

Extraction and Purification of Xylooligosaccharides from Coffee Peel Using Endo- β -1,4-D-Xylanase XynBTN63D and Adsorption for Prebiotics

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ABSTRACT

This research aims to extract and purify xylooligosaccharides (XOS) from coffee peel xylan using recombinant endo- β -1,4-D-xylanase (XynBTN63D) and adsorption-based methods, and to assess their prebiotic potential. The xylan was hydrolyzed with XynBTN63D at 40°C for 24 hours. The hydrolysate was purified using activated carbon with ethanol (10%–50%) as the eluent, followed by column chromatography. XOS composition was analyzed before and after purification using thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). Both pre- and post-purification products contained xylobiose (X2) and xylotriose (X3), while ethanol concentrations of 10%, 20%, and 30% were effective in removing residual xylan. Higher ethanol concentrations were less effective in XOS recovery. Activated carbon significantly improved the purity by eliminating impurities and enhancing X2 and X3 separation. The results confirm that the enzyme operates with high specificity, producing oligosaccharides with potential applications in prebiotics and functional food industries. This study demonstrates the potential of coffee peel a widely available agricultural waste as a low-cost, sustainable lignocellulosic source for prebiotic XOS production. The unique application of XynBTN63D in combination with optimized ethanol-based adsorption contributes to the development of efficient XOS purification protocols.

Key words: Prebiotic; enzymatic hydrolysis; bioconversion; purification; lignocellulosic biomass.

1 INTRODUCTION

Xylan, a major polysaccharide component of hemicellulose, is an integral part of plant cell walls and commonly associated with cellulose and lignin (Curry; Peña; Urbanowicz, 2023). It is abundantly present in lignocellulosic agricultural byproducts such as cassava peel, cassava bagasse, and coffee peel (Ratnadewi et al., 2016; Samanta et al., 2015). Given its widespread availability and underutilization, xylan serves as a sustainable and renewable feedstock for the biotechnological production of high-value compounds such as xylooligosaccharides (XOS) through enzymatic hydrolysis (Mano et al., 2018).

XOS are short-chain oligosaccharides obtained from the partial hydrolysis of xylan and have gained substantial interest due to their health-promoting properties. They are known for their prebiotic activity, selectively stimulating beneficial gut bacteria, and exhibiting antioxidant, anticarcinogenic, and anti-inflammatory effects (Chang et al., 2020; Valladares-Diestra et al., 2023; Ali et al., 2025). Furthermore, their low glycemic index supports applications as functional sweeteners suitable for diabetic patients (Gonçalves et al., 2023; Palaniappan; Antony; Emmambux, 2021).

Enzymatic hydrolysis using endo- β -1,4-D-xylanase, particularly those from glycoside hydrolase family 11, is preferred over chemical methods due to greater specificity and mild reaction conditions. This enzyme cleaves the internal

β -(1 $\rightarrow$ 4) linkages in the xylan backbone to produce XOS with varying degrees of polymerization (Ali et al., 2025). Recently, recombinant forms of endo- β -1,4-D-xylanase such as pYHM-1 and pESC have been developed and demonstrated high catalytic efficiency, including enhanced production of low-DP XOS such as xylobiose (X2) and xylotriose (X3) (Lei et al., 2025).

Studies have shown that coffee peel is a rich source of hemicellulose (13.1 g/100 g), making it a promising substrate for XOS production (Curry; Peña; Urbanowicz, 2023). Ratnadewi et al. (2020) previously reported XOS production from coffee peel using recombinant xylanase from termite gut systems, confirming its potential as a low-cost biomass for functional oligosaccharide production. However, most prior studies have focused on the hydrolysis step without thorough optimization of purification techniques, which are crucial for enhancing XOS quality and applicability.

A critical barrier to the downstream application of XOS lies in the presence of residual xylan, protein contaminants, and colored by-products. Activated carbon adsorption is a widely used purification method due to its capacity to remove high-molecular-weight impurities, pigments, and proteins. However, the efficient elution of low-DP XOS such as xylobiose and xylotriose requires optimization of ethanol gradients to achieve selective recovery (Jiang; Li, 2024).

Recent comprehensive reviews, including that by Ali et al. (2025) in *Food Research International*, emphasize that while the enzymatic production of XOS is well-established, there is a research gap in purification strategies particularly in combining activated carbon with optimized elution protocols to selectively enrich short-chain XOS.

This study addresses that gap by employing two recombinant xylanases (pYHM-1 and pESC) to hydrolyze coffee peel-derived xylan into XOS, followed by purification using activated carbon coupled with gradient ethanol elution. The hydrolysate and purified fractions were evaluated using TLC and HPLC to confirm XOS profile and recovery efficiency. The novelty of this research lies in (1) comparing two recombinant xylanases for XOS production from coffee peel, and (2) systematically optimizing ethanol concentrations for selective elution of X2 and X3 during purification. This integrated bioprocess offers a more efficient and sustainable route for producing functional oligosaccharides from agricultural waste, contributing to both waste valorization and prebiotic development in the food and health industries.

2 MATERIAL AND METHODS

2.1 Materials

Coffee peel was sourced from the Coffee and Cocoa Research Center in Jember, Indonesia. The endo- β -1,4-D-xylanase enzymes, specifically produced from *Saccharomyces cerevisiae*, were stored in glycerol stocks at the CDAST Laboratory, University of Jember. All reagents, media, and chemicals used in this study were of analytical grade. Commercial activated carbon was utilized. Standards for TLC and HPLC analyses (X1-X6) were purchased from Waco-Chemical, Osaka, Japan, and Megazyme, Ireland, Europe. TLC plates (Silica Gel 60 F₂₅₄) were provided by Merck, Germany.

2.2 Production of Recombinant Endo- β -1,4-D-xylanase pESC and pYHM-1

1% inoculum of *Saccharomyces cerevisiae* BJ1824 was transferred into 50 mL YPG medium supplemented with 2% galactose, and incubated for 3 days with shaking. The culture was centrifuged at 7000 rpm for 15 minutes at 4°C. The pellet and supernatant were separated and stored at 4°C (Ratnadewi et al., 2024).

2.3 Xylan Extraction from Coffee Peel

Coffee peel was cleaned with distilled water to remove surface dust particles, dried in an oven at 50°C, blended, and then sieved to obtain a 60-mesh coffee peel powder.

For delignification, 4 grams of fine coffee peel powder were soaked in 40 mL of 0.5% NaOCl solution for 5 hours. The solution was then rinsed with distilled water, filtered, and dried in an oven. The delignified coffee peel powder was treated with 12% NaOH solution (1:10, v/v) and soaked for 24 hours. After centrifugation at 10000 rpm for 10 minutes, the supernatant was neutralized with 6 N HCl. Centrifugation was applied to the neutral sample at 10,000 rpm for 10 min and the supernatant of this process was mixed with ethanol 95 % (w/w) with volume ratio 1 : 3 (supernatant : ethanol). The mixture was centrifuged at 10000 rpm for 10 min and the precipitate was collected and dried. The xylan extract was dissolved to a concentration of 0.8% in phosphate-citrate buffer (pH 5.5) to a final volume of 2 mL (Ratnadewi et al., 2016). Crude xylan content was calculated using the equation below:

Yield of xylan = (mass of dried crude xylan / mass of coffee peel) $\times$ 100%

2.4 Enzyme Activity and Protein Content Determination

The enzyme activity was assessed using the Miller method. 125 μ L aliquot of enzyme was mixed with 125 μ L of 0.8% (w/v) coffee peel xylan substrate in phosphate-citrate buffer (pH 5.5). Controls were prepared with heat-inactivated enzymes (heated at 100°C for 60 minutes). The samples and controls were analyzed using the DNS (Miller) method with absorbance measured at 540 nm. Enzyme activity was calculated using a xylose standard curve with the formula: Enzyme Activity (U/mL) = (S-K $\times$ Vtotal) / (BM xylose $\times$ t $\times$ Venzyme $\times$ fp)

Protein content was determined using the Bradford method. A 100 μ L enzyme sample was mixed with 1000 μ L of Bradford reagent and homogenized. The mixture was incubated for 2-5 minutes, and absorbance was read at 595 nm. Protein concentration was determined using a BSA standard curve based on the Bradford Assay (Banerjee et al., 2019).

2.5 Xylan Hydrolysis of Coffee Peel Extract by Endo- β -1,4-D-xylanase

Xylan hydrolysis was performed by adding 125 μ L of endo- β -1,4-D-xylanase to 125 μ L of xylan substrate. The mixture was incubated for 24 hours at 40°C. Control samples used endo- β -1,4-D-xylanase enzymes mixed with phosphate-citrate buffer (pH 5.5) and heated at 100°C for 60 minutes. Total reducing sugar was determined by using the DNS reagent, and absorbance was measured at 540 nm. The hydrolysis products (XOS) were analyzed by thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC).

2.6 Purification of XOS Using Activated Carbon Adsorption

Purification of the hydrolysis products was carried out using 60-80 mesh activated carbon, activated by heating in an oven at 70-80°C for 2 hours. A layer of cotton was placed at the bottom of the column, followed by 2 x 1 cm bed volume of the adsorbent. Ethanol was added 1 cm above the adsorbent. The column was opened, and the eluent was collected. A 6 mL sample was loaded, followed by sequential addition of ethanol in gradients of 10% to 50%. The eluents exiting the column were collected in microtubes. Fractions were tested by TLC, and fractions likely containing prebiotics were analyzed by HPLC (Zhao; Xu; Hao, 2023).

3 RESULT

3.1 Xylan Extraction from Coffee Peel

Xylan was successfully extracted from coffee peel, a lignocellulosic waste material, using sodium hydroxide (NaOH) as the extraction solvent. NaOH is widely recognized for its ability to disrupt the lignocellulosic matrix by breaking the hydrogen bonds between hemicellulose and cellulose, allowing the selective dissolution of xylan while minimizing cellulose solubilization. The extraction process involved soaking coffee peel powder in a 0.5% NaOH solution for 24 hours at room temperature, enabling sufficient interaction between the alkaline solvent and the coffee peel's structural components. After the incubation period, the extracted solution was neutralized using 6 N hydrochloric acid (HCl) to restore pH balance, ensuring that the xylan remained stable in solution. Precipitation was then carried out using ethanol, a well-established method for isolating xylan by reducing its solubility in the aqueous phase (Lin et al., 2023).

Following the completion of the extraction process, the total xylan yield was determined. From an initial mass of 4.058 ± 0.05 grams of dried coffee peel powder, the delignification process yielded 3.645 ± 0.06 grams of delignified biomass. Subsequently, 0.128 ± 0.03 grams of xylan were successfully recovered, resulting in an overall xylan yield of $3.023 \pm 0.78\%$ (Table 1). This yield is consistent with findings from previous studies that have employed alkaline extraction techniques for xylan recovery from various lignocellulosic biomass sources (Shivalingamurthy; Kuppasamy, 2021). The relatively moderate yield suggests that a significant portion of the xylan fraction remains embedded within the residual biomass, potentially due to incomplete extraction or losses incurred during processing, such as inefficiencies in precipitation or washing steps.

Table 1: Comparison of Xylan Extraction Yields from Various Biomass Sources.

Study Source	Xylan Yield (%)	Xylan Recovery (%)
This Study (Coffee Peel)	3.023 ± 0.78	45.07
Sugarcane Bagasse (SES 159 – NaOH) ^a	23.85 ± 2.08	72.39
Sugarcane Bagasse (SES 159 – H ₂ O ₂) ^a	17.43 ± 1.84	52.89
Sugarcane Bagasse (SES 159 – DMSO) ^a	0.45 ± 0.07	1.36
Coffee Peel (NaOH 12%) ^b	9.73	43.00

^aShivalingamurthy and Kuppasamy, (2021); ^bRatnadewi et al. (2020).

3.2 Enzyme Activity and Protein Content

The activity of endo- β -1,4-D-xylanase (XynBTN63D), an enzyme essential for the hydrolysis of xylan into xylooligosaccharides (XOS), was evaluated to determine its efficiency in catalyzing the breakdown of xylan. The specific activity of XynBTN63D was measured for two different constructs, yielding values of 4.317 U/mg for the pESC-XynBTN63D construct and 4.222 U/mg for the pYHM1-XynBTN63D construct. These values indicate the enzyme's ability to efficiently hydrolyze xylan into its oligomeric sugar components.

The total protein content of the enzyme preparations was quantified using the Bradford assay, a well-established method for protein concentration determination (Löptien et al., 2024). The protein concentrations were found to be 1.228 mg/mL for pESC-XynBTN63D and 1.161 mg/mL for pYHM1-XynBTN63D. These values align with previous findings by Ali et al. (2025), who reported similar protein concentrations in recombinant xylanase preparations, further supporting the reliability of the obtained data.

Enzyme activity was assessed using the Miller method, which employs 3,5-dinitrosalicylic acid (DNS) as a reagent to detect the presence of reducing sugars. In this assay, the hydrolysis of xylan results in the formation of reducing sugars, which react with DNS to produce a yellow-brown complex, measurable spectrophotometrically. This method has been widely used in enzymatic studies due to its sensitivity and reproducibility in detecting enzyme activity (Sokač et al., 2023). The hydrolysis products were further analyzed for sugar content to confirm the enzymatic conversion of xylan into XOS.

3.3 XOS Production from Coffee Peel Xylan

Xylooligosaccharides (XOS) were successfully produced from the extracted xylan derived from coffee peel using recombinant endo- β -1,4-D-xylanase. The enzymatic hydrolysis was conducted under optimized reaction conditions, specifically at pH 5.5 and an incubation temperature of 40°C for a duration of 24 hours. The resulting hydrolysis products

exhibited a distinct dark brown coloration, which suggests the presence of residual lignin and indicates that the hydrolysis process may not have been entirely complete. The hydrolysis products were analyzed using thin layer chromatography (TLC) (Figure 1) and high-performance liquid chromatography (HPLC) (Figure 2). TLC analysis revealed distinct spots that corresponded to xylobiose (X2) and xylotriose (X3), while no detectable xylose (X1) was observed in the chromatogram.

To confirm the presence of XOS, the Retardation factor (Rf) values of the hydrolysis products were systematically compared with those of standard XOS compounds (Table 2).

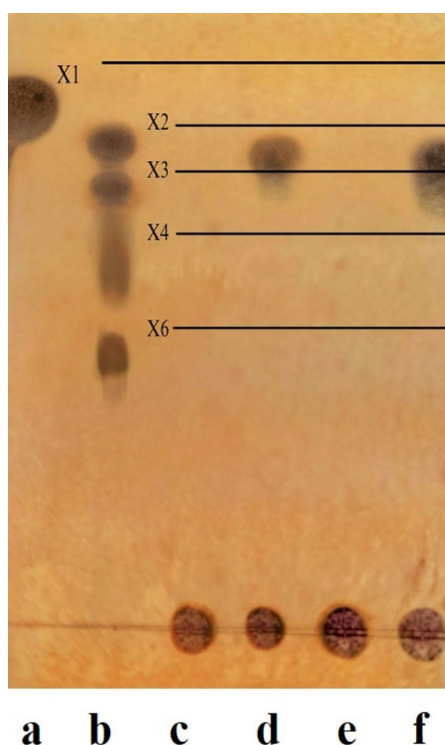


Figure 1: Chromatogram of XOS products from coffee peel xylan hydrolysis by endo- β -1,4-D-xylanase. (a) Xylose standard; (b) XOS standard; (c) Xylan; (d) Xylan + endo- β -1,4-D-xylanase (pYHM-1); (e) Xylan + inactive enzyme; (f) Xylan + endo- β -1,4-D-xylanase (pESC).

The Rf values of the hydrolysis products obtained from enzymatic treatment closely matched the standard values for xylobiose (X2) and xylotriose (X3), thereby demonstrating that the enzymatic hydrolysis specifically produced these oligosaccharides. The clear visual similarity in migration between samples and standards supports this identification. This analysis was carried out based on a single TLC run for each sample. Due to time and material constraints, replicate runs were not performed, and therefore statistical analysis such as calculation of standard deviation or significance testing could not be applied to the Rf values. Despite this limitation, the TLC profiles provided consistent and interpretable results. Future studies should consider conducting repeated measurements to support quantitative statistical evaluation and improve the robustness of the data.

Further confirmation of XOS production was achieved via HPLC analysis (Figure 2). The retention times of the hydrolysis products obtained from coffee peel xylan were found to be closely aligned with those of the XOS standards (Figure 3), reinforcing the conclusion that enzymatic hydrolysis selectively yielded xylobiose (X2) and xylotriose (X3).

The HPLC chromatogram of the XOS products generated from the hydrolysis of coffee peel xylan by endo- β -1,4-D-xylanase revealed five distinct peaks with retention times of 3.939, 5.335, 5.661, 6.432, and 6.696 minutes (Table 3).

The standard chromatogram for XOS components (Figure 3) indicates retention times for xylose (X1) at 5.505 minutes, xylobiose (X2) at 5.425 minutes, xylotriose (X3) at 5.354 minutes, and xylotetraose (X4) at 5.160 minutes. When comparing these retention times to those in the chromatogram of the hydrolyzed coffee peel xylan sample, the peaks at 5.335 minutes and 5.661 minutes were found to be the closest matches to the standard retention times of xylobiose (5.425 minutes) and xylotriose (5.354 minutes), respectively. This strong alignment of retention times further supports the conclusion that the enzymatic hydrolysis of coffee peel xylan resulted predominantly in the formation of xylobiose (X2) and xylotriose (X3).

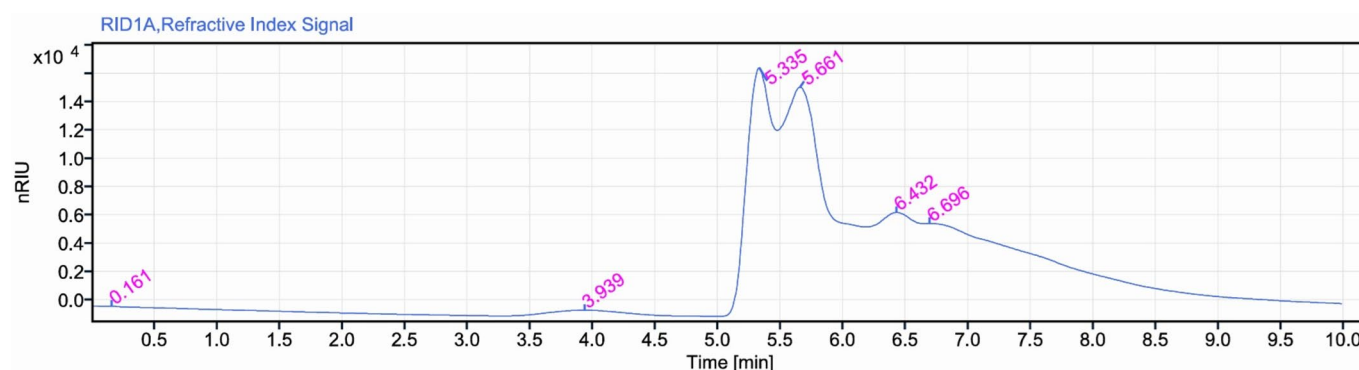


Figure 2: HPLC chromatogram of XOS products from coffee peel xylan hydrolysis by endo- β -1,4-D-xylanase.

Table 2: Calculation of Rf values for XOS standard and XOS sample before purification.

Description*	Eluent Distance (cm)	Sample Distance (cm)	Rf Value
X1	9.5	7.4	0.77
X2	9.5	6.4	0.67
X3	9.5	5.8	0.61
X4	9.5	5.1	0.54
X5	-	-	-
X6	9.5	3.8	0.4
XOS (pYHM-1)	9.5	6.3	0.66
XOS (pESC)	9.5	6.1	0.64

*X1–X6 correspond to X1 (Xylose); X2 (Xylobiose); X3 (Xylotriose); X4 (Xylotetraose); X5 (Xylopentaose); and X6 (Xylohexaose).

Table 3: Quantitative calculation of HPLC chromatogram of XOS products from coffee peel xylan hydrolysis by endo- β -1,4-D-xylanase.

Peak	Retention Time (min)	Width (min)	Height	Area %
1	3.939	1.734	417.8	1.31
2*	5.335	0.460	17510.1	18.46
3*	5.661	0.704	16076.5	32.24
4	6.432	0.473	7095.4	13.56
5	6.696	3.338	6254.9	34.22

*Peaks 2 and 3 correspond to xylobiose (X2) and xylotriose (X3), respectively, based on comparison with standard retention times.

3.4 XOS Purification by Activated Carbon Adsorption

The purification of XOS was carried out using activated carbon adsorption to remove impurities such as macro-lignin, protein residues, and polysaccharides, which contribute to the turbidity of the hydrolysate. This method effectively utilizes the non-polar surface of activated carbon to adsorb these contaminants while allowing the XOS to elute. The purification process occurred in two stages: the adsorption of the hydrolysate on a carbon column followed by desorption using ethanol at varying concentrations (10%-50%).

The effectiveness of the purification process was evaluated using Thin Layer Chromatography (TLC) (Figure 4).

The chromatographic analysis showed successful separation of XOS components. Specifically, the 10% ethanol fraction primarily contained xylobiose (X2), while the 20% ethanol fraction contained xylotriose (X3). Fractions with higher ethanol concentrations (30%-50%) contained larger oligosaccharides such as xylotetraose (X4). The increasing ethanol concentration facilitated the elution of XOS with larger degrees of polymerization (DP).

Additionally, residual xylan was observed in the higher ethanol concentration fractions. This was likely due to the lower solubility and higher molecular weight of xylan, which inhibited its complete desorption in the presence of ethanol. The TLC chromatograms of the purified fractions revealed distinct spots corresponding to the XOS standards, confirming the successful purification and separation of the oligosaccharides.

3.5 HPLC Analysis of Purified XOS

High-Performance Liquid Chromatography (HPLC) was employed to further analyze and quantify the purified XOS. The HPLC analysis provided precise identification and quantification of individual XOS components. The HPLC chromatogram of XOS purification from xylan hydrolysis + endo- β -1,4-D-xylanase pYHM-1 and xylan hydrolysis + endo- β -1,4-D-xylanase pESC are shown in Figure 5.

For the XOS hydrolyzed by endo- β -1,4-D-xylanase pYHM-1, the HPLC chromatogram exhibited two distinct peaks at retention times of 5.233 minutes and 6.108 minutes. The peak at 5.233 minutes was slightly shifted compared to the standard retention times of X2 (5.425) and X3 (5.354), suggesting the presence of impurities or changes in the composition during the purification process. The peak at 6.108 minutes did not align with the retention times of any standard, indicating the presence of additional oligosaccharides or byproducts in the sample.

In contrast, the XOS hydrolyzed by endo- β -1,4-D-xylanase pESC resulted in a chromatogram with four peaks at retention times of 5.326, 5.830, 6.068, and 6.654 minutes. The peak at 5.326 minutes closely matched the retention times of X2 (5.425) and X3 (5.354), confirming the presence of these components. However, the additional peaks at 5.830, 6.068, and 6.654 minutes did not correspond to any standard retention times, indicating the presence of additional components or structural variations resulting from the purification process.

The combined TLC and HPLC analyses consistently identified X2 and X3 as the major components in the purified XOS derived from coffee peel xylan hydrolysis using endo- β -1,4-D-xylanase.

4 DISCUSSION

The findings from this study provide valuable insights into the extraction, enzymatic hydrolysis, and purification of xylan from coffee peel, a promising agricultural waste material. The results align with existing literature, offering further evidence for the potential use of coffee peel as a sustainable source of xylan, a valuable polysaccharide with various industrial applications.

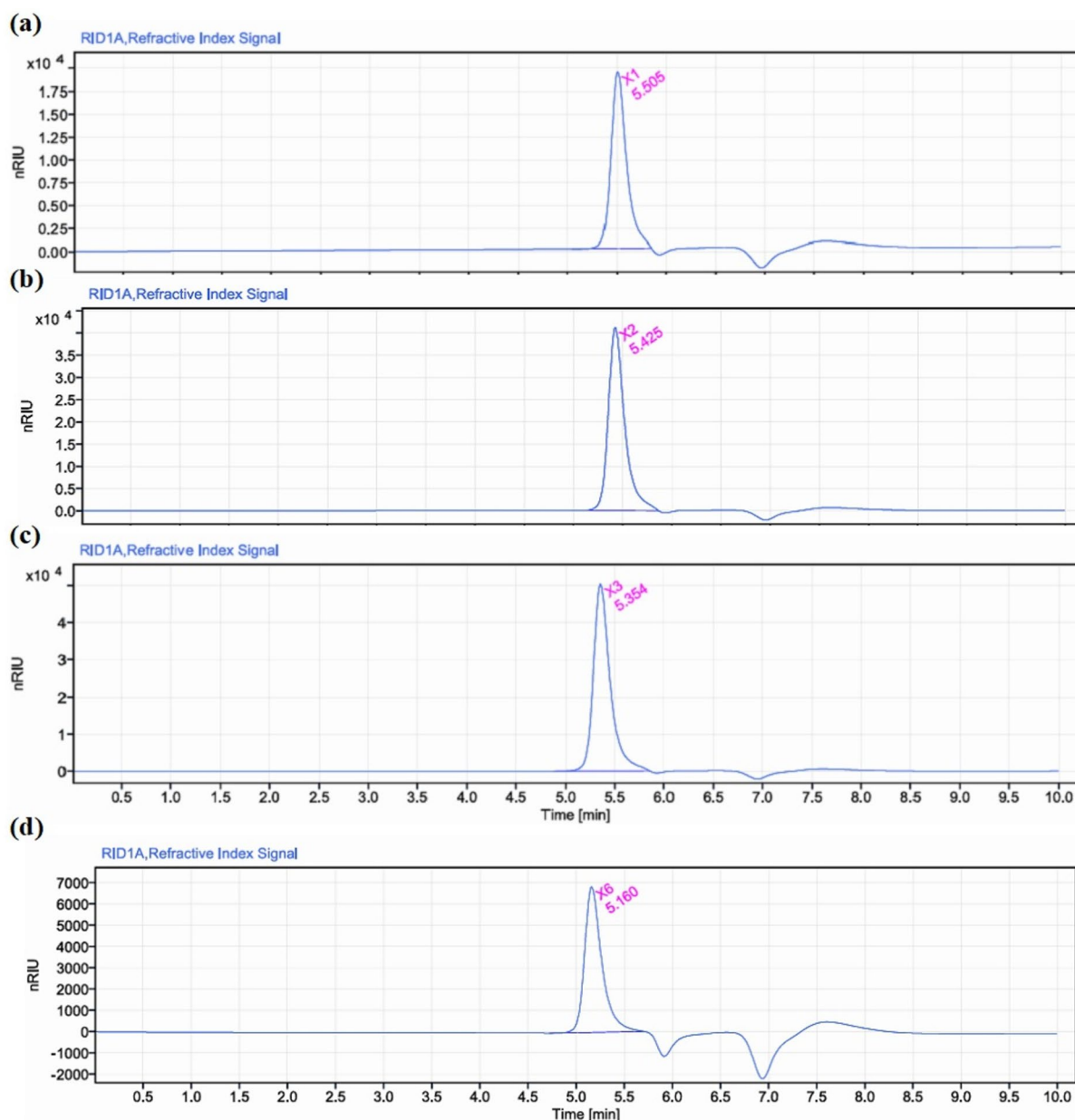


Figure 3: HPLC chromatogram of XOS standard. (a) Monomeric xylose (X1); (b) Xylobiose (X2); (c) Xylotriose (X3); (d) Xylotetraose (X6).

4.1 Xylan Extraction from Coffee Peel

The results of this study align with existing literature on alkaline extraction methods, which emphasize the effectiveness of NaOH in disrupting hemicellulose-cellulose interactions. Sodium hydroxide functions by breaking the ester and ether linkages within the lignocellulosic structure, facilitating the selective solubilization of xylan while allowing other structural polysaccharides, such as cellulose, to remain largely intact (Ali et al., 2025). This targeted breakdown is essential for maximizing xylan recovery while preserving its structural integrity for downstream applications.

The yield of 3.023% (Table 1) obtained in this study is comparable to previously reported values for xylan extracted

from various agricultural residues using alkaline treatments. For example, Valladares-Diestra et al. (2021) demonstrated that alkaline extraction methods are particularly advantageous due to their ability to efficiently break hemicellulose-cellulose interactions, thereby enhancing xylan solubility. However, the relatively low yield observed in this study suggests that process parameters, such as NaOH concentration, extraction time, and temperature, may require further optimization to improve overall efficiency. Studies have shown that increasing the concentration of NaOH can enhance xylan solubilization, but excessive alkalinity may also lead to unwanted degradation of the xylan polymer, reducing its molecular weight and limiting its functional applications. Similarly, extending the extraction time may improve yield but could also increase the risk of

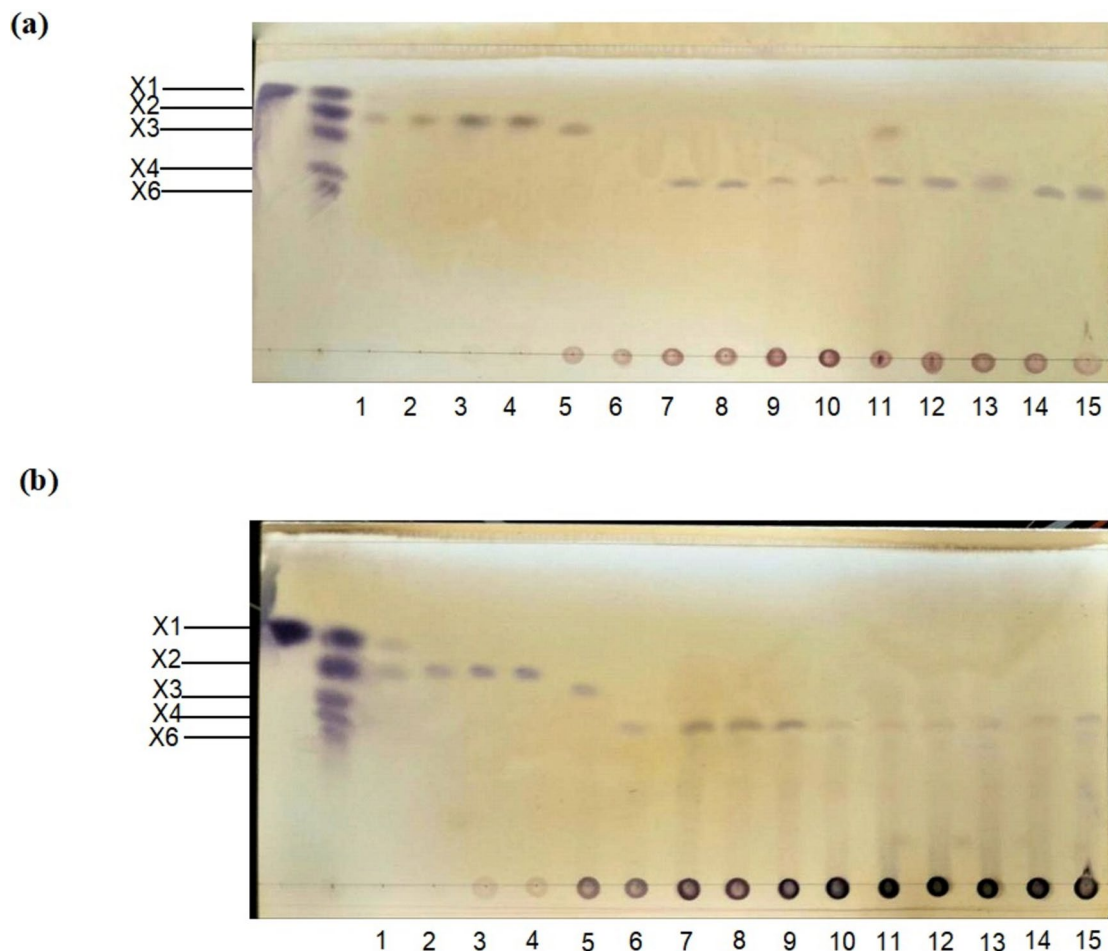


Figure 4: Chromatogram of XOS purification (a) Purification of XOS resulting from xylan hydrolysis + endo- β -1,4-D-xylanase pYHM-1; (b) Purification of XOS resulting from xylan hydrolysis + endo- β -1,4-D-xylanase pESC.

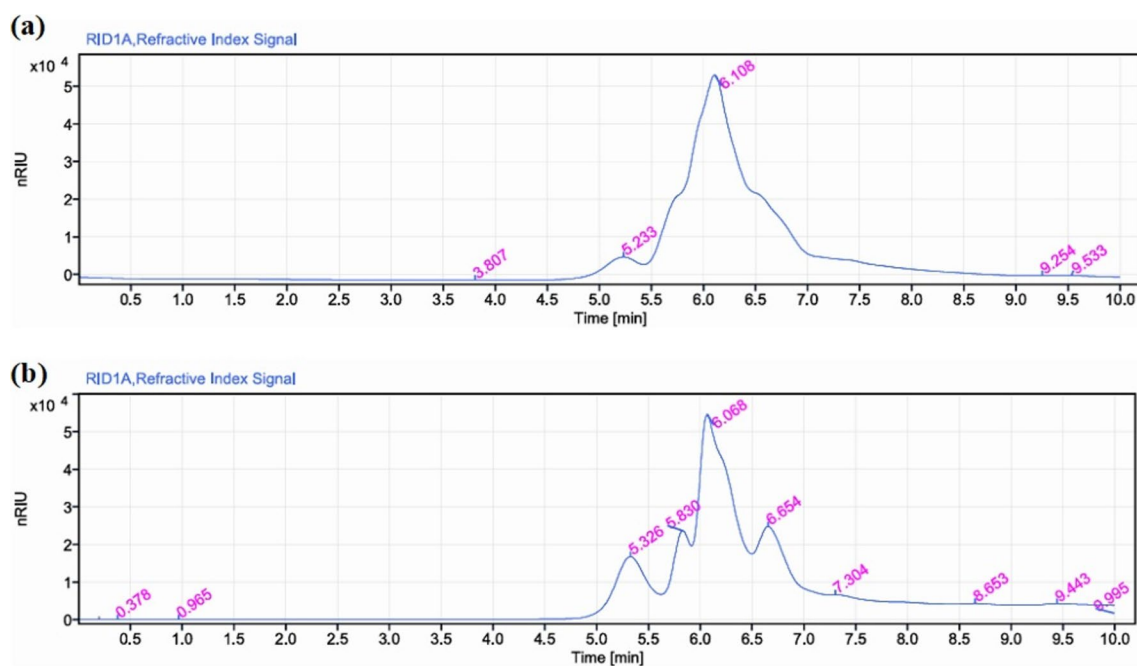


Figure 5: HPLC chromatogram of XOS purification resulting from (a) xylan hydrolysis + endo- β -1,4-D-xylanase pYHM-1; (b) xylan hydrolysis + endo- β -1,4-D-xylanase pESC.

unwanted side reactions, such as lignin dissolution, which may interfere with xylan purity.

Another important factor to consider is the efficiency of the precipitation step. Ethanol precipitation is a widely used technique for xylan recovery, as it reduces the solubility of xylan in solution, leading to its selective aggregation and isolation. However, the precipitation efficiency can be influenced by factors such as ethanol concentration, temperature, and the presence of other solubilized compounds. It is possible that some xylan fractions remained dissolved in the supernatant after precipitation, contributing to the observed yield limitation. The results of this study highlight the potential of coffee peel as a viable source of xylan. By optimizing the extraction process, the recovery of xylan from coffee peel could be further improved, contributing to the sustainable utilization of agro-industrial waste and the development of value-added bioproducts.

4.2 Enzyme Activity and Protein Content

The specific activity values of 4.317 U/mg for pESC-XynBTN63D and 4.222 U/mg for pYHM1-XynBTN63D indicate a high level of enzymatic efficiency in hydrolyzing xylan. These values are within the expected range for recombinant xylanases and suggest that both constructs produce functionally active enzymes. The slight difference in specific activity between the two constructs may be attributed to variations in expression levels, folding efficiency, or post-translational modifications.

The protein concentrations obtained through the Bradford assay further validate the effectiveness of the expression systems used. The measured values of 1.228 mg/mL for pESC-XynBTN63D and 1.161 mg/mL for pYHM1-XynBTN63D indicate a successful production of recombinant xylanase. These protein content values are consistent with previous studies Valladares-Diestra et al. (2023), confirming that the expression and purification processes were effective in producing soluble and active enzyme preparations. The minor variation in protein concentrations between the two constructs could be due to differences in transcriptional regulation, codon usage, or protein stability.

The Miller method, based on the use of DNS reagent, provides a reliable measure of enzyme activity by quantifying the reducing sugars released during xylan hydrolysis. The observed enzyme activity levels confirm the catalytic efficiency of XynBTN63D in degrading xylan into xylooligosaccharides, supporting its potential application in industrial processes. However, the enzyme's hydrolytic performance could be further optimized by adjusting factors such as temperature, pH, substrate concentration, and reaction time. Previous studies have demonstrated that xylanase activity can be enhanced under optimized conditions, leading to improved substrate conversion rates and higher yields of XOS.

These findings highlight the functional potential of XynBTN63D for xylan hydrolysis and its relevance for applications in the production of biofuels, prebiotics, and other biotechnological processes. The comparable enzyme activity and protein content observed in this study suggest that both pESC-XynBTN63D and pYHM1-XynBTN63D constructs are viable expression systems for recombinant xylanase production, with potential for further optimization to maximize enzymatic performance.

4.3 XOS Production from Coffee Peel Xylan

The results from both TLC (Figure 1) and HPLC (Figure 2) analyses confirm that the enzymatic hydrolysis of coffee peel xylan using recombinant endo- β -1,4-D-xylanase selectively produced xylobiose (X2) and xylotriose (X3), with no detectable presence of xylose (X1). The absence of xylose in the hydrolysis products suggests that the enzyme predominantly cleaves internal glycosidic bonds in xylan, rather than acting in an exo-fashion to produce monomeric xylose. This observation aligns well with previous findings by Ratnadewi et al. (2020), which also identified xylobiose as the principal product in similar enzymatic hydrolysis reactions.

Furthermore, these results agree with the findings reported by Kallel et al. (2014), who also observed that selective enzymatic hydrolysis of xylan using endo- β -1,4-D-xylanase led to the predominant formation of higher-degree polymerization (DP) oligosaccharides such as xylobiose and xylotriose, while avoiding excessive degradation into xylose monomers. This selective hydrolysis is particularly advantageous for industrial and functional applications, as xylooligosaccharides with a DP of two or greater have been widely recognized for their prebiotic potential.

The dark brown coloration of the hydrolysis products suggests that some residual lignin or other phenolic compounds remained in the sample despite the extraction and hydrolysis processes. This phenomenon is commonly observed in lignocellulosic biomass-derived oligosaccharides and may require additional purification steps, such as activated carbon treatment or ultrafiltration, to remove these non-carbohydrate impurities for applications requiring high-purity XOS.

The high specificity of the enzyme toward xylan, as demonstrated by the selective production of xylobiose and xylotriose, underscores its potential application in various biotechnological fields, particularly in the production of prebiotics, functional food ingredients, and bioactive compounds. Since XOS are known to promote the growth of beneficial gut microbiota such as *Bifidobacterium* and *Lactobacillus*, the ability to produce them efficiently from coffee peel xylan highlights a promising valorization pathway for agricultural waste. The enzymatic hydrolysis of coffee peel xylan using recombinant endo- β -1,4-D-xylanase effectively yielded xylobiose and xylotriose as major products. The

results confirm that the enzyme operates with high specificity, producing oligosaccharides with potential applications in prebiotics and functional food industries.

4.4 Purification and Analysis of XOS Using Thin Layer Chromatography (TLC) and High-Performance Liquid Chromatography (HPLC)

The purification of XOS through activated carbon adsorption followed by ethanol desorption was highly effective in removing contaminants such as macro-lignin, protein residues, and polysaccharides, which were present in the initial hydrolysate. The purification process yielded XOS fractions with varying degrees of polymerization, demonstrating the selective nature of ethanol in desorbing oligosaccharides. The increasing ethanol concentration facilitated the elution of XOS with higher degrees of polymerization, as evidenced by the TLC results, which revealed distinct spots corresponding to the XOS standards.

The observed residual xylan in the higher ethanol concentration fractions supports the notion that xylan, due to its higher molecular weight and lower solubility, was not completely removed during the purification process. The fact that xylan was more efficiently desorbed with increasing ethanol concentration further indicates the selective nature of the purification method. These results align with those reported by Kim et al. (2022), who found that the purification of XOS from various plant materials using activated carbon and ethanol as desorbents efficiently removed impurities while maintaining the integrity of the oligosaccharides.

The HPLC analysis provided further insight into the purity and composition of the XOS fractions. The chromatogram of XOS purified from the hydrolysis using endo- β -1,4-D-xylanase pYHM-1 (Table 4) revealed the presence of peaks that did not correspond exactly with the standards for X2 and X3 (Figure 3), suggesting the possible formation of impurities or structural variations during purification. In contrast, the XOS purified using endo- β -1,4-D-xylanase pESC (Table 5) showed retention times that were closer to those of X2 and X3 (Figure 3), confirming the presence of these oligosaccharides. However, the presence of additional peaks suggests that the purification process might have introduced other oligosaccharides or byproducts that are yet to be identified.

These findings are consistent with previous research, such as the work by Nordberg et al. (2018), which highlighted the effectiveness of activated carbon in removing impurities from oligosaccharide preparations. Their study also corroborated the notion that the quality of XOS can be significantly improved through efficient purification techniques such as those employed in this study.

Table 4: Quantitative calculation of HPLC chromatogram of XOS purification resulting from xylan hydrolysis + endo- β -1,4-D-xylanase pYHM-1.

Peak*	Retention Time (min)	Width (min)	Height	Area %
1	5.233	1.114	6029.1	5.01
2	6.108	3.653	54262.6	94.29

*Peak 1 corresponds to XOS (xylobiose (X2) and xylotriose (X3)); Peak 2 likely represents oligosaccharides with longer xylose chains or minor impurities.

Table 5: Quantitative calculation of HPLC chromatogram of XOS purification resulting from xylan hydrolysis + endo- β -1,4-D-xylanase pESC.

Peak*	Retention Time (min)	Width (min)	Height	Area %
1	5.326	0.889	16515.8	11.59
2	5.830	0.288	23625.2	8.56
3	6.068	0.599	52553.2	38.62
4	6.654	0.750	24483.0	19.13

*Peaks 1–4 correspond to xylobiose (X2) and xylotriose (X3) as major components, with additional peaks likely representing other purified XOS fractions.

The successful isolation and purification of X2 and X3 oligosaccharides from coffee peel xylan hydrolysis demonstrate the effectiveness of the purification methodology in producing high-purity XOS. The findings of this study have significant implications for the large-scale production of XOS as prebiotics, and the methodology described herein provides a promising approach for obtaining high-quality XOS from renewable biomass sources such as coffee peel.

5 CONCLUSIONS

This study demonstrates the successful purification of xylooligosaccharides (XOS) from coffee peel xylan using endo- β -1,4-D-xylanase and adsorption methods. Xylobiose (X2) and xylotriose (X3) were efficiently isolated with activated carbon and ethanol gradients, with 10–30% ethanol effectively removing residual xylan. These results highlight coffee peel as a promising sustainable source of XOS, though further biological and statistical studies are needed to validate its prebiotic potential and industrial applications.

6 AUTHOR CONTRIBUTION

Conceptual Idea: Ratnadewi, A.A.I.; Methodology design: Ratnadewi, A.A.I.; Anggraini, R.; Yulvia, A.; Kusuma,

A.A.N.N.; Data collection: Anggraini, R.; Data analysis and interpretation: Ratnadewi, A.A.I.; Anggraini, R.; and Writing and editing: Yulvia, A.; Kusuma, A.A.N.N.

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