA, USA) was applied to the experimental data obtained. The degree of agreement between experimentally obtained values and considered mathematical model was evaluated after applying the above mathematical model together with values of kinetic constants K_m and V_m.

P. rubens xylanase-assisted production of xylooligosaccharides from soybean hulls

Hydrolysis of the hemicellulose portion of soybean hulls and production of xylooligosaccharides with different degrees of polymerization were performed using partially pure xylanase produced by *P. rubens*.

Isolation of hemicellulose from soybean hulls for the production of xyloogosaccharides

Soybean hulls were pretreated with a mechanical mill (Mixer Mill MM400, Retsch, Germany). In this process, 1 g of the substrate was ground into smaller particles with a size of 40-60 mesh using a standard ball for 120 s at 25 Hz. Subsequently, 10 g of the substrate powder was resuspended in 100 mL of 1.5 M NaOH (*S*/*L* ratio 1:10), and alkaline extraction of the hemicellulose fraction was performed as described (*15*). Briefly, the suspension was incubated at 80 °C for 60 min. The suspension was then cooled and the solid fraction was removed by centrifugation at 1870 × *g* (Eppendorf™ refrigerated centrifuge 5430 R, Thermo Fisher Scientific, USA) for 10 min. The remaining supernatant (50 mL) was acidified to pH 5.5 with 6 M acetic acid and then cold ethanol (96

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%, 150 mL) was added. After centrifugation 1870 × *g* for 10 min, the precipitate rich in hemicellulose was collected. The resulting precipitate was resuspended in distilled water and precipitated again. The extracted hemicellulose was freeze-dried for 24 h at a critical temperature of - 40 °C in a laboratory freeze dryer (Beta 1-8 Freeze Dryer, Martin Christ, GmbH, Germany).

Enzymatic hydrolysis of extracted hemicellulose from soybean hulls

In this work, the enzymatic hydrolysis of extracted and hydrothermally pretreated hemicellulose with partially pure xylanase from *P. rubens* was studied under the previously defined optimal reaction conditions for specific xylanase. First, a suspension of 100 mg dried hemicellulose in 10 mL citrate buffer (0.1 M; pH=6) was prepared. Also, 100 mg of dried hemicellulose was resuspended in 10 mL of citrate buffer (0.1 M; pH=6) and incubated at 121 °C and 1.3 bar for 1 h to achieve hydrothermal pretreatment (hot water and elevated pressure) of the substrate. Then, the enzyme 10 $U/g_{substrate}$ was added and both reactions were carried out at 45 °C for 5 h under constant mixing at 150 rpm (KS 4000 i control, IKA, Staufen, Germany). At the specified time intervals, samples were taken from the reaction mixtures and incubated at 100 °C (Thermo Scientific™ Digital Heating Shaking Drybath, Waltham, USA) for 5 min to achieve enzyme deactivation, and the xylose concentration was determined by DNS assay.

Qualitative evaluation of soybean hull hemicellulose hydrolysates by thin layer chromatography

Thin layer chromatography using silica plates (L x W - 5 x 10 cm, St. Louis, Sigma Aldrich) was used for qualitative characterization of hydrolysates. The mobile phase for TLC consisted of n-butanol:acetic acid:water (2:1:1). The separation was performed in a TLC chamber B x H (10.5 x 12.5 cm) for 90 min. The plates were then dried in an oven at 90 °C and immersed in the staining solution consisting of 40 mL acetone, 2 mL concentrated sulfuric acid, and 20 mg orcinol. After staining, plates were dried at 90 °C for 5 min to aid in chromatogram development.

Quantitative evaluation of soybean hull hemicellulose hydrolysates by high performance liquid chromatography

Samples were analyzed using Dionex Ultimate 3000, Thermo Scientific (Waltham, USA) HPLC system. Analysis was performed using deionized water as mobile phase with an elution rate of 0.6 mL/min, on carbohydrate column (Hi-Plex Ca^{2+}, 300 mm x 7.7 mm, 8 mm, Agilent, Santa Clara, CA, USA) incubated on 80 °C. The sample injection volume was 20 µL and the analysis time was 25 min. Detection of product was achieved using the RI detector (RefractoMax 520, ERC GmbH,

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Riemerling, Germany) preheated on 40 °C, and all data acquisition and processing was done using Chromeleon 7.2 Software (Thermo Scientific, Waltham, USA). Analytical standards: glucose, fructose, sucrose, arabinose, xylose, xylobiose, xylotriose, xylotetraose, and xylopentaose were used to generate calibration curves. Based on the obtained slope, the quantification of the target component in the obtained mixture was determined.

Statistical analysis

All experiments in this study were performed in triplicate and the results are presented by mean ± SD. One-way analysis of variance ANOVA (Minitab®17 software, Minitab Ltd., Coventry, UK) was used for comparison of the results obtained. The Tukey test was applied for comparing differences between mean values at a confidence interval of 95 % (p<0.05).

RESULTS AND DISCUSSION

Kinetics of xylanase production

Xylanases are extremely important industrial enzymes and are estimated to have a large share of the global enzyme market (*16*). Therefore, a significant amount of xylanases with exceptional properties, such as enzyme activity and stability in difficult process environments, must be readily available for industrial use (*17*). Given the importance of xylanases, data are continuously being collected on the microorganisms that produce them, as well as on the growth conditions and composition of the culture medium, in order to maximize the yield of xylanase enzymes. The Department of Biochemical Engineering and Biotechnology at the Faculty of Technology and Metallurgy has a large and diverse collection of fungal strains; therefore the selection of a competent xylanase producer was considered as the main task of this study. The first step in confirming the potential of the selected strain was to use selective agar plates on which a xylanase enzyme activity index greater than 1.5 cm was observed (*18*). The strain was then identified by sequencing the genes ITS and NL. After amplification of the 28s ribosomal RNA region with a size of 600 bp and sequencing and comparison of the sequences with reference strains deposited in the NCBI database, the strain was found to belong to *Penicillium* spp. and was most likely *Penicillium rubens*. The identified strain was further used for the first time in the study of xylanase production using SSF and wheat bran as substrate.

The best inducer of xylanase synthesis is xylan. However, due to the prohibitive price of xylan, the use of pure xylan as a carbon source for the production of xylanases is considered to be an unattractive approach. Therefore, it has been recognized that using agricultural waste as the sole

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carbon source for xylanase synthesis could be a good solution. Although wheat bran is considered a waste, there is strong evidence that the bran is recognized as a suitable substrate for xylanase production because of its hemicellulose nature (presence of nonstarch polysaccharides, 41 to 60 %), favorable degradability, and the presence of some nutrients (*19,20*). Furthermore, biochemical composition shows that after hydrolysis of wheat bran, significant amounts of soluble sugars such as glucose (42.5 % dry mass), xylose (15.4 % dry mass), arabinose (3.1 % dry mass) and galactose (2.7 % dry mass) are produced, which are necessary to initiate microorganism growth and promote its further growth (*21*). Wheat bran xylans composed of β-D-(1,4)-linked xylopyranosyl skeleton and can be replaced by α-L-arabinofuranosyl as the main group of pendants for O2 and/or O3, making it an appropriate source for the production of xylanase (*22*). For this purpose, *P. rubens* was incubated for 192 h on wheat wheat bran at 30 °C and harvested regularly, and data on the activity of xylanase in crude extracts were collected and shown in Fig. 1.

Fig. 1.

With few exceptions, xylanase production by filamentous fungi occurs in cultures with an initial pH below 7.0 (*23*). Under these fermentation conditions, the xylanase activity titer in the extract increases with increasing fermentation time until it reaches its peak value after 168 h, as shown in Fig. 1. Thus, the maximum xylanase production with *P. rube*ns was measured after 168 h of fermentation and reached a value of 21 U/g of substrate. These results indicate that wheat bran is a suitable substrate for the cultivation of *P. rubens* and the production of xylanase because in addition to cellulose, it contains hemicellulose at high concentrations, which is also recognized as an inducer of xylanase production (*24*). Xylanase synthesis on wheat bran is thought to be significantly affected by unquantified particular trace elements, including minerals, in addition to cellulose and hemicellulose (*25*). Fig. 1 shows a sharp increase in xylanase activity from 12 to 168 h, particularly from 96 to 168 h of fermentation, in addition to a sharp decrease in activity after 168 hours of fermentation. The decrease in the amount of inducer ingested during the earlier growth stages of the microorganism could be the cause of this decrease in activity. In addition, overexposure of lignin through the consumption of xylan increases the likelihood of occurrence of phenolic compounds that inhibit xylanase activity (*25*).

The production of xylanases by various fungal strains using wheat bran as a carbon source in the fermentation process on a solid substrate is well documented in the literature. *Penicillium echinulatum*, for example, produced 10 U/g of xylanase under solid-state conditions using wheat bran (*24*). When cultured under SSF in the presence of wheat bran, *A. niger* XY-1 exhibited remarkable xylanase levels. The xylanase activity reached 14.64 U/g dry substrate after 48 h of fermentation (*26*).

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After optimization of process parameters, wheat bran promoted high xylanase secretion (7.83 U/g dry carbon source) by *H. lanuginosa* in SSF (*27*). Although wheat bran was found to be an excellent substrate for xylanase production, further investigation and data analysis revealed that slight enrichment of the substrate had a significant effect on increasing xylanase yield. The enrichment of wheat bran with a modified inorganic Czapex-Dox medium is one such example. More specifically, wheat bran moistened with a modified inorganic Czapex-Dox medium was used for the production of xylanase using the strain *Rhizopus oryzae* SN5. The maximum yield of xylanase, 273.83 U/gds, was obtained at 30 °C and pH=6.0 after 5 days of incubation (*28*). In addition, *A. niger* NFCCI 4113 was used to produce xylanase using wheat bran as a carbon source and yielded 771.37 U/gds after 144 h of fermentation at 30 °C, pH=5, and an initial moisture content of 75 % (*29*). Similarly, Dobrev *et al.* (2007) observed that the use of wheat bran as a substrate in SSF resulted in good xylanase production (*30*). Wheat bran offers several possibilities for combination with other substrates to increase the yield of the target enzyme, in this case xylanase, and can also be used as the sole carbon source for microbial growth during fermentation. Since the selected strain *P. rubens* and the substrate wheat bran proved to be suitable for the production of xylanase, one of the research directions could be the optimization of the parameters of the fermentation process together with the use of a consortium of microorganisms, which would increase the use of the substrate and thus a better yield of the activity of the enzyme units can be expected (*31*).

Partial purification of xylanase

The potential environmental impact of enormous waste streams in the extraction of enzymes from complex fermentation medium has already been greatly minimized by choosing fermentation on solid-state instead of submerged fermentation for xylanase production. Precipitation with ammonium sulfate or organic solvents is often used as the first step in enzyme purification processes from complex fermentation media. However, if we want to produce enzymes on an industrial scale, we need to consider the purification steps or more precisely, we need to reduce the consumption of chemicals. Therefore, in this study, xylanase produced by culturing the new fungal strain on wheat bran was partially purified using membrane separation techniques and column chromatography. In the first stage of purification protocol, membranes with pore size of 10 kDa were used to concentrate the crude xylanase extract obtained. In this way, two streams, retentate and permeate, are separated. The presence of xylanase in the retentate was detected by DNS activity assay, indicating that the molecular mass was above 10 kDa. From Table 1, which summarizes the purification of xylanase, it can be deduced that the membrane separation procedure chosen can be considered as the first stage

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of purification of the xylanase produced. This is evidenced by the fact that only 5 % of the activity of the preparation was lost after the first purification step, although the purity of the preparation was increased fourfold. In comparison, the use of ammonium sulfate in the first stage of xylanase purification from *A. fumigatus* resulted in an activity yield of 51 %, demonstrating that the membrane technique is superior to the use of ammonium sulfate in the first steps of xylanase purification (26). To simplify chromatography and obtain representative results, the prepared xylanase preparation was then highly concentrated using centrifugal filters (3 kDa) and chromatographed on a Sephadex G-100 gel column.

Table 1.

Fig. 2 shows the chromatogram of partially purified xylanase sample obtained after using membrane separation units, indicating two peaks. However, it was determined which fractions contained xylanase by studying the xylanase activity and protein concentration of each fraction. The activity study showed that xylanase was in fractions F_20 to F_40.

Fig. 2.

Gel filtration chromatography was used for the molecular weight estimation of partially purified xylanase. Based on the elution volumes of the enzyme and standard markers, the molecular mass of xylanase was calculated to be approximately 36 kDa. By observing the electrophoretic mobility (Fig. 2 A) and the positioning of the bands in all samples obtained in all purification steps, this estimate was confirmed. The molecular mass of partially purified xylanase was determined to be 35 kDa based on the collected fractions and the activity and position of the bands in the electrophoregram. The molecular sizes of xylanases isolated from fungi range from negligible to very large; however, the results show good agreement with the molecular sizes of xylanases isolated from *A. oryzae* and *Beauveria bassiana* (37 kDa) (*32*,*33*). The fractions in which xylanase activity was detected were pooled, freeze-dried, and used for further enzyme characterization.

Biochemical characterization of partially pure xylanase

The success of enzyme catalysed reaction determines the charge on the substrate and the arrangement of charged groups on the enzyme. In this context, it is necessary to investigate how pH affects a particular reaction. The effect of pH on partially pure xylanase is presented in Fig. 3. The xylanase activity of *P. rubens* was highest at pH=6 (Fig. 3), but also xylanase showed significant activity at pH 5 and 7, reaching 70 % in this pH range. Since the enzyme retains less than 20 % of its activity at pH values above 8 and below 5, these conditions are not acceptable for the xylanase tested. The results of this study are fully consistent with the data already collected on the pH ranges in which

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fungal-derived xylanases function best. There are a few xylanases that prefer an alkaline environment, but the vast majority of fungal-derived xylanases are acidophilic (*32*).

Fig. 3.

The temperature optimum of partially pure xylanase from *P. rubens* was determined by performing a conventional hydrolysis reaction of xylan from beechwood at different temperatures, and the influence of temperature on xylanase activity is presented on Fig 4. A significant increase in xylanase activity was observed at temperature between 25 and 45 °C (Fig. 4). Xylanase optimum activity was observed at 45 °C. However, it is also very important to emphasize that a high percentage of activity was maintained at temperatures above 45 °C. Xylanase was found to retain 80 % of its activity at a temperature of 50 °C. Further increasing the reaction temperature above 50 °C is not beneficial for the selected xylanase as a significant decrease in activity was observed under these conditions.

Fig. 4.

Similar results were found in other studies on the biochemical characterization of xylanase from *Penicillium* strains: *P. purpurogenum* (pH=7.0, 50°C) (*34*), *P. sclerotiorum* (pH=4.5, 50°C) (*35*), and *P. ocitanis* (50°C) (*36*). In summary, stable xylanases are desirable for various industrial processes, especially those requiring extreme conditions.

Kinetic analysis of partially purified P. rubens xylanase

The kinetic properties of partially pure xylanase were studied by changing the initial concentration of the substrate xylan from beechwood and modeling the experimental data with a mathematical model reflecting Michaelis–Menten kinetics. The data obtained show that the enzyme obeys Michaelis–Menten kinetics (R^2 = 0.9930). In the present study, the kinetic parameters for xylanase produced by *P. rubens* were calculated and it was found that K_m and V_m for beechwood xylan were 43.74 mg/mL and 0.64 μmol/(min·mg), respectively. These values are consistent with the kinetic parameters values of other fungal xylanases, which range from 0.09 to 40.9 mg/mL for K_m, depending on the substrate used. The data obtained in this study show that the partially pure xylanase has a high affinity for the substrate, which is important for the industrial use of the enzyme (*37*).

Production of xylooligosaccharides from soybean hulls using partially purified P. rubens xylanase

Data are constantly being collected on the efficiency of using xylanase in the conversion of lignocellulosic feedstocks as this process alleviates the problem of agricultural waste while providing numerous industrially important products. Documented data on the production of xylooligosaccharides from lignocellulosic materials are mainly related to techniques using commercial xylanases. Therefore, this study investigated the feasibility of using partially pure xylanase from *P.*

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rubens in the hydrolysis of the hemicellulose fraction from soybean hulls extracted by alkaline treatment. In addition, the separated hemicellulose fraction was subjected to high-temperature and pressurized water treatment, known as hydrothermal treatment, to assess whether pretreatment before enzymatic hydrolysis was necessary to maintain or increase the yield of xylooligosaccharides. The DNS method was used to follow the progress of the hydrolysis reaction and the results are expressed as xylose equivalents (Fig. 5a). Then qualitative evaluation of the resulting hydrolysate was performed by thin-layer chromatography (Fig. 5b) to determine the existence of the xylooligosaccharides and the degree of polymerization (DP). Subsequently, the sugar content was identified by HPLC (Table 2).

As can be seen from Fig. 5, the content of reducing sugars expressed as xylose equivalents increases during the hydrolysis of both untreated and hydrothermally treated hemicellulose, indicating the affinity of the partially purified xylanase for the selected substrate. More precisely, in the hydrolysate obtained with pretreated hemicellulose from soybean hulls significant increase ($p < 0.05$) in reducing sugars content was observed (47.36±1.21 mg of reducing sugar per gram of initial biomass) than in the hydrolysate obtained by hydrolysis of non-pretreated hemicellulose from soybean hulls (41.98±0.77 mg of reducing sugar per gram of initial biomass), indicating that the hydrolysis process with pretreatment is preferable in terms of the output of total reducing sugars. The data reported are consistent with previously reported data on the effect of hydrothermal treatment on the extractability of XOS (*6*). Indeed, the hydrothermal treatment led to solubilization and fragmentation of xylan into soluble xylooligomers, which were converted by xylanase into functional XOS with different degrees of polymerization (*6*). Comparing the yield of XOS obtained by two different routes, the process using non-pretreated hemicellulose for XOS production yielded up to 10.62±0.42 mg, while hydrothermal treatment resulted in almost the same yield 10.47±0.34 mg of XOS per gram of starting biomass. Although the XOS content in both hydrolysates are not significantly different ($p<0.05$), the XOS profile differs significantly (Table 2).

Table 2

The profile of reducing sugars in the hydrolysate obtained by hydrolysis of the non-pretreated hemicellulose is characterized by the dominant content of glucose (6.47±0.37 mg of glucose per gram of initial biomass) and xylotriose (10.99±0.11 mg of xylotriose per gram of initial biomass). On the other hand, the hydrolysate obtained by the hydrolysis of hemicellulose previously treated with hot water under high pressure was rich in xylobiose, xylotriose, xylotetraose, and xylopentaose with the highest content of short XOS, xylobiose (4.29±0.08 mg of xylobiose per gram of initial biomass). The combination of hot water and high pressure destroys hemiacetal bonds in hemicellulose, thus

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promoting the extraction of oligosaccharides from hemicellulose or, more specifically, enzymatic hydrolysis (*34*). After deacetylation, special regions in the hemicellulose structure become accessible to the enzyme, which is reflected in the change of XOS profile in the hydrolysate (*6*). Moreover, the presence of arabinose was detected in the hydrolysate obtained after hydrothermal treatment, which was not present in the hydrolysate of hemicellulose that had not been hydrothermally pretreated. It seems that the partially pure xylanase preparation, which has been shown to contain, in addition to xylanase, *α*-L-arabinofuranosidase and *β*-xylosidase (data not shown), contributes to the saccharification of the hemicellulosic fraction of soybean hulls, as evidenced by an increase in the yield and type of monosaccharides such as arabinose and xylose, which have been confirmed to be antiglycemic agents and to have an exceptional impact on human health (*36).* From these results, it can be concluded that the observed process warrants hydrothermal treatment prior to enzymatic hydrolysis.

CONCLUSIONS

Production of xylanase was performed by SSF of wheat bran with *P. rubens*. A purification process based on membrane separation techniques and column chromatography was developed for the enzyme produced, reducing the use of chemicals and environmental impact. The enzyme exhibits optimal activity in a pH range of 5 to 7 and a temperature range of 37 to 50 °C. The hydrolysates prepared by different strategies resulted in XOS with different degrees of polymerization, which was confirmed by thin-layer and HPLC chromatography. Based on the obtained results, hydrolysates containing XOS can be considered as promising mixtures since they contain a high percentage of xylobiose, which is known as a prebiotic. The use of a new xylanase with improved catalytic properties over lignocellulosic substrates could be a viable technique to increase process yield and efficiency and is therefore ideal for inclusion in a global biorefinery plan for soybean hull upgrading.

FUNDING

The authors acknowledge the generous funding support of Ministry of Science, Technological Development and Innovation of the Republic of Serbia within the Projects: Contract No. 451-03-47/2023-01/200135 and Contract No. 451-03-47/2023-01/200287, EUREKA project E! 13082 BIOFLOSBAKE-LAVGLU, and Science Fund of the Republic of Serbia, #GRANT No. 7751519, Multifunctional leaf protein and assembled nanocarrier structures delivered by enzyme technology - MultiPromis.

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CONFLICT OF 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.

AUTHORS' CONTRIBUTION

The authors of the paper have all made a significant scientific contribution to the completion of all experimental plans on the given subject. Specifically, N. Šekuljica was responsible for designing and conducting the research, defining the methodology, analyzing the data, and writing a draft of the publication. S. Jakovetić Tanasković contributed to the critical revision of the original draft and data processing. J. Mijalković was involved in the development of the enzymatic hydrolysis procedure for the recovery of xylooligosaccharides. M. Simović was involved in the development of the method for detection of XOS and analysis of sugar profile in the hydrolysates. N. Pavlović qualitatively characterized hydrolyzates by thin-layer chromatography. I. Gazikalović, J. Bakrač, and N. Luković performed the selection of the producing microorganism. N. Đorđević performed the sequencing and identification of the selected strain. A. Culetu contributed to the conception of the paper. Z. Knežević-Jugović provided resources, conceptualized the research experimental design, and contributed to visualization, monitoring, writing, and editing of the original draft.

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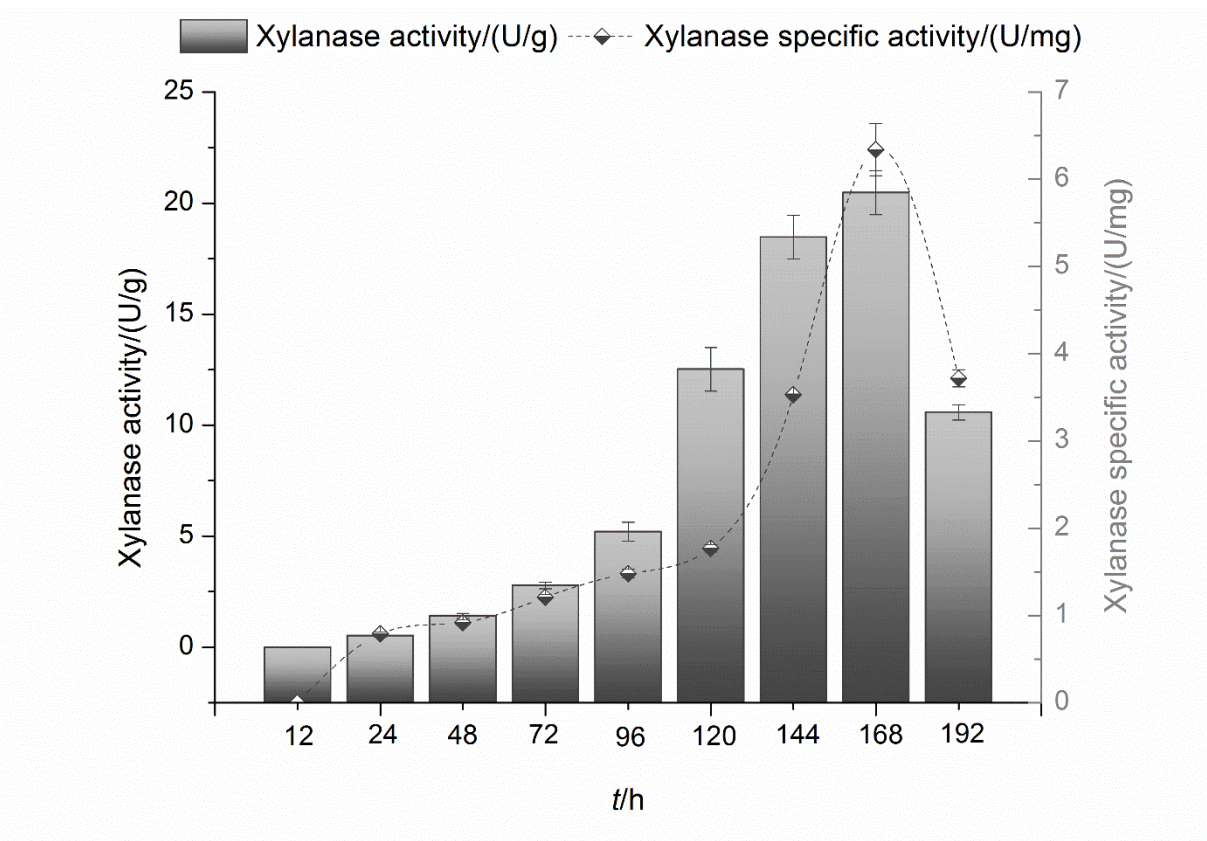

Fig 1. Kinetics of xylanase production by culturing *P. rubens* on wheat bran under the solid state fermentation regime (fermentation conditions: [T] = 30 °C; [pH] = 5.0); each point represents the mean (n = 3) ± SD

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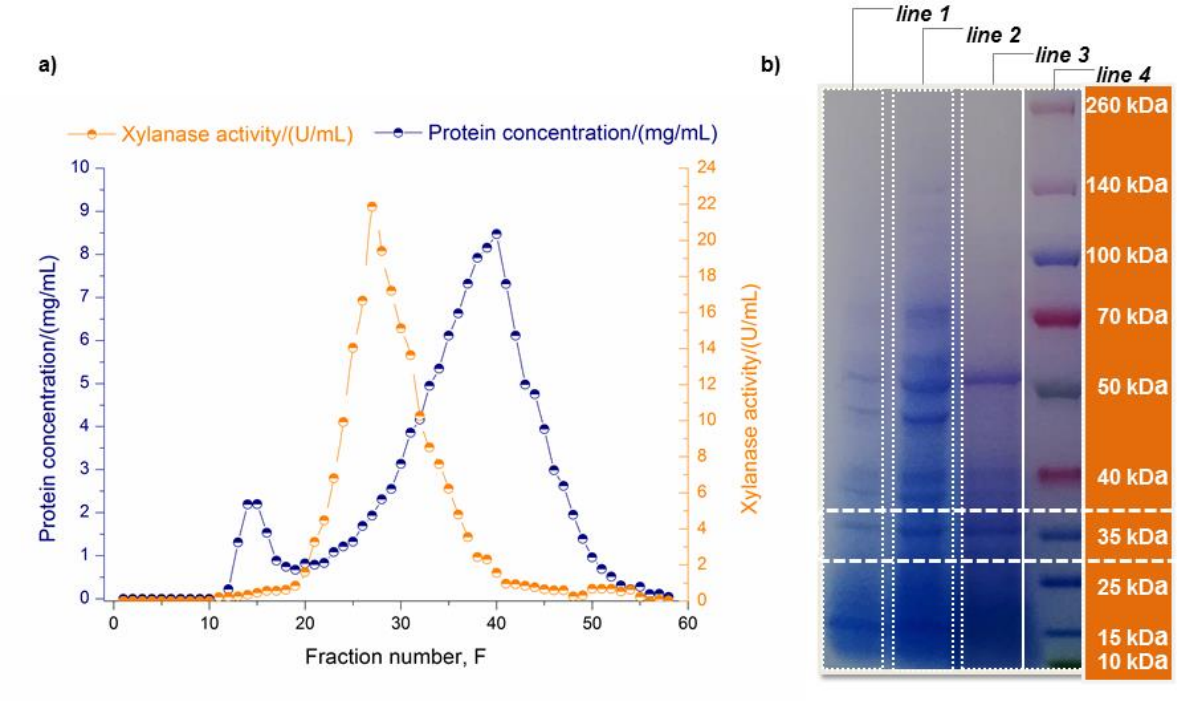

Fig. 2. a) Size elution profile of xylanase; b) An electrophoretic illustration of the various steps in the purification of the xylanase on a 4 % stacking and 20 % resolving poliacrylamide gel: line 1 – crude xylanase extract; line 2 – partially purified xylanase using a membrane with molecular weight cut-off 10 kDa; line 3 – a fraction with maximal xylanase activity collected from size exclusion column; line 4 – molecular weight markers

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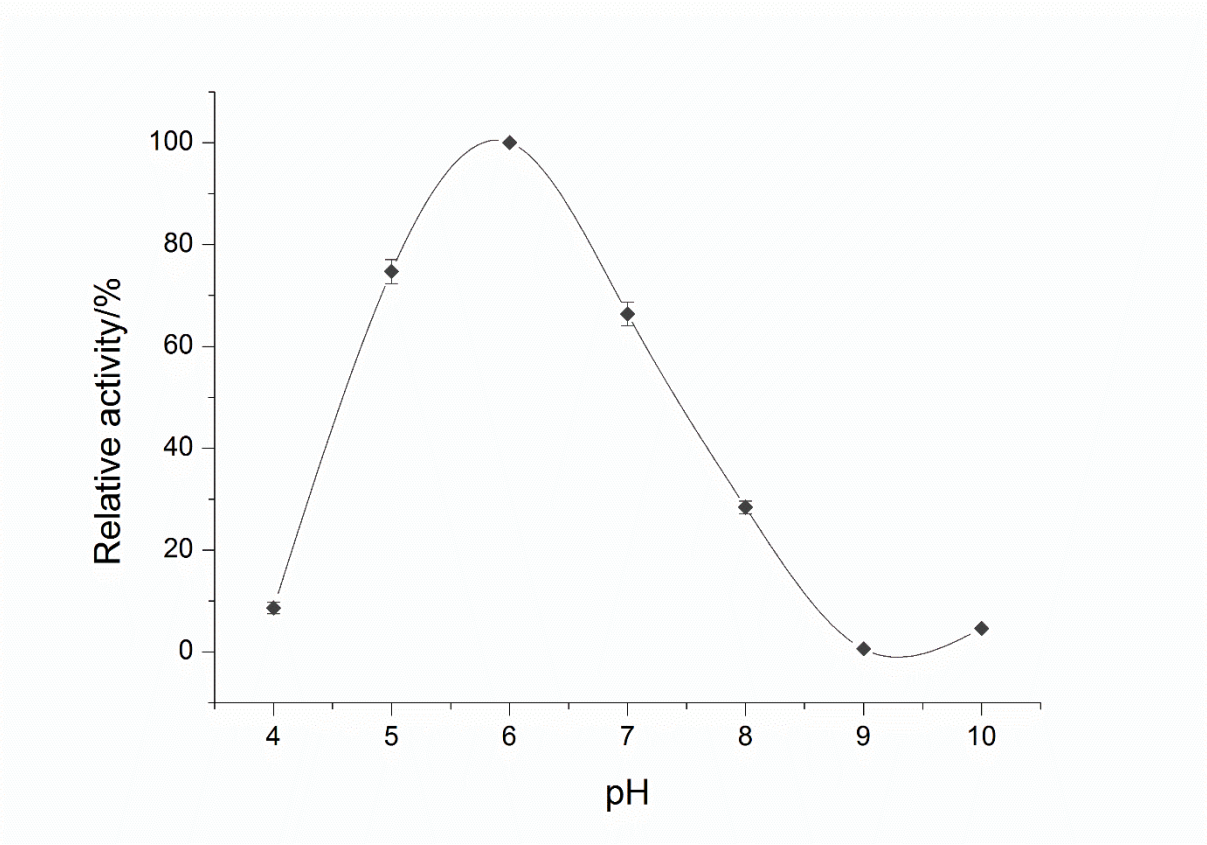

Fig. 3. Effect of pH on the activity of *P. rubens* xylanase; each point represents the mean (n = 3) ± SD

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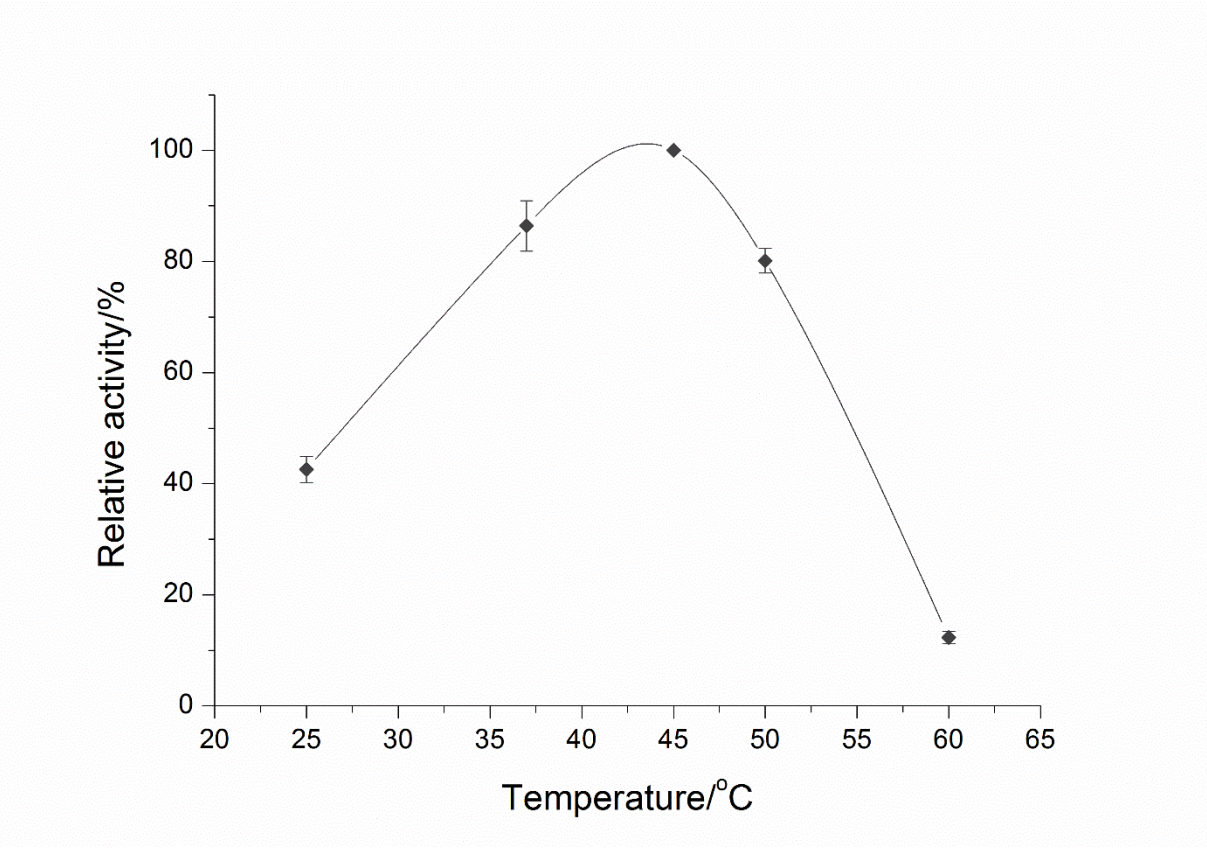

Fig. 4. Effect of temperature on the activity of *P. rubens* xylanase; each point represents the mean (n = 3) ± SD

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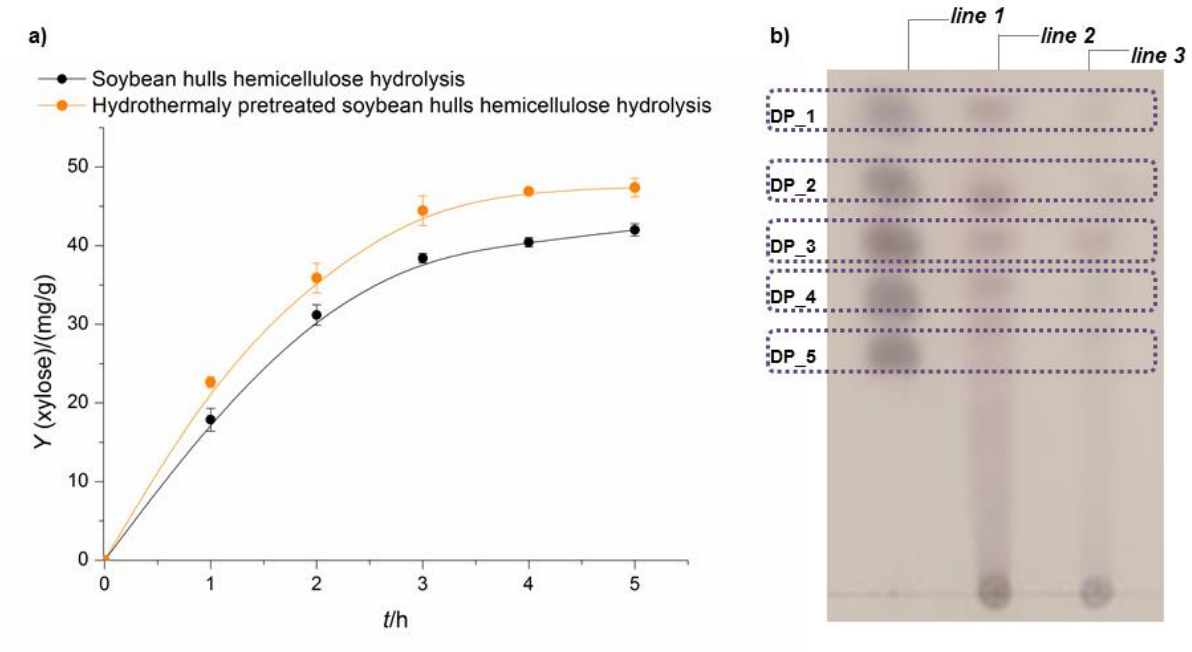

Fig. 5. a) Time course of soybean hull hemicellulose hydrolysis catalyzed by partially purified xylanase from *P. rubens* (reaction conditions: $[T]_r$ = 45 °C, $[pH]_r$ = 6.0, [Xylanase] = 10 U/$g_{biomass}$, [S] = 1% *m*/*V*) ; b) Thin-layer chromatography of hydrolysis products: line 1 – standard xylooligosaccharides markers (xylose, xylobiose, xylotriose, xylotetraose, xylopentaose); line 2 – hydrolysate of soybean hull hydrothermally pretreated; line 3 – soybean hull hydrolysate; each point represents the mean (n = 3) ± SD

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Table 1. Purification of xylanase from *P. rubens*

Purification step	A/(U/mL)	A_{tot}/(U)	Y/(%)	C/(mg/mL)	PF
Crude extract	1.05±0.01	195.26±1.23	100.00±0.00	7.74±0.45	1.00±0.00
Membrane, 10 kDa	16.86±0.98	185.49±3.21	94.99±2.34	25.98±1.30	4.76±0.12
Membrane, 3 kDa	61.39±1.21	128.93±2.23	66.03±2.33	84.65±2.11	5.32±0.23
SEC, 100 kDa	9.08±0.56	95.34±1.45	48.83±1.56	5.17±0.22	12.89±0.56

A → Xylanase activity, A_{tot} → Total activity of xylanase, Y → Activity Yield, C → protein content, PF → Purification Factor

Table 2. A summary of XOS yields after enzymatic hydrolysis (calculated depending on the initial biomass content)

	Hydrolysis strategy	
Yield/(mg monosaccharide/g of initiall biomass)	Without hydrothermal pretreatment	With hydrothermal pretreatment
Arabinose	/	1.88±0.05
Glucose	6.47±0.37	6.67±0.31
Xylose	/	2.02±0.03
	Yield/(mg XOS/g of initiall biomass)	
Xylobiose	/	4.29±0.08
Xylotriose	10.69±0.27	2.49±0.05
Xylotetraose	/	2.24±0.16
Xylopentaose	/	1.69±0.02