PRODUCTION OF XYLOOLIGOSACCHARIDES FROM BANANA PSEUDOSTEM AND IN VITRO EVALUATION OF THEIR ANTIOXIDANT, ANTIDIABETIC AND ANTIHYPERTENSIVE ACTIVITIES

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ABSTRACT

In this study, banana pseudostem presented a chemical composition of 56% ± 0.05 cellulose, 19% ± 0.2 hemicellulose, 18% ± 0.3 lignin, 5.96% ± 0.4 ash and 0.07% ± 0.02 extractives. It was possible to obatin a xylan with an yield equivalent to 42.5% (w/w) using an alkaline pretreatment. The enzymatic activity of the thermostable recombinant endoxylanase from *Cryptococcus flavescens* expressed in *Komagataella phaffii* was 600 IU/mL in 2% of substrate (xylan from banana pseudostem). Enzymatic hydrolysis with a load of 200 IU of endoxylnase per gram of xylan (2%) for 48 h at 50 °C under stirring of 250 rpm led to a production of 1 g/L of XOS. The results indicate a marked inhibitory activity of XOS due to its antioxidant (49% ± 1 and 65 ± 0.6), antidiabetic (9.5% ± 0.2 and 17% ± 0.05 for α-amylase, 11% ± 0.8 and 19.4% ± 0.3 for α-glucosidase) and antihypertensive (15% ± 0.1 and 24% ± 0.04) action at concentrations of 0.5 and 1 mg/mL. It should be noted that the proposed bioprocess uses enzymatic hydrolysis, which is a clean ("eco-friendly") technology that enables the production of functional biomolecules from the sustainable use of agro-industrial waste with interesting properties.

Keywords: Biorefinery. DPPH. Amilase. Glucosidase. Angiotensin.

1 INTRODUCTION

Oligosaccharides can be obtained through the bioconversion of different lignocellulosic biomasses, such as sugar cane bagasse (ZHOU and XU, 2019), corn straw (QIAN et al., 2020), banana pseudostem (FREITAS et al., 2021), among others. Banana pseudostem is one of the interesting raw materials for obtaining high value-added products such as oligosaccharides due to feasibility, since in 2021 Brazil exported around 100,000 tons of bananas, which represents an increase of 26.33% when compared to 2020 (CONAB, 2021), being the fourth largest producer of the fruit in the world in 2020 (FAO, 2020). It is estimated that for every 1 ton of bananas produced, 3 tons of pseudostem waste are generated (SILVA et al., 2020).

Among the benefits of oligosaccharides in human health are the reduction of cholesterol levels, maintenance of gastrointestinal health, increased bioavailability of calcium, reduced risk of colon cancer, cytotoxic effects on human leukemia cells and the beneficial effect on type 2 diabetes mellitus (ANDO et al., 2004). Some researchers point to non-digestible oligosaccharides as dietary supplements that can be added to drinks, dairy products, yogurts and symbiotic foods (MANNING and GIBSON, 2004; GIBSON and ROBERFROID, 1995).

In this context, the present study presents an approach to developing high value-added xylooligosaccharides (XOS) with antioxidant, antidiabetic and antihypertensive properties. These oligosaccharides were obtained from a single biomass (xylan from banana pseudostem) using heterologously expressed endoxylanase produced and partially purified from *Komagataella phaffii* through an eco-friendly process, with low use of chemical products, exploiting a renewable biomass that is still underexploited.

2 MATERIAL & METHODS

LIGNOCELLULOSIC BIOMASS

The banana pseudocaulde was provided by São Paulo State University (UNESP) from Rio Claro/SP, Brazil. The material first went through an alkaline extraction stage to fractionate the hemicellulose-rich portion. The banana pseudostem was subjected to chemical characterization, determining the content of cellulose, hemicellulose and lignin according to the methodology proposed by NREL (SLUITER et al., 2008).

CHEMICAL CHARACTERIZATION

The chemical composition of the banana pseudostem in natura was analyzed in order to quantify the cellulose, hemicellulose, lignin and ash content. The cellulose, hemicellulose and lignin content was determined by hydrolysis with 72% (w/w) sulfuric acid according to the analytical procedure recommended by NREL (SLUITER et al., 2008). Subsequently, the dried samples (300 mg) were hydrolyzed with 72% sulfuric acid (3 mL) at 30 °C for 1 h, followed by dilution to a final concentration of 4% (m/v) and 84 mL of distilled water. The mixture was autoclaved at 121 °C for 1 h, cooled and filtered through a porous bottom crucible (4-5 µm). Insoluble lignin was quantified as the amount of insoluble solid obtained from the mass of insoluble ash. Soluble lignin was measured in an alkaline medium by reading it in a spectrophotometer at 280 nm.

Finally, the ash content was determined by burning 1 g of the sample in a porous bottom crucible for 6 h using a 600 °C muffle furnace until constant weight, according to the NREL /TP-510-42622 procedure (SLUITER et al., 2005).

HEMICELLULOSE EXTRACTION

The xylan was previously extracted from the material according to the method proposed by Zilliox and Debeire (1998) with modifications proposed by Akpinar et al. (2010), with a reduction in the use of KOH (from 24 to 12%), in a reaction with aid of an autoclave (using steam at 121 °C by 30 min), followed by a precitation in ethanol/acetic acid followed by oven drying at 60 °C overnight.

PRODUCTION OF ENDOXYLANASE FROM *Komagataella phaffii*

The thermostable recombinant endoxylanase from *Cryptococcus flavescens* expressed in *Komagataella phaffii* (MARTINS et al., 2020) was used to produce XOS. For this purpose, the yeast was cultivated in 500 mL flasks containing 50 mL of YPD medium (1% Yeast, Extract, 2% Peptone, 0.00004% Biotin (Sigma-Aldrich), 2% Glucose) at 30 ºC and 200 rpm, overnight. When the inoculum reached ª cell density (OD 600), it was transferred to 1 L conical flasks containing 500 ml of medium made up of 16.6 g/L glucose, 8.33 g/L peptone, 4.16 g/L yeast extract and 2, 08% defoamer C (Sigma-Aldrich) previously sterilized (121 °C for 20 min) and 0.8% saline solution (6 g/L $CuSO_4$, 0.08 g/L KI, 3 g/L $MnSO_4$, 0.2 g/L Na_2MoO_4, 0.02 g/L, H_3BO_3, 0.5 g/L $CoCl_2$, 20 g/L $ZnCl_2$, 65 g/L $FeSO_4$. $7H_2O$, 0.2 g/L Biotin, 5 mL/L H_2SO_4 98%) sterilized with a 0.22 µm Millipore filter. Cultivation was conducted at 30 °C and initial pH 6.0. At the end of the process, the cells were separated by centrifugation at 1300 g for 5 min. The supernatant containing the recombinant endoxylanase was concentrated using an ultrafilter (Millipore, 10 kDa).

DETERMINATION OF ENZYMATIC ACTIVITY AND XOS PRODUCTION

Endo-1,4-xylanase activity was assessed against 500 µL of 2% soluble xylan and 0.1 mL of diluted supernatant, which were mixed at 45 °C for 30 min in 50 mM sodium acetate buffer, pH 5.2, in a total volume of 1 mL (DA SILVA et al., 2011). The resulting reducing sugars were analyzed using a DNS assay (MILLER, 1959). A xylanase unit was defined as the amount of enzyme required to produce 1 µmol of reducing sugars under the reaction conditions. The enzymatic hydrolysis for the generation of XOS was carried out using 200 IU/g of endoxylanase setting the temperature at 50 °C, 48 h of reaction, under stirring of 250 rpm using xylan as substrate at 2% (w/v) in 50 mM sodium acetate buffer, pH 5.2.

QUANTIFICATION AND IDENTIFICATION OF THE PRODUCTS GENERATED

The supernatants resulting from the enzymatic hydrolysis of the biomasses studied (XOS) were quantified by ion exchange chromatography with pulsed amperometric detection (HPLC-PAD), using a Dionex® chromatograph (Sunnyvale, CA, United States) equipped with a Carbopac PA-100 column (4 x 250 mm) and a Carbopac PA-100 guard column (4x50 mm), GP50 pump, and an ED40 electrochemical detector which adopted a linear gradient of A (NaOH 50 mM) and B (NaOAc 500 mM, NaOH 50 mM) using the PEAKNET software. The integrated peak areas were adjusted based on standards purchased from Megazyme® xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentose (X5), xilohexose (X6).

ANTIOXIDANT ACTIVITY

For this analysis, the DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical capture method was applied according to (ÁVILA et al., 2020). A reaction was carried out with 50 µL of hydrolysate containing XOS (0.5 and 1 mg/ml) (ethanol as a negative control) to 50 µl of DPPH solution (0.1 mM) diluted in ethanol (95% v/v), and incubated in the dark for 2 h, followed by reading at 517 nm. The activity was calculated using Equation 1, where Ac corresponds to the absorbance of the XOS reaction with the radical, and Ao corresponds to the absorbance of the reaction control.

$$(\%)\ \text{Inhibition DPPH} = (1 - Ac/Ao) * 100 \quad \text{(Equation 1)}$$

ANTIDIABETIC ACTIVITY

The antidiabetic effects of XOS were evaluated by investigating their action in inhibiting the activity of the enzymes α-amylase from *Bacillus licheniformis* and α-glucosidase from *Saccharomyces cerevisiae*, according to Casarin, Rasera and De Castro (2021).

For α-amylase activity, 0.5 mL aliquots of the reaction mixture containing α-amylase solution (0.5 U/mL) and samples (0.5 and 1 mg/mL) in 50 mmol/L phosphate buffer (pH 7) were prepared and left to stand for 30 min. To each reaction tube was added 0.5 mL of starch solution (0.5% p:v) which has been incubated at 70 °C in the thermostatic bath for 30 min. The reaction was stopped by adding 1 mL of 3,5-dinitrosalicylic acid (DNS), followed by incubation in a boiling bath for 5 min. The tubes were cooled, 8.0 mL of distilled water added and the absorbance measured at 540 nm on a spectrophotometer. The control was composed of the enzymatic reaction without the addition of the extract containing XOS, while the reaction blank was comprised of a mixture of the DNS reagent and the substrate.

For α-glucosidase activity, the samples were diluted in a 100 mmol/L phosphate buffer solution (pH 6.9) to a final concentration of 0.5 and 1 mg/mL and aliquots of 50 µL will be added to 100 µL of enzyme solution (0.1 U/mL), followed by incubation at 25 °C for 10 min. Aliquots of 50 µL of the substrate 4-nitrophenyl-α-d-glucopyranoside were added to the reaction mixtures and kept at 37 °C for 5 min. The absorbance was measured at 405 nm in a microplate reader. All assays were compared with the control reaction, which was prepared by adding 50 µL of buffer solution in place of the XOS.

The results were expressed as a percentage of inhibition, as shown in Equation 2.

(%) Inhibition = (Abs control - Abs sample/Abs control) * 100 (Equation 2)

ANTIHYPERTENSIVE ACTIVITY

The antihypertensive effect of XOS was measured as a function of Angiotensin Converting Enzyme (ACE) inhibitory activity according to the method described by Siow and Gan (2013). Aliquots of 50 µL of the samples containing XOS (0.5 and 1 mg/mL) were mixed with 50 µL of ACE (0.005 U/mL) and 150 mL of 4.15 mM hippuryl-histidyl-leucine (substrate) in borate buffer containing 0.3 M NaCl, pH 8.3. The reaction mixture was incubated at 37°C for 30 min and stopped by adding 500 µL of 1M HCl.

The extraction of hippuric acid from the action of ACE on the substrate was carried out by adding 1.5 mL of ethyl acetate, vortexing for 1 min and resting for 5 min. An aliquot of 800 µL of the upper phase of the reaction mixture was dried for 1 h at 80°C. The resulting residue was then solubilized in 1.0 mL of distilled water. The concentration of hippuric acid was determined at 228 nm. The control reaction was prepared by adding 50 µL of distilled water instead of the sample. The antihypertensive potential was expressed as a function of the percentage inhibition of ACE activity according to Equation 2, mentioned above.

3 RESULTS & DISCUSSION

The xylan extraction yield was equivalent to 42.5%. The banana pseudostem showed a chemical composition containing 56% ± 0.05 of cellulose, 19% ± 0.2 of hemicellulose, 18% ± 0.3 of lignin, 5.96% ± 0.4 of extractives and 0.07% ± 0.02 of extractives. The endoxylanase activity of *Cryptococcus flavescens* expressed in *Komagataella phaffii* in the presence of 2% of xylan was equal to 600 IU/mL. Using an endoxylanase load of 200 IU/g of xylan, it was possible to obtain a yield of 1g/L of XOS (65% of X2, 5.3% of X3, 16% of X4, 6.3% of X5 and 7.4% of X5).

Through in vitro functional properties evaluation approaches, it was possible to observed that the XOS (1 mg/mL) presented a maximum antioxidant activity of 65% ± 0.6 based on the reduction of the free DPPH radial molecule (Table 1). For comparison purposes, a recent study in the literature indicated an antioxidant activity of 75% for XOS (2.5 mg/mL) obtained from sugar cane bagasse biomass (PASCHOA et al., 2024), where the same method was used. Another study used 2 mg/ml of XOS obtained from the xylan of sugar cane straw and observed an antioxidant activity of 78%, also using the same methodology employed in this study. In the literature, the antioxidant activity of XOS is attributed to the presence of hydroxycinnamic acid derivatives linked to esters, such as ferulic, caffeic and coumaric acid, as well as the syringic acid residues and methyl glucuronic acid branches present in the xylan chain (ÁVILA et al., 2020), from which XOS are derived.

Table 1 In vitro evaluation of functional properties of XOS.

XOS (mg/mL)	DPPH inhibition (%)	α-amilase inhibition (%)	α-glucosidase inhibition (%)	ACE inhibition (%)
0.5	49 ± 1	9.5 ± 0.2	11 ± 0.8	15 ± 0.1
1	65 ± 0.6	17 ± 0.05	19.4 ± 0.3	24 ± 0.04

In addition to antioxidant activity, this study evaluated the antidiabetic activity of XOS obtained from banana pseudostem, observing the percentage inhibition of α-amylase and α-glucosidase activities in the presence of XOS (0.5 and 1 mg/mL). The results indicate that at a concentration of 1 mg/mL of XOS, there is an inhibition of α-amylase and α-glucosidase activities equivalent to 17% ± 0.05 and 19.4% ± 0.3, successively (Table 1). In the literature, one study observed that at a concentration of 10 mg/mL, neoagarooligosaccharides (NAO) obtained from the enzymatic hydrolysis of commercial agar showed an inhibition of α-glucosidase activity equivalent to 42.3%, and additionally showed an inhibition of α-amylase of less than 15% at a concentration of 1 mg/mL of NAO. Mune et al. (2024) highlighted that analyzing the enzymatic coupling of XOS with α-amylase and α-glucosidase that there is a moderate strength of interactions, both hydrogen bonds and non-bonded contacts, in the active site of these enzymes.

The results of the in vitro analysis for antihypertensive potential of XOS showed a maximum inhibition percentage in relation to ACE enzyme equal to 24% ± 0.04 when present at a concentration of 1 mg/mL (Table 1). A study in the literature used the alga *Ulvan lectura* to produce ulvan extracts and obtain ulvan oligosaccharides through enzymatic hydrolysis, observing that the ulvan oligosaccharides obtained showed an in vitro inhibition of the ACE enzyme corresponding to 81.86, 50.18, and 38.78%, at a concentration of 12.5 mg/mL, 8.0 mg/mL, and 6.0 mg/mL (HUNG et al., 2021).

The hypothesis of this study is that higher concentrations of XOS than those evaluated (0.5 and 1 mg/mL) could lead to higher percentages of inhibition of the enzymes a-glucosidase, a-amylase and ACE, since a directly proportional relationship is observed between increasing inhibition with the inhibitor concentration, as observed by Mune et al. (2024), and confirmed in the studies by Hong et al. (2017) and Hung et al. (2021).

4 CONCLUSION

By means of this study, it was possible to obtain 1 g/L of XOS using as raw material a renewable biomass that has been underexplored in the literature. Through an eco-friendly bioprocess with reduced use of chemical reagents in the alkalin pretreatment, the xylan has been converted into XOS using a heterologously expressed endoxylanase. It was possible to highlighted that the, XOS produced in this study presented interesting properties such as antioxidant, antidiabetic and

antihypertensive action. Future steps include evaluating of others properties from XOS, as well as prebiotic and antimicrobial activities, also investigating a cytotoxicity assessment and a technical-economic analysis of the bioprocess, which will contribute to the original and innovative state of this work.

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ACKNOWLEDGEMENTS

This study is funded by The São Paulo Research Foundation (FAPESP), grants numbers (2022/08607-0; 2022/15221-1 and; 2022/05731-2. The authors thank the Brazilian National Council for Scientific and Technological Development (CNPq) for grants (403675/2021-9 and 311333/2023-0).