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Microwave-assisted Xylanase Hydrolysis Produced by *Trichoderma asperellum* AW2 for the Production of Bioactive Water-soluble Rice Straw Hydrolyzate

Wafaa A Helmy[1], Amira A Hassan[1], Shaimaa A Nour[1], Shaymaa A Ismail[1*]

[1]*Department of Chemistry of Natural and Microbial Products, Pharmaceutical and Drug Industries research institute, National Research Centre, El Bohouth Street, Dokki, Giza 12622, Egypt*

Abstract

Conversion of lignocellulosic biomasses into value-added commodities relies on the depolymerization of their constitutive components. Herein, submerged fermentation of rice straw using *Trichoderma asperellum* AW2 was initially carried out with the production of xylanase activity of 96.638 U/mL. The produced xylanase was characterized in which the highest enzymatic activity was estimated at 50 °C, in the working pH range 5-6 using beech wood xylan concentration of 17.5 mg/mL, with half-life time of 86.6 min at this temperature. In addition, it was an endo-acting enzyme with the capability for releasing xylooligosaccharides. The applicability of the produced xylanase in the hydrolysis of untreated rice straw was evaluated. The result estimated the releasing of 19.9 mg/g of reducing sugars after 8 h that was improved by about 70% by microwave pre-treatment of rice straw with the releasing of mono-sugars in addition to xylooligosaccharides of different degrees of polymerization as it confirmed with the aid of thin layer and high-performance liquid chromatographic analysis. Moreover, the scanning electron microscopy of the waste residue confirmed the potency of the produced xylanase for depolymerizing the hemicellulosic fraction of rice straw. Finally, the antioxidant and the Fibrinolytic activity of the hydrolyzate were estimated in comparison to xylanhydrolyzate.

Keywords: *Trichoderma asperellum* AW2*;* Rice straw; Xylanase; Microwave pre-treatment; Bioactive hydrolyzate

1. Introduction

Biocatalysts in general are highly specific enzymes produced by all domains of life that can promote the bio-conversion of a targeted substrate. In the last two decades, the enzyme industry had attracted a growing interest, as it had been extensively exploited in various industrial processes, with a specific great scope on the microbial sources [1]. Xylanases are enzymes having the ability to catalyze the hydrolysis of xylan, a polysaccharide of β-1,4-glycosidic linked xylose chains branched with different residues in the side chain including D-glucuronic and methyl-glucuronic acid in addition to L-arabinose and acetic acid [2]. Xylan-degrading enzymes have drawn a great interest owing to their prevalence applications in various fields including ethanol production [3], clarification of juice [4], animal feed production [5], detergent industries [6], textile industry [7], pulp bleaching [8], impart a plant immunogenicity [9] and possess antifungal activity [10]. Moreover, xylanases are widely utilized for the hydrolysis of xylan producing xylooligosaccharides (XOS), 2-6 linked xylose units bonded by β-1,4 glycosidic bonds, that possess various biological activities [11].

In general, hemicellulose constitutes about 20-35 % of various lignocellulosic biomasses in which xylan participates as its major component [12]. Therefore, lignocellulosic biomasses are the most attractive producers of XOS [13]. The application of high temperature and high pressure (auto hydrolysis) as well as the use of acids and enzymes are the most commonly applied methods for the decomposition of lignocellulosic biomasses. Enzymatic hydrolysis of biomasses is considered as the optimal method due to its specificity, employed mild conditions and minimal environmental impacts. The high productivity cost of the applied enzymes with the low yield of the products are the major disadvantages that obstacles its industrial applications [14], reflecting the importance of the economic production of highly specific enzymes. Additionally, the application of combined strategies for physical or chemical pre-

*Corresponding author e-mail: shaymaaabdallaismail@gmail.com.; (Shaymaa A Ismail).

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treatments with the use of enzymes has attracted a great interest as they can reduce the recalcitrance of lignocellulosic composite facilitating the XOS productivity [15-17].

Various lignocellulosic biomasses were reported for the economic production of xylanases using various microorganisms, bacteria [18], fungi [19] and yeast [20]. Among different microorganisms, fungi have been reported as potent xylanase producers where the genus *Aspergillus* and *Trichoderma* are commercially applied for their industrial production. Generally, there are two main techniques for the microbial production of enzymes; submerged and solid-state fermentation in which the former is characterized by accelerated production rate, high yield and adequate oxygen supply in addition to its suitability for fungi in need of high moisture content for their growth [21-22]. In fermentation technology, the conditions of the fermentation process crucially affect the growth of microorganisms and consequently its product of interest, reflecting the requirements of optimized conditions for producing the desired product and reducing its cost [6].

The selection of the suitable waste for the appropriate growth of the microorganism and the production of enzyme by applying the fermentation technology depends on a plethora of physico-chemical parameters related to its cost and availability [22]. Additionally, the utilization of lignocellulosic biomasses as substrates in the fermentation process provided a whole range of benefits in terms of productivity, cost efficiency, time and media components with the reduction of the pollution load on the environment. Herein, rice straw waste was utilized for producing xylanase under submerged fermentation applying *Trichoderma asperellum* AW2 followed by optimization of the fermentation conditions for the highest enzymatic productivity. The enzymatic activity of the produced fraction was optimized regarding the effect of the temperature, pH, and the concentration of the substrate, followed by determining its kinetic and thermal constants. The hydrolytic potentiality of the produced enzyme was initially evaluated by using beech wood xylan followed by establishing its applicability for the hydrolysis of rice straw. The impact of microwave pre-treatment on the enzymatic hydrolysis of rice straw was also examined. Finally, the antioxidant and Fibrinolytic activities of the dried hydrolyzate resulted from the enzymatic hydrolysis of rice straw were tested in comparison to beech wood xylanhydolyzate.

2. **Materials and methods**

2.1. Microorganism

The examined fungus had been isolated by the research team in the Chemistry of Natural and Microbial Products Department, National Research Centre, Dokki, Giza, Egypt during a screening program focused on the isolation of microorganisms producing cellulose and hemicellulose degrading enzymes utilizing some locally available lignocellulosic biomasses. Identification of the fungus was carried out depending on its cultural and light microscope examined characteristics followed by 18S rDNA sequencing performed in Sigma Scientific Services Co. applying ITS1 and ITS4 primers. The phylogenetic analysis was performed on the base of neighbor joining method followed by construction of the tree using MEGA x [23].

2.2. Enzyme production

It was carried under submerged fermentation conditions of rice straw that had been collected directly from the fields after air drying followed by cutting into small pieces and grinding (initial moisture content was zero). Initially, the isolated strain was cultured on PDA slants (Merck, Darmstadt, Germany) for 7 days at 30 ^{o}C, then each slant cultivated an inoculum medium of 50 mL composed of (g %), dextrose, 2; yeast extract, 0.05; peptone, 0.5; $MgSO_4.7H_2O$, 0.05; and KH_2PO_4, 0.1 followed by incubation at 30 °C for 48 h. The inoculum (10% v/v) had been used to cultivate 0.5 g of rice straw suspended in 50mL tape water that was incubated for 8 days at 30 °C and agitation speed of 180rpm. The culture media were centrifuged at 4500rpm (4°C) for 10min where the enzymatic activity of the clear solution was assayed.

Xylanase activity was estimated using 1 % beech wood xylan (SERVA, Heidelberg, Germany) dissolved in phosphate buffer of pH 6 (0.05 M). Xylan solution (500 µL) was added to 500 µL of the cell free supernatant and incubated at 50 °C for 30 min [24]. The released reducing sugars were estimated according to Miller, [25] in which 2.5 mL of DNS solution was added immediately at the end of the incubation period to the reaction mixture then placed in a boiling water bath for 10 min. After cooling, the optical density was determined at 540 nm. One enzyme unit was identified as the capability of the enzyme for releasing one µmol of reducing sugars per minute under the assaying conditions using xylose as the standard.

2.3. Optimization of the enzymatic productivity

2.3.1. One-factor-at-a-time

Replacement and supplementation were carried out in each experiment utilizing the previous conducted optimum conditions. The impact of the fermentation time, different concentrations of the carbon source,

different nitrogen sources with an equivalent content of nitrogen to the constituent nitrogen of Mandel's medium [26] that composed of (g/L) $CaCl_2$; 0.3, $MgSO_4.7H_2O$; 0.3, KH_2PO_4; 2, urea; 0.63, peptone; 0.75 and $(NH_4)_2SO_4$; 1.4 was evaluated. In addition, the effect of different concentrations of the selected nitrogen source was examined.

2.3.2. Statistical optimization

Two sequential steps were applied in which the significant variables affecting the enzyme productivity were initially identified based on Plackett-Burman design [27]followed by optimization of the selected variables according to Box-Behnken design.

In Plackett-Burman design, seven independent variables namely; concentration (%) of rice straw, peptone, KH_2PO_4, $CaCl_2$ and $MgSO_4.7H_2O$, initial pH, and the fermentation period, were screened in separate eight experimental runs where each variable was examined at two levels, high (+1) and low (-1). The influence of each variable was estimated by calculating its effect according to the following equation:

$$E_{(Xi)} = 2(\Sigma M_{i+} - M_{i-})/N \quad \text{Eq. (1)}$$

$E_{(Xi)}$ is the effect of the variable under investigation, M_{i+} and M_{i-} represent the xylanase activity where the variable (Xi) adjusted at +1 and -1 values, respectively where N is the number of trials.

In Box-Behnken design, different levels of the significant variables, low (-), basal (0) and high (+), were examined in 15 experimental runs with 3 central points. Correlation between the enzyme activity and the examined variables was interpreted according to the following equation:

$$Y = B_0 + \Sigma B_i X_i + \Sigma B_{ii} X_i^2 + \Sigma B_{ij} X_i X_j \quad \text{Eq. (2)}$$

where Y is the predicted enzyme activity; $ß_0$, $ß_i$, $ß_{ii}$and $ß_{ij}$are the intercept of the model, linear, quadratic and cross product coefficients, respectively where X_i and $X_j$are the coded levels of the variables under investigation.

2.4. Enzyme partial purification

The enzyme of the culture supernatant was fractionally precipitated (by applying acetone or ethanol as solvents) in addition to ammonium sulfate salting out covering the concentration range from 20 to 90% with 10% intervals. The enzymatic activity of each fraction was assayed as above and the protein content was estimated according to Lowry *et al*., [28]. The enzyme fraction that possessed the proper specific activity was then subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as described in details by Laemmli [29].

2.5. Characterization of the produced fraction

2.5.1. Influence of pH

The influence of the pH on the enzymatic activity was evaluated by the determination of the enzymatic activity within the pH ranges from 4 to 5 for 0.05 M acetate buffer, and from 6 to 8 applying 0.05 M phosphate buffer solutions. In addition, the residual activity was estimated after its incubation at the optimum pH for different incubation periods up to 120 min in which the primary enzymatic activity at the zero time was assigned as the 100% activity.

2.5.2. Effect of temperature

The thermal effect on the enzymatic activity was studied at different temperatures (35-70 ^oC) where the activation energy (E_a) of the enzyme was estimated based on the slope of the Arrhenius plot as following:

$$\text{Slope} = -E_a/R \quad \text{Eq. (3)}$$

Moreover, the thermal stability of the enzyme was also examined at different temperatures (40-50 ^oC) through monitoring of the residual enzymatic activity under the conducted optimum conditions after its pre-incubation (in absence of its substrate) for different incubation periods up to 120 min. The primary enzyme activity at the zero time was assigned as the 100% activity. The thermostability parameters of the enzyme were estimated as follow:

Slope of Arrhenius plot ($\ln K_d$ versus 1/T) $= -E_d/R$ Eq. (4)

$$T_{1/2} = \ln(2)/K_d \quad \text{Eq. (5)}$$

Decimal reduction time (D-value) $= \ln(10)/ K_d$ Eq. (6)

$$\text{Enthalpy}(\Delta H_d) = E_d - RT \quad \text{Eq. (7)}$$

$$\text{Free energy } (\Delta G_d) = -RT*\ln(K_d h/K_b T) \quad \text{Eq. (8)}$$

$$\text{Entropy } (\Delta S_d) = (\Delta H_d - \Delta G_d)/T \quad \text{Eq. (9)}$$

where K_d is the thermal deactivation rate constant, T is the temperature (K), E_d is the decay activation energy ($KJmol^{-1}$), R is the gas constant ($8.3145\ Jmol^{-1}K^{-1}$), h is Planck constant ($6.626*10^{-34}$ J.S) and $K_b$is Boltzman constant ($1.38*10^{-23}\ JK^{-1}$).

2.5.3. Substrate specificity

The enzyme activity was estimated using substrates other than beech wood xylan, specified for the determination of cellulytic and hemicellulytic enzymes activity, purchased from Sigma-Aldrich, Saint Louis, USA. Locust bean gum, carboxy-methyl cellulose (CMC), and pectin were used at concentration of 1.0 % (see section 2.2), while the enzyme activity applying other synthetic substrates including (p-nitrophenyl-β-D-xylopyranoside, p-nitrophenyl-β-D-galacto-pyranoside, and p-nitrophenyl-β-D-glucopyranoside) was determined at substrate concentration level of 0.1 % [30].

2.5.3. Effect of beech wood xylan concentration
The enzymatic activity was evaluated at the conducted optimized conditions applying different concentrations of beech wood xylanranging from 2.5 to 25 mg/mL.

2.6. Hydrolytic activity of the produced enzyme
2.6.1. Hydrolysis of beech wood xylan
The applicability of the produced xylanase fraction in the hydrolysis of beech wood xylan was examined by adding 1mL of the enzyme solution (2 U/mL) to 1 mL of the substrate (17.5 mg/mL) followed by incubation at 40°C for different hydrolysis intervals (1-6 h). At the end of each hydrolysis period, denaturation of the added protein was performed by boiling of the reaction mixture for 10 min and centrifugation at 4000rpm for 10 min at 4°C. The released amounts of reducing sugars from the enzymatic reaction were estimated according to Miller, [25]. The hydrolysis percentage was estimated based on the following equation:

$$\text{Hydrolysis percentage} = (A_S / A_T) * 100 \qquad \text{Eq. (10)}$$

where A_S was the released amount of reducing sugars in the sample and A_T was the added amount of xylan in the reaction.
After conducting of the optimum period of hydrolysis, the effect of the enzyme activity (0.78-50 U/mL) added in the reaction mixture was examined.

2.6.2. Hydrolysis of rice straw
The produced xylanase fraction was examined in the direct hydrolysis of rice straw. One gram of rice straw was initially suspended in 5 mL of 0.2 M acetate buffer at pH 5 and 5 mL of the enzyme with the optimum enzyme activity conducted during the hydrolysis of beech wood xylan was added then incubated at 40°C for different hydrolysis periods (2-22 h). By the end of the hydrolysis process, boiling for 10 min, filtration with a piece of cotton mesh and centrifugation for 10 min at 4000 rpm and 4 °C then air drying of the clear supernatant were performed. Microwave pre-treatment of the suspended rice straw for 1 min was also examined and the released reducing sugar was quantified as usual. Surface scanning of rice straw before and after treatment was performed under field emission high resolution scanning electron microscope (Quanta 250, HRFEG, Czech) with magnification of 3000 x and accelerating voltage of 20 KV.

2.6.3. Thin layer chromatographic analysis
Thin layer chromatography (TLC) analysis of the clear supernatants (50 µL each) was carried out using silica gel 60 TLC plates (Merck, Darmstadt, Germany). The applied mobile phase was propanol:water at ratio of 8.5:1.5 (v/v) and the spraying reagent used for visual detection of the produced sugars was phenol-sulfuric acid [31].

2.6.4. Monosaccharide constituent
The mono-constituent sugars of the dried samples were determined according to Block et al., [32]. Initially, acid hydrolysis was performed in a boiling water bath using 0.1 M HCl for 1 h then the hydrolyzed products were analyzed by descending paper-chromatography using What man No. 1 sheets, n-BuOH–MeCO–H_2O (4:5:1 v/v/v) as a solvent [33] and aniline phthalate as a spraying reagent [34]. Quantification of the separated sugars was estimated according to Wilson, [35].

2.6.5. Fourier transforms infrared spectroscopy
The nature of the chemical bonds and active functional groups of the dried hydrolyzate were evaluated by Fourier transform infrared (FTIR) spectroscopy (Vertex 80v, Bruker) analysis.

2.6.6. High performance liquid chromatography
The produced XOS in the dried hydrolyzate was assayed by high performance liquid chromatography (HPLC) using xylose, xylobiose, xylotrise and xylotetrose (Megazyme, Wicklow, Ireland) as standards. HPLC analysis was carried out applying Agilent Technology 1100 series liquid chromatograph accompanied with a refractive index detector. Shim-pack SCR-101N column with the use of ultrapure water adjusted at flow rate 0.7 mL/min as the mobile phase was applied.

2.7. Biological activity
The antioxidant as well as the fibrinolytic activity of rice straw dried hydrolyzate was examined and compared to beech wood xylanhydrolyzate sample.

2.7.1. Antioxidant activity
It had been initially evaluated for beech wood xylanhydrolyzate in which different concentrations of the sample (20-200 mg/mL) were tested for their scavenging activity of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical (Sigma-Aldrich, Saint Louis, USA). The sample solution (0.1 mL) was added to the methanol solution (3.9 mL) of DPPH radical (1.1×10^{-4} mol/L) and left in dark place for 30 min and the decrease in the absorbance at 515 nm was monitored spectrophotometrically. The results were expressed as the µg Trolox Equivalents (TE)/mg of the dry sample [36]. Moreover, the antioxidant activity of rice straw hydrolyzate was also examined at the concentration possessed the highest DPPH scavenging activity.

2.7.2. Fibrinolytic activity

The Fibrinolytic activity of the hydrolyzate sample was optically observed in which 1mL of the sample solution (0.2% w/v) was added to a prepared clot followed by incubation at 37°C for 24h. The clot was prepared by incubating a mixture composed of 0.8mL saline solution, 0.2mL of $CaCl_2$ solution (2% w/v) and 1mL plasma in a water bath at 37°C [37].

2. Results and discussion

2.1. Microorganism

The cultural features of the isolated fungal strain grown on PDA medium showed its greenish coloration (Fig. 1A) with light microscope examination illustrated in figure (1B). Next, the 18S rDNA sequencing analysis estimated its similarity to *Trichoderma asperellum*and the constructed phylogenetic tree was illustrated in figure (1C). By submitting the sequence data to NCBI, the isolated strain named *Trichoderma asperellum*AW2 had received accession number of ON909038. Although several microorganisms had been reported as xylanase producers, filamentous fungi are the main source for its commercial production in which *Trichoderma* sp. had been reported as a suitable candidate [10, 38, 39, 40 and 41].

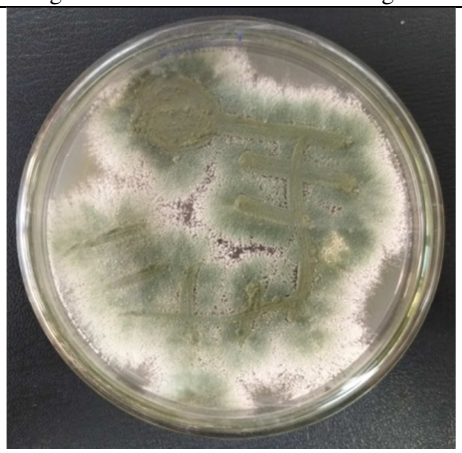
3.2. Xylanase production

In general, lignocellulosic biomasses are renewable resources that composed mainly of cellulosic (35-50 %) and hemicellulosic (20-35 %) fractions which are covalently linked with lignin fraction (5-30 %) [42]. The constitutive polysaccharides of lignocellulosic biomasses offer promising futures for the manufacturing of microbial enzymes with various biotechnological applications [43-47]. Xylanases are essential enzymes which contribute in the biodegradation of the hemicellulosic constitute of lignocellulosic biomasses, specifically xylan, producing various bioactive products. Recently, various lignocellulosic biomasses including corn cobs [6], sugarcane bagasse [10], tangerine peel [48], wheat straw and wheat bran [19] had been used for xylanases production. In the current study, xylanase production with initial activity of 4.774 U/mL was estimated by submerged fermentation of rice straw.

3.3. Optimization of xylanase production

3.3.1. One-variable-at-a-time

The effect of the incubation period as well as the concentration of rice straw and the nitrogen content was tested and the results indicated that the highest enzymatic activity (74.352 U/mL) was monitored after 8 days fermentation period through the fermentation of 2.0 % rice straw suspended in 50 mL of modified Mandel's medium (peptone (0.25 %) was the sole nitrogen source) (Table 1). Production of xylanase utilizing rice straw as a substrate had been previously reported by Ketsakhon*et al*., [49] and by examining the effect of its concentration; the optimum concentration for the enzyme productivity was similar to that estimated in the current study (2 %). Additionally, the nitrogen source is a crucial variable for enzymes production. Therefore, the efficiency of various nitrogen sources on the productivity of *Trichoderma asperellum*AW2 xylanase was examined. The results indicated that the maximum productivity was achieved (64.860 U/mL) by using peptone that increased to 74.352 U/mL by adjusting the peptone concentration at 0.25 %. This result agreed with that mentioned by Dhaver*et al*., [38] and Bakry *et al*., [50]. In general, the incubation period varied by the variation in the species as well as the used substrate. The optimum incubation period had been estimated as 3 days for the fermentation of a mixture of corn cobs and wheat bran using *Trichoderma orientalis*[40], 5 days for the fermentation of wheat bran using *Trichoderma harzianum*[38] and 7 days for the fermentation of wheat chaff using *Trichoderma reesei*[51].



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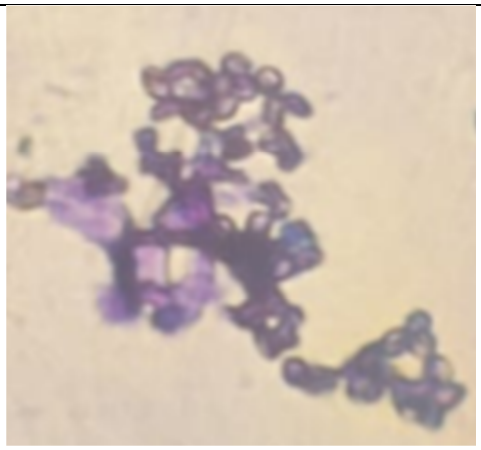

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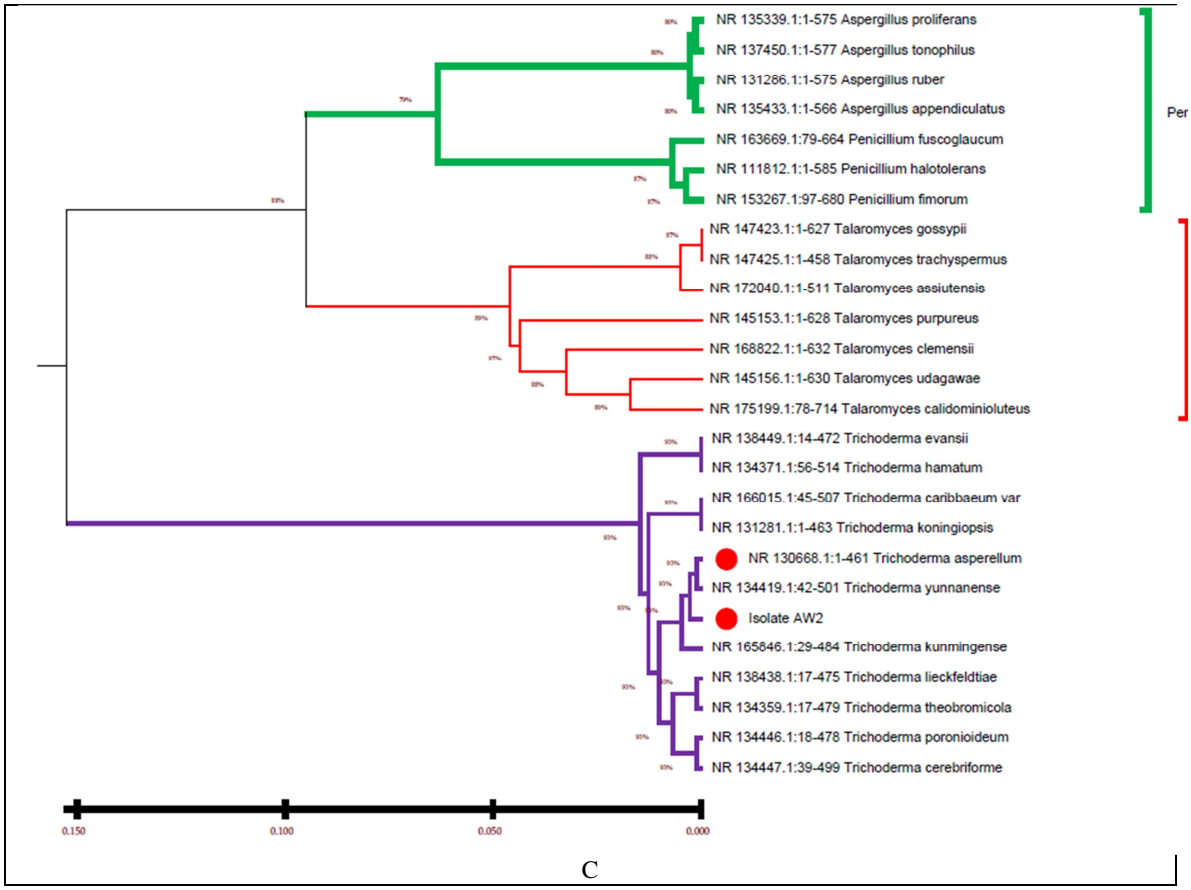


Fig. 1: (A) The isolated fungus grown on PDA cultural medium, (B) microscopic features, and (C) the phylogenetic tree constructed using MEGA X

Table 1: One-variable-at-a-time optimization of the culture media

Incubation period (days)	**4**		**8**		**12**		**15**
Xylanase activity (U/mL)	2.559 ± 0.196		4.774 ± 0.318		2.468 ± 0.071		2.189 ± 0.002
Concentration of rice straw (%w/v)	**0.5**	**1**	**2**		**4**		**6**
Xylanase activity (U/mL)	2.171 ± 0.391	5.132 ± 0.094	7.129 ± 1.364		6.258 ± 0.396		5.983 ± 0.158
Nitrogen source	**Control**	**Mandel's medium**	**Peptone**	**Urea**	**$(NH_4)_2SO_4$**	**Yeast extract**	**Corn steep liquor**
Xylanase activity (U/mL)	7.58 ± 0.212	1.871 ± 0.000	64.860 ± 1.951	0.486 ± 0.294	18.662 ± 0.329	-	8.926 ± 0.094
Concentration of peptone (%w/v)	**0.1**	**0.25**	**0.5**		**1**	**1.5**	**2**
Xylanase activity (U/mL)	25.173 ± 1.496	74.352 ± 4.828	64.860 ± 2.426		2.515 ± 0.134	0.678 ± 0.185	0.190 ± 0.049

3.3.2. Statistical optimization

Statistical technique is a popular method applied successfully in the optimization of various microbial enzymes. It examines the interactive effects of various parameters, maximizing the productivity and saving time that consequently reduces the overall cost of enzyme production [52-54]. Moreover, it has been

usually applied for the optimization of xylanase production [6, 39, 48 and 50, 55].

Herein, the production of *Trichoderma asperellum*AW2 xylanase was statistically optimized by applying two sequential designs. Initially, Plackett-Burman design was applied in which the effect of seven independent variables was examined. The means of the obtained results were tabulated (Table 2) indicating a wide range of variation. The highest activity (89.561 U/mL) was monitored at the fourth run by adjusting the composition of the fermentation medium as follow; concentration of rice straw, 3 %; peptone concentration, 0.4 %; KH_2PO_4 concentration, 0.2 %; $CaCl_2$ concentration, 0.3 % and $MgSO_4.7H_2O$, 0.03 % with initial pH 5 and incubated for 6 days. The overall significance of the design was derived according to the analysis of variance (ANOVA) indicating the Prob> F value of 1.33 E^{-4}. Additionally, the multiple regression analysis of the data indicated the significance (*P*-value < 0.05) of the concentration of rice straw, peptone and KH_2PO_4 while the other variables were insignificant (Supplementary table 1). Moreover, the correlation coefficient (R^2) value was 0.94, indicating that 94 % variation in the experimental results was attributed to the examined independent variables. Edwards *et al*., [56], reported that high R^2 values (more than 0.9) manifested the accuracy of the applied design. As it illustrated in figure 2, the concentration of rice straw and peptone exerted a positive effect while the concentration of KH_2PO_4 exerted a negative effect. The positive value indicated that the higher effect was observed when the variable was at the positive level (+1) while the negative value was vice versa.

In the second step, the significant variables were optimized based on Box-Behnken design. The obtained results indicated that the highest xylanase productivity (96.638 U/mL) was monitored at rice straw concentration, 4 %; peptone concentration, 0.3 % and KH_2PO_4 concentration, 0.2 % (Table 3). The overall significance of the design was estimated from the ANOVA indicated that the estimated Prob> F value was 3.22E^{-9}. The multiple regression coefficient of the data indicated the acceptable accuracy of the proposed model as the R^2 value was 0.92 (Supplementary table 2). The predicted xylanase activity was estimated based on the following second order polynomial equation:

$$Y= -250.999+ 99.69728X_1 + 587.3278X_2 +377.298X_3 –15.1362X_1^2 –818.388X_2^2 –1198.02X_3^2 – 1.73461X_1X_2 + 45.06858X_1X_3 +202.9838X_2X_3$$

Eq. (11)

where, Y expressed the predicted enzymatic activity and X_1, X_2, and X_3 were the concentration of rice straw, peptone and KH_2PO_4, respectively. Additionally, by plotting of the residual plot, the design was manifested to be correct on average for all of the reported results (Fig. 3).

It can be concluded that the achieved highest enzymatic activity (96.638 U/mL) was 20.2-fold higher than the estimated initial activity and higher than the optimized values reported for xylanase production under submerged fermentation of various lignocellulosic biomasses using fungal strains, 0.1676 U/mL reported for *T. reesei*QM 9414 on the fermentation of wheat chaff [51], 84 U/mL reported for *Aspergillus niger* on the fermentation of wheat bran [57] and 34.63 U/mL reported for *Fusarium* sp. on the fermentation of wheat bran [58]. On the other hand, Dhaver*et al*., [38] reported the production of 153.8 U/mL for *T. harzianum* on the fermentation of wheat bran and Ellatif*et al*., [10] estimated the production of 181.01U/mL for *T. harzianum*on the fermentation of sugarcane bagasse.

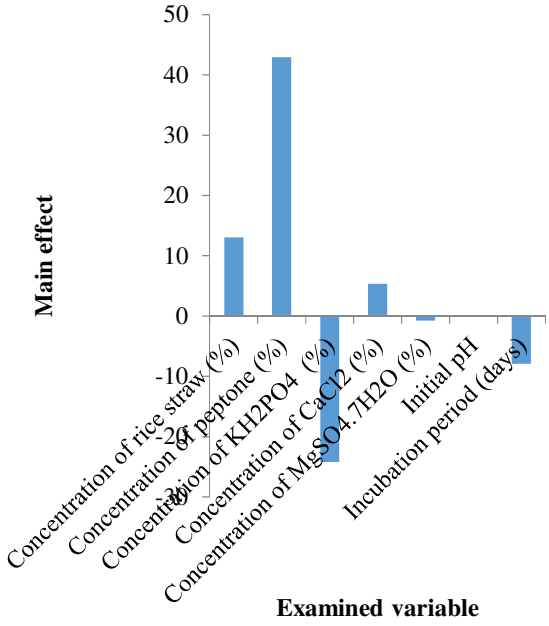


Fig. 2: The main effect of the variables examined in Plackett Burman design

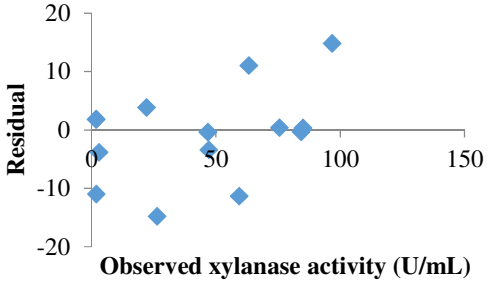


Fig. 3: Residual plot

3.4. Partial purification of the enzyme

The fractional precipitation of the crude enzyme in the culture supernatant applying acetone or ethanol as well as ammonium sulfate salting out was examined. The result estimated that the 40 % fraction salted out by ammonium sulfate exhibited specific enzymatic activity of 178.06 U/mg protein, with 4.91 purification fold and 25.29 % recovered activity yield. In addition, the 50 % fraction showed a specific enzymatic activity of 159.28 U/mg protein with 4.39 purification fold and 26.94 % recovered activity yield, respectively. Collection of both fractions (the specific activity was 168.67 U/ mg protein) had been used in the next experiments. Figure (4) presented the SDS-PAGE for the collected fraction. In the current study, the specific activity of the precipitated fraction (40-50 %) was extremely higher than 20.53 U/mg reported by Ameen, [19].

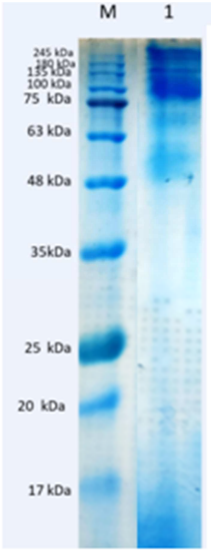


Fig. 4: SDS-PAGE analysis of 40-50% ammonium sulfate salted out fraction (1) in which M is the marker

Table 2: Plackett Burman design

Run number	Concentration of rice straw (% w/v)	Concentration of peptone (% w/v)	Concentration of KH_2PO_4 (% w/v)	Concentration of $CaCl_2$ (% w/v)	Concentration of $MgSO_4.7H_2O$ (% w/v)	Initial pH	Fermentation period (days)	Xylanase activity (U/mL)
1	-(2)	-(0.1)	-(0.2)	+(0.3)	+(0.3)	+(7)	-(6)	32.75495
2	+(3)	-(0.1)	-(0.2)	-(0.03)	-(0.03)	+(7)	+(10)	33.32667
3	-(2)	+(0.4)	-(0.2)	-(0.03)	+(0.3)	-(5)	+(10)	62.45698
4	+(3)	+(0.4)	-(0.2)	+(0.3)	-(0.03)	-(5)	-(6)	89.56127
5	-(2)	-(0.1)	+(2)	+(0.3)	-(0.03)	-(5)	+(10)	1.476498
6	+(3)	-(0.1)	+(2)	-(0.03)	+(0.3)	-(5)	-(6)	16.26923
7	-(2)	+(0.4)	+(2)	-(0.03)	-(0.03)	+(7)	-(6)	46.89269
8	+(3)	+(0.4)	+(2)	+(0.3)	+(0.3)	+(7)	+(10)	56.70641

Table 3: Box-Behnken Design

Run number	Independent variable			Xylanase activity (U/mL)	
	Concentration of rice straw (% w/v) X_1	Concentration of peptone (% w/v) X_2	Concentration of KH_2PO_4 (% w/v) X_3	Observed	Predicted
1	- (2)	- (0.3)	0 (0.2)	46.70952	47.09867
2	+ (4)	- (0.3)	0 (0.2)	96.63847	81.8455
3	- (2)	+ (0.5)	0 (0.2)	26.25502	41.04766
4	+ (4)	+ (0.5)	0 (0.2)	75.49013	75.10064
5	- (2)	0 (0.4)	- (0)	1.817869	0.0
6	+ (4)	0 (0.4)	- (0)	3.008504	6.824584
7	- (2)	0 (0.4)	+ (0.4)	22.03646	18.22038
8	+ (4)	0 (0.4)	+ (0.4)	59.28196	70.64772
9	0 (3)	- (0.3)	- (0)	1.873376	12.84931
10	0 (3)	+ (0.5)	- (0)	1.759586	0.0
11	0 (3)	- (0.3)	+ (0.4)	47.09807	50.52566
12	0 (3)	+ (0.5)	+ (0.4)	63.22298	52.24708
13	0 (3)	0 (0.4)	0 (0.2)	84.26031	84.5932
14	0 (3)	0 (0.4)	0 (0.2)	84.9264	84.5932
15	0 (3)	0 (0.4)	0 (0.2)	84.59335	84.5932

3.5. Characterization of the produced xylanase

3.5.1. Influence of pH

The enzymatic activity of the produced xylanase fraction had been examined at various pH values (4-8). The optimal enzymatic activity for Trichoderma asperellum AW2 xylanase was recorded at the pH range 5-6 that decreased to about 38 % at pH 6.5. At neutral pH, the enzyme activity decreased by 66 % that approximately diminished at pH 8 (Fig. 5A). Moreover, the impact of the pre-incubation period of the enzyme at the selected pH value revealed maintenance of the initial enzyme activity for more than 2 h (Fig. 5B). The achieved results consisted with that reported for xylanases produced by Trichoderma sp., indicated the acidic conditions as the optimum for the enzyme activity [10, 38 and 59].

3.5.2. Effect of temperature

The enzymatic activity had been evaluated at various incubation temperatures ranged from 35 to 70 °C. The experimental data indicated that the optimum activity for *Trichoderma asperellum*AW2 xylanase was monitored at 50 °C which decreased by about 5 % at 55 °C (Fig. 6A). In addition, thermal stability at temperature values up to 50 °C had been illustrated in figure (6B). The enzyme half-life at its optimum temperature was 86.6 min (Table 6). The presented results agreed with that indicated by Ezeilo*et al*., [59] in which the optimum activity for *Trichoderma asperellum* UC1 xylanase was estimated at 50 °C while Ellatif*et al*., [10], reported that the incubation at 40 °C as the optimum degree for xylanase produced by *Trichoderma harzianum*.

Based on Arrhenius plot illustrated in Figure 6C, D, the estimated thermal activation and inactivation energy values were 38.047 and 219.17 KJ mol^{-1}, respectively. Moreover, the thermal denaturation constants and the thermodynamic denaturation parameters including ΔH, ΔG and ΔS at temperature from 40 to 50 °C were illustrated in table (4).

3.5.3. Substrate specificity

By applying beech wood xylan as a reaction substrate, the produced xylanase fraction possessed its highest activity (167.851 U/mg protein) but a very low activity was observed toward the p-nitrophenyl-β-D-xylopyranoside (exo-xylanase activity substrate). Additionally, it possessed a very low glucosidase activity (5.678 U/mg protein) without the detection of any carboxy-methyl cellulase activity (Table 5). These results manifested the endo-xylanolytic

activity of the enzyme as well as its very low cellulytic activity. Production of xylanolytic enzymes with low cellulolytic activity is an attractive approach for efficient hydrolysis of the hemicellulosic fractions of lignocellulosic biomasses without a significant effect on the cellulose fibers [10, 60-61].

3.5.4. The impact of xylan concentration

The enzymatic hydrolysis of xylan was carried at various substrate concentrations. The obtained results indicated the highest enzymatic activity using 17.5 mg/mL beech wood xylan (Fig. 7A). Based on Lineweaver-Burk plot (Fig.7B), the estimated K_m and V_{max} values were 7.14 mg/mL and 666.67 U/mg protein/min, respectively. The K_m and V_{max} values significantly influence the enzyme-substrate sensitivity i.e. high V_{max} value and low K_m value indicated high enzyme-substrate sensitivity (Horn *et al*., 2006). Silva *et al*., (2015) estimated the V_{max} values for *Trichoderma inhamatum*xylanases ranged from 462.2 to 5443.7 U/mg protein while the corresponding K_m values ranged from 1.6 to 14.5 mg/mL.

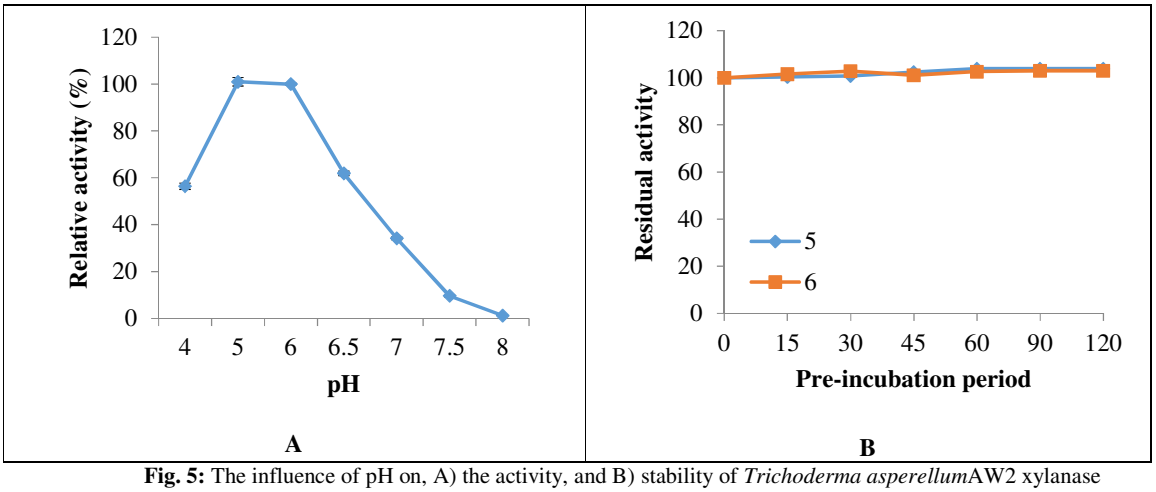
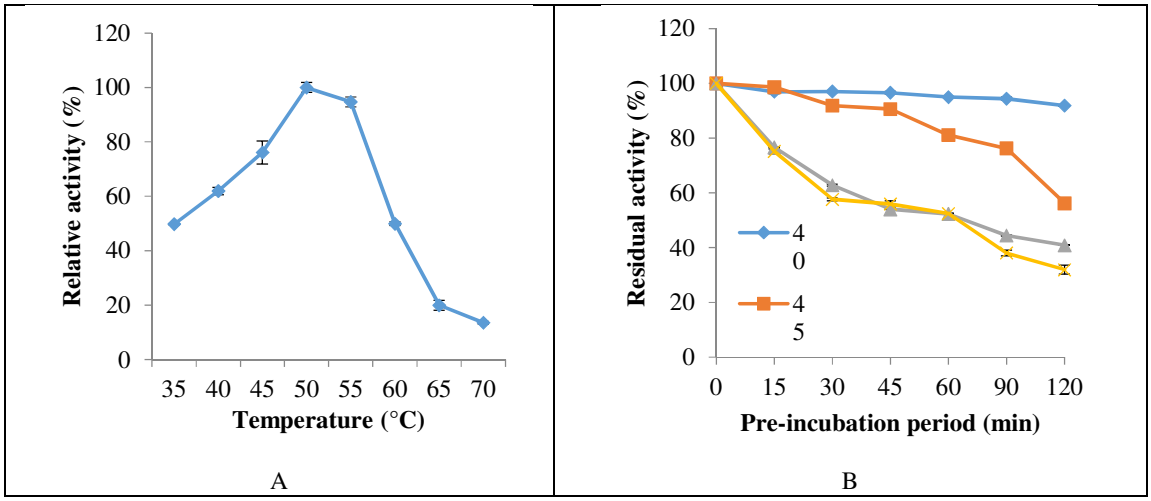


Fig. 5: The influence of pH on, A) the activity, and B) stability of *Trichoderma asperellum*AW2 xylanase



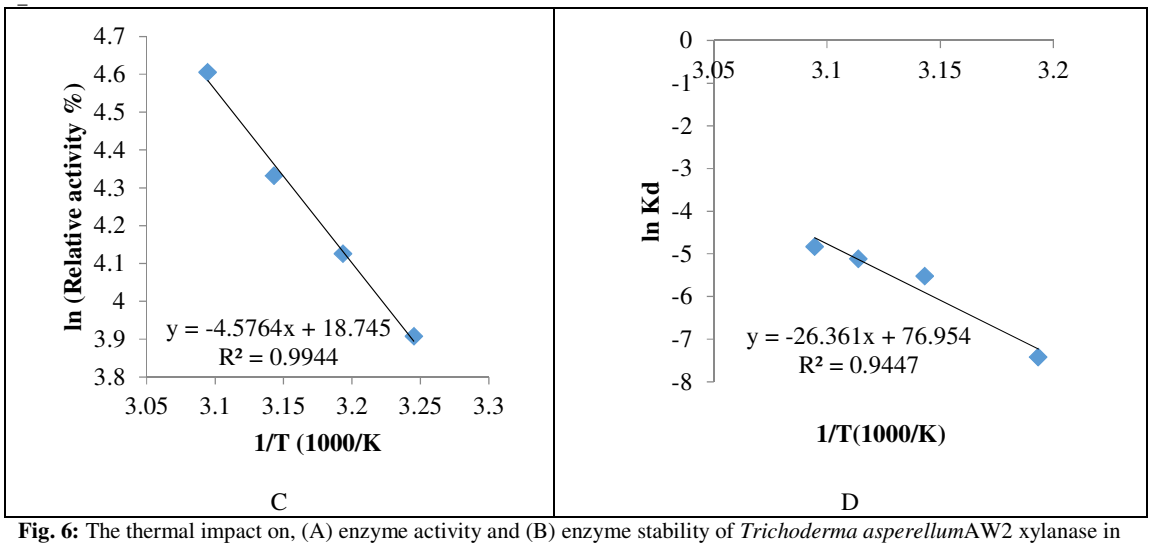


Fig. 6: The thermal impact on, (A) enzyme activity and (B) enzyme stability of *Trichoderma asperellum*AW2 xylanase in addition to Arrhenius plot for thermal (C) activation, and (D) denaturation

Table 4: Denaturation thermal constants and thermodynamic parameters for *Trichoderma asperellum*AW2 xylanase

Temperature (°C)	$T_{1/2}$ (min)	*D*-value (min)	ΔH (KJ mol^{-1})	ΔG (KJ mol^{-1})	ΔS (KJ mol^{-1} K^{-1})
40	1155.245	3837.642	216.567	96.142	0.385
45	173.287	575.646	216.525	92.701	0.389
48	115.525	383.764	216.5	92.518	0.386
50	86.643	287.823	216.483	92.337	0.384

Table 5: Substrate specificity of *Trichoderma asperellum*AW2 xylanase

Enzyme substrate	Specific activity (U/mg protein)
Beech wood xylan	167.851
Locust been gum	-
Pectin	-
Carboxy-methyl cellulose	-
p-nitrophenyl-β-D-galactopyranoside	-
p-nitrophenyl-β-D-xylopyranoside	4.55
p-nitrophenyl-β-D-glucopyranoside	5.678

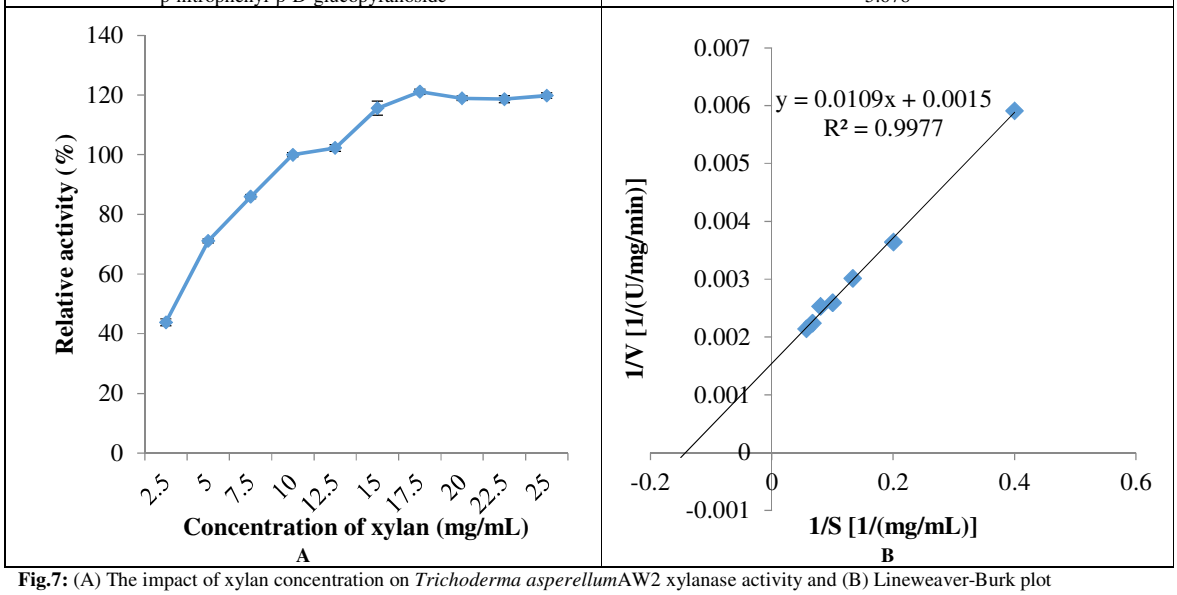


Fig.7: (A) The impact of xylan concentration on *Trichoderma asperellum*AW2 xylanase activity and (B) Lineweaver-Burk plot

3.6. Hydrolytic activity of the produced enzyme

In general, the applicability of xylanases in the hydrolysis of xylan and production of xylooligosaccharides is very expressive owing to their prevalence biological activities including prebiotic [7, 13], antioxidant [6], anti-inflammatory [62] and anti-tumor [63].

3.6.1. Xylan hydrolysis

The applicability of the *Trichoderma asperellum*AW2 xylanase for xylan hydrolysis was evaluated after various incubation intervals. The results shown in figure (8A) demonstrated a rapid increase in the hydrolysis percentage up to 3 h reaching 23 % without any further increase by increasing the time. Moreover, performing the hydrolysis process using different enzyme activities indicated the enhancement of the hydrolysis percentage up to 28% at enzyme activity of 25 U/mL without any further increase at higher enzyme activity (Fig. 8B). Performing the TLC analysis for the produced hydrolyzate possessing the highest hydrolysis percentage indicated the release of xylooligosaccharides mixture (Fig.8C). The presented results agreed with that reported by Ismail *et al*., [6] for xylan hydrolysis.

3.6.2. Hydrolysis of rice straw

Direct hydrolysis of rice straw by the produced enzyme indicated a noticeable improvement in the released reducing sugars to reach its maximum value (19.9 mg/g) after 8 h incubation period. In addition, microwave pre-treatment of rice straw increased the released amount of reducing sugars by more than 70 % reaching 28 mg/g (Fig. 9A). Microwave pre-treatment is a promising method for the conversion of various biomasses as it can uniformly transfer heat energy among the biomass matrix assisting the exploitation of its constitutive components [17, 52, 64-65]. Previously, microwave pre-treatment of rice straw had been reported as a promising method for facilitating arabinoxylan extraction that subjected to enzymatic hydrolysis releasing reducing sugars with maximum yield of 25.44 mg/g [66].

TLC analysis of the hydrolyzate estimated the release of oligosaccharide higher than disaccharide that estimated by the hydrolysis of un-treated samples (Fig. 9B, C). Moreover, scanning electron microscope (SEM) examination of the samples before and after treatment as well as the enzyme hydrolyzed microwave pre-treated sample indicated a remarkable change in their surface structure (Fig. 10).

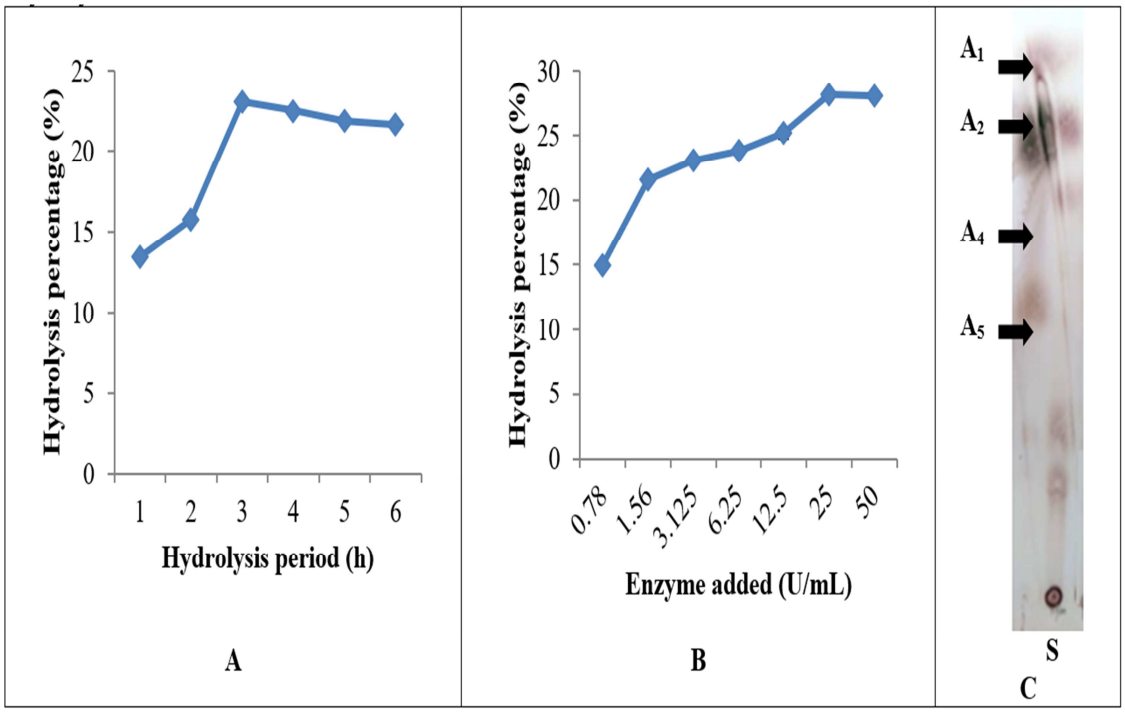


Fig. 8: The impact of (A) different hydrolysis period and (B) the added xylanase activity on the hydrolysis percentage in addition to (C)TLC analysis of xylan-hydrolyzate sample (S) in which A_1, A_2, A_4 and A_5 are standards of mono-, di-, tetra- and penta-sugars

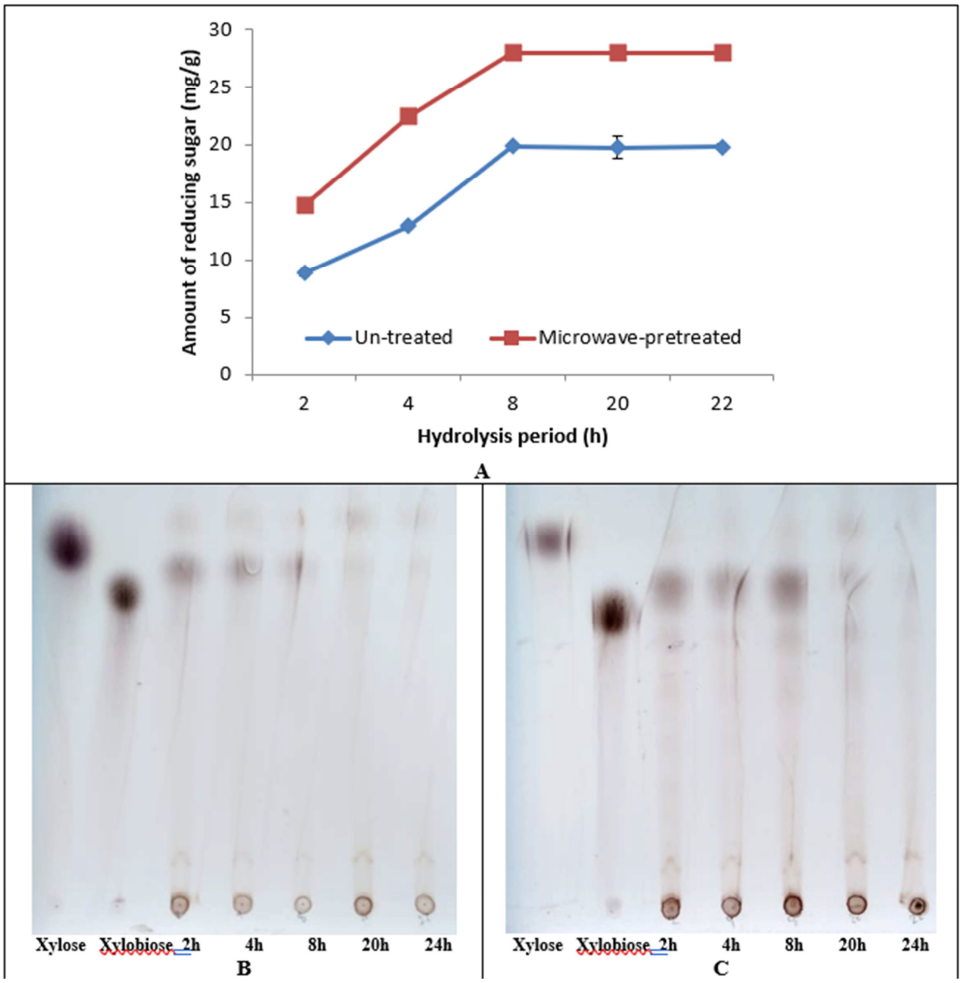


Fig. 9: A) Released reducing sugars via enzymatic hydrolysis of un-treated and microwave pre-treated rice straw, (B) TLC of the un-treated, and (C) the microwave pre-treated samples

3.6.3. Monosaccharide constituent

The contents of the mono-constituent sugars of the dried hydrolyzate produced through the hydrolysis of rice straw compared with those released from xylan hydrolysis were tabulated in Table (6). Rice straw-hydrolyzate was rich in xylose and arabinose similar to xylan-hydrolyzate in addition to high content of glucose.

3.6.4. Fourier transform infrared spectroscopy

Performing the FTIR for the hydrolyzate produced by the hydrolysis of microwave pre-treated rice straw in compare to the one resulted from beech wood xylan indicated the presence of the major bands of xylan; the band around 3200 cm^{-1} attributed to OH stretching, band around 2900 cm^{-1} corresponding to stretching of C–H bonds, band around 1600 cm^{-1} may be attributed to the bending of the attached water molecules. Moreover, bands around 1400 cm^{-1} were related to the bending of C–H, C–O or C–OH bonds, while those around 1030 cm^{-1} were attributed to the stretching of C–C, C–O or C–OH bond, band around 900 cm^{-1} attributed to the beta glucosidic linkage, around 620 cm^{-1} attributed to stretching of C–C–H and finally around 500 cm^{-1} attributed to bending of C–O–C (Fig. 11). Additionally, these results were similar to those reported for various XOS [6, 17, 67-69].

3.6.5. High performed liquid chromatography

The HPLC analysis of microwave pre-treated rice straw hydolyzate in compare to that resulted from beech wood xylan confirmed the release of a mixture of XOS (composed of xylobiose, xylotriose and xylotetrose) in addition to xylose and glucose as mono-sugars (Fig. 12). Similar results had been reported via the enzymatic depolymerization of arabinoxylan extracted from rice straw [66].

3.7. Biological activity

3.7.1. Antioxidant activity

The DPPH scavenging activity of different concentrations of beech wood xylanhydrolyzate was examined (Fig.13). The scavenging activity was

enhanced via increasing the concentration of the hydrolyzateup to the maximum activity of 35.37 µg TE/mg at the concentration 100 mg/mL. Therefore, the activity of the dried microwave pre-treated rice straw hydrolyzate was determined at that concentration and the result was 51.61 ± 0.25 µg TE/mg without the detection of any activity for xylose and glucose at this concentration. The xylan source and the used enzyme are significant variables that influence the variation in the structure of the resulted hydrolyzate as well as its antioxidant activity [6, 70-71]. Antioxidant active compounds are attractive propositions for overcoming the free radical's deleterious impacts in the biological systems and minimizing cell destruction that exaggerate the pathogenesis of various diseases such as diabetes, cancer, and cardiovascular disorders [6, 43, 55, 72].

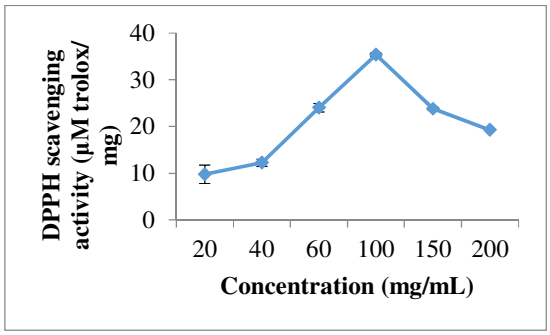


Fig. 13: Antioxidant activity of different concentrations of beech wood xylanhydrolyzate

3.7.2. Fibrinolytic activity

In a previous study reported by Ragab *et al*., [73], the hemicellulose extracted from rice straw did not show any fibrinolytic activity that improved by its sulfation. Herein, another attempt for producing natural Fibrinolytic products derived from rice straw was examined via hydrolyzing of microwave pre-treated rice straw using xylanase followed by evaluating the Fibrinolytic activity of the resulting hydrolyzate. The result indicated that the hydrolyzate showed a moderate activity similar to beech wood xylanhydrolyzate (Figure 14). Aly *et al*., [74] reported that XOS possessed weak to moderate Fibrinolytic activity.

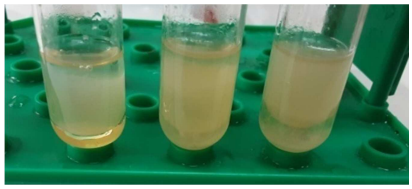
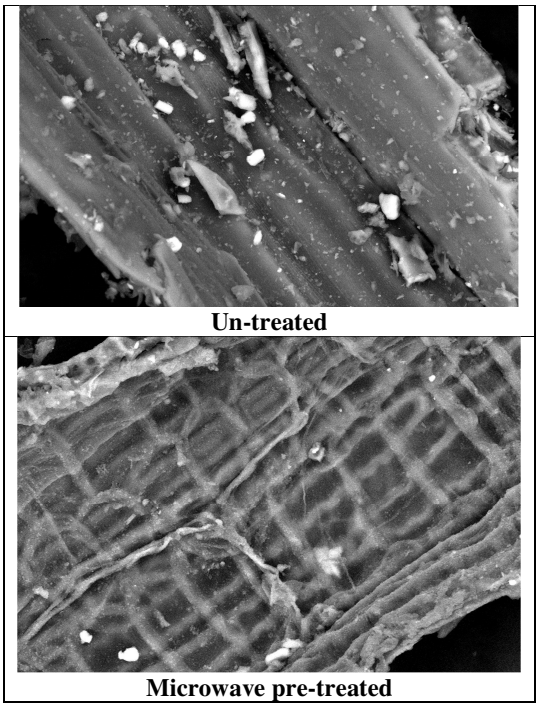


Fig. 14: Fibrinolytic activity of the hydrolyzate of (S_1) beech wood xylan and (S_2) microwave pre-treated rice straw

Table 6: Mono-constituent of the hydrolyzates

Hydrolyzate	Xylose (%)	Arabinose (%)	Glucose (%)	Glucouronic acid (%)
Beech wood xylan	45.9	24.5	7.8	21.8
Rice straw	35.6	28.8	35.6	-



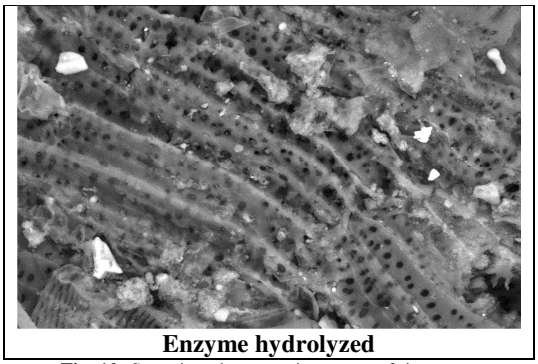
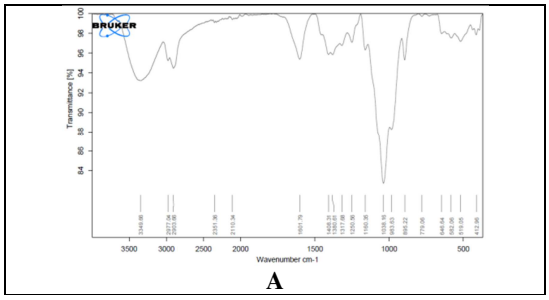


Fig. 10: Scanning electron microscope of rice straw

3. Conclusion

The present study focused on utilizing rice straw for the economic production of xylanase under Smf using *Trichoderma asperellum* AW2. The highest enzymatic activity (96.638 U/mL) was 20.2-fold higher than the estimated initial activity. Moreover, the partial pure enzyme was optimally active at pH ranged from 5 to 6 and temperature 50 °C with half-life time of 86.6 min at this temperature. The enzyme possessed high specific activity for xylan hydrolysis

with K_m and V_{max} values of 7.14 mg/mL and 666.67 U/mg protein/min, respectively. Its hydrolytic activity was mainly an endo-acting with the releasing of a XOS mixture, suggesting its applicability for the bioconversion of lignocellulosic biomasses. The produced enzyme was applied for the direct hydrolysis of rice straw releasing 19.9 mg/g of reducing sugars after 8 h which was improved by 70 % after microwave pre-treatment with the release of a mixture of mono-sugars in addition to the production of XOS. Further investigations would be directed toward the optimization and purification of the produced XOS from the microwave pre-treated rice straw using the produced enzyme. Finally, the produced hydrolyzate possessed an antioxidant activity of 51.61 ± 0.25 µg TE/mg and moderate Fibrinolytic activity.



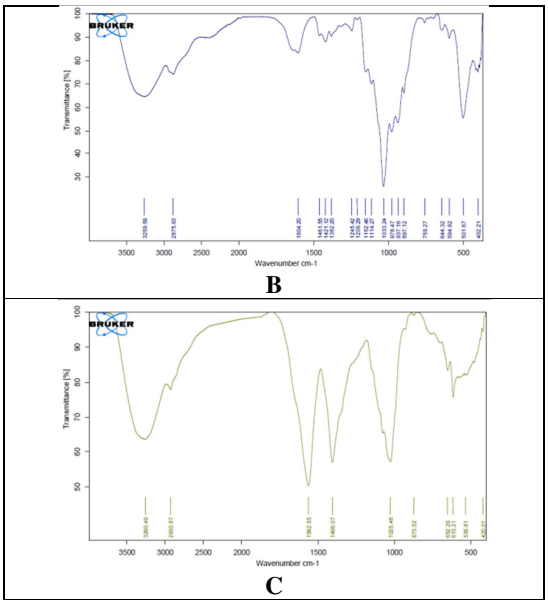
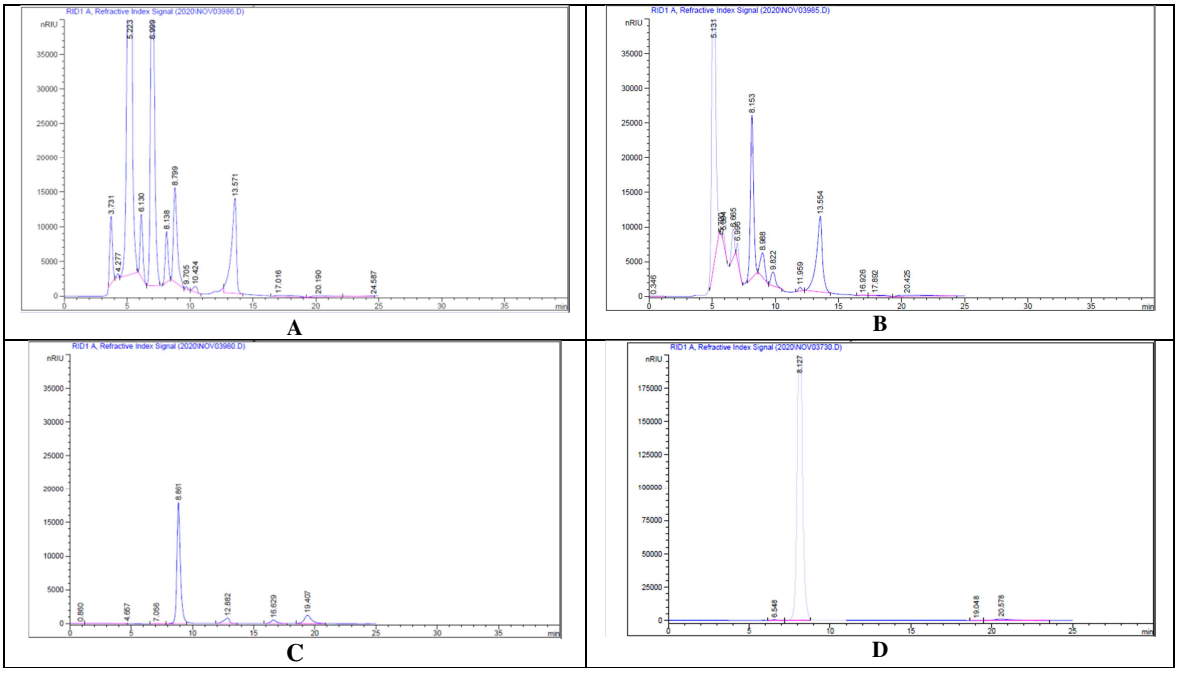


Fig. 11: FTIR spectra of, (A) xylan, (B) xylan-hydrolyzate, and (C) microwave pre-treated rice straw hydrolyzate



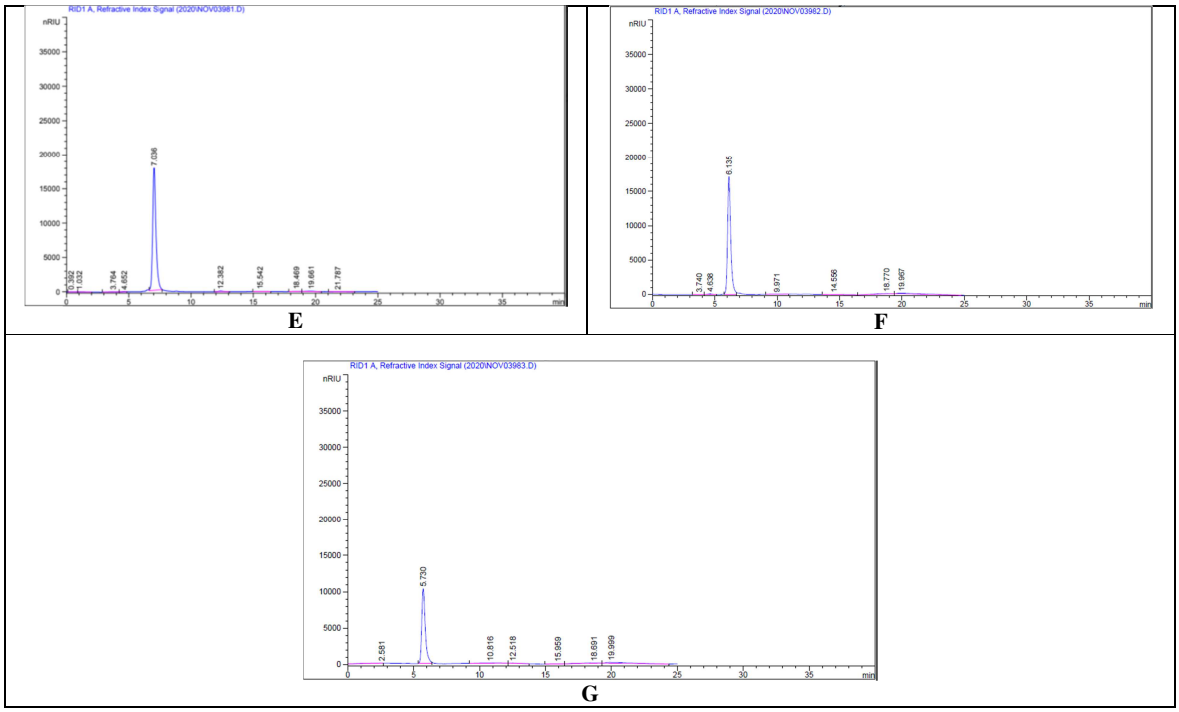


Fig. 12: HPLC analysis of, (A) the hydrolyzate of beech wood xylan, (B) microwave pre-treated rice straw hydrolyzate, (C) xylose, (D) glucose, (E) xylobiose, (F) xylotriose, and (G) xylotetrose

4. CRediT authorship contribution statement

Wafaa A Helmy: Project administration, Funding acquisition, Methodology. **Amira A Hassan:** Methodology, writing- review & editing. **Shaimaa Nour:** Methodology. **Shaymaa A Ismail:** Conceptualization, Methodology, Formal analysis, data interpretation, Writing-original draft.

5. Conflict of interest

There is no conflict of interest to declare.

6. Acknowledgment

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