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Xylanase Production by *Cellulomonas phragmiteti* Using Lignocellulosic Waste Materials

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Abstract: Lignocellulosic biomass holds promise as a renewable feedstock for various applications, but its efficient conversion requires cost-effective degradation strategies. The main objective of this study was to investigate the effect of the growth conditions of *Cellulomonas phragmiteti* in the production of (hemi)cellulosic supernatants. To meet this aim, different lignocellulosic residues were used as carbon sources for growth using defined mineral or nutritive culture media. Cell-free culture supernatants with xylanolytic activity were produced in all the conditions evaluated, but the highest xylanase activity (15.3 U/mL) was achieved in Luria–Bertani (LB) medium containing 1% waste paper. Under these conditions, almost negligible β-glucosidase, cellobiohydrolase, β-xylosidase, and α-arabinofuranosidase activity was detected. The xylanolytic supernatant showed tolerance to salt and displayed maximal catalytic efficiency at pH 6 and 45 °C, along with good activity in the ranges of 45–55 °C and pH 5–8. As it showed good stability at 45 °C, the supernatant was employed for the hydrolysis of birchwood xylan (50 g/L) under optimal conditions, releasing 10.7 g/L xylose in 72 h. Thus, *C. phragmiteti* was found to produce a xylanolytic enzymatic supernatant efficiently by utilizing the cheap and abundant lignocellulosic residue of waste paper, and the produced supernatant has promising attributes for industrial applications.

Keywords: bacterial enzymes; hemicellulases; enzyme fermentation; waste valorization; xylanase characterization; enzyme stability

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1. Introduction

Lignocellulosic biomass serves as a vital and abundant resource for the production of renewable energy and bioproducts [1]. Industrial and agricultural residues and forestry by-products usually remain as non-utilized lignocellulosic wastes; however, they contain substantial amounts of carbohydrates and other valuable substances. In order to valorize these waste materials into energy and bioproducts in an environmentally friendly way, the efficient biodegradation of their carbohydrates is crucial [2]. However, due to the complex polymeric structure of lignocellulose, accessing the sugar components that built it up is still a major challenge [3]. Lignocellulose represents a complex highly recalcitrant structure comprising cellulose surrounded by hemicellulose and lignin. The complete deconstruction of lignocellulose requires the simultaneous action of many different enzymes, such as cellulases, hemicellulases, and lignin-decomposing enzymes [4,5]. Among hemicellulases, xylanases hold particular significance due to the abundance of xylan hemicellulose in many lignocellulosic residues having great potential for biotechnological valorization. Xylanases are hydrolytic enzymes that break xylan into smaller units [6]. These enzymes have wide applications in the food industry, animal feed production, and paper manufacturing, and

in recent years, they have garnered increasing attention for their role in the production of biofuels and biochemicals [7,8].

Bacterial xylanase production using lignocellulosic waste shows great promise for cost-effective enzyme production. Various microbial strains, including *Clostridium* sp., *Cellulomonas* sp., *Bacillus* sp., *Thermomonospora* sp., and *Streptomyces* sp., exhibit the capability to produce xylanases that can be used in lignocellulose degradation [9]. *Bacillus* species such as *B. halodurans* and *B. pumilus* have been widely studied as efficient xylanolytic enzyme producers [10,11]. *Cellulomonas* strains are also promising sources of xylanolytic enzymes [12]; however, less attention has been dedicated to them so far. *Cellulomonas fimi* and *Cellulomonas flavigena* have been reported to have extracellular xylanase activity [9,13]. Fourteen genes potentially encoding xylanases were found in the *C. flavigena* genome sequence [14]. Ontañon et al. investigated *C. fimi* and *Cellulomonas* sp. B6 and their use for biomass deconstruction. Different lignocellulosic residues (e.g., sweet corn cob, waste paper, and wheat bran) were tested. Both strains showed high xylanolytic activity in culture supernatants, although *Cellulomonas* sp. B6 was more efficient [15].

The production of xylanases from lignocellulosic waste materials offers a dual advantage. On one hand, it utilizes inexpensive substrates, thereby reducing the production costs of xylanases significantly; on the other hand, it contributes to the eco-friendly management of these waste materials [16,17]. Lignocellulosic wastes such as agricultural residues, forestry by-products, and industrial remnants can serve as low-cost and efficient carbon sources for the production of xylanases and other high-value enzymes [18]. Xylanases produced from lignocellulosic wastes can have versatile applications. They can be employed in different tasks in various industries, including biofuel production, bioremediation, and food processing, making them a high-value bioproduct that can be produced from low-cost substrates [19].

In this context, this article explores the production of bacterial xylanases by *Cellulomonas phragmiteti* using lignocellulosic residues as substrates and characterizes the produced enzyme preparation (e.g., pH and temperature optima, NaCl tolerance, thermostability).

2. Materials and Methods

2.1. Microorganism

Cellulomonas phragmiteti NCAIM B.02303 was kindly donated by the National Collection of Agricultural and Industrial Microorganisms (Budapest, Hungary). *Cellulomonas phragmiteti* is a Gram-positive, facultatively anaerobic bacterium with motile rod-shaped cells. Its colonies on King's B agar plates became smooth, yellow to pale orange, and circular within 3 days of cultivation at 25 °C [20]. *C. phragmiteti* grows well at 15–37 °C (optimum: 25 °C) and pH 7.0–9.0 (optimum: pH 8.0), and in the presence of 2.0–7.0 w/v% NaCl (optimum: 5.0 w/v%) [20]. *C. phragmiteti* was maintained at 4 °C on Luria–Bertani (LB) [21] agar in Petri dishes containing the following: 0.5 w/w% yeast extract, 1 w/w% NaCl, 1 w/w% trypton and 1.5 w/w% agar.

2.2. Lignocellulosic Materials

Wheat bran (WB), brewer's spent grain (BSG), rye bran (RB), rice straw (RS), distillers dried grain with solubles (DDGS), and waste paper (WP) were examined as carbon sources for xylanase enzyme production by *C. phragmiteti*. Xylan from birchwood was used in enzymatic hydrolysis tests, and it was purchased from Sigma-Aldrich (St. Louis, MI, USA). The WB was provided by Gyermelyi Ltd. (Gyermely, Hungary); the RB was kindly donated by the Research Group of Cereal Science and Food Quality, Budapest University of Technology and Economics (Budapest, Hungary); the RS was provided from Suranaree University of Technology (Nakhon Ratchasima, Thailand); the DDGS was provided from Hungarian University of Agriculture and Life Science (Budapest, Hungary); and the WP was provided from a local paper and stationery shop (Budapest, Hungary). The WP consisted of unprinted, pressed, recycled, corrugated cardboard paper sheets, which were cut into small pieces (smaller than 0.5 cm) (Figure S2), mixed with distilled water to set

10 *w*/*w*% dry matter content, homogenized with a hand blender for 10 min, and autoclaved (121 °C) for 30 min before being used to prepare culture media for cell cultivation or xylanase fermentation. WB, BSG, RB, and DDGS were mixed with distilled water to set 10 *w*/*w*% dry matter and then autoclaved (121 °C) for 30 min before being used in preparing culture media for cell cultivation or xylanase fermentation. RS was cut into small pieces (0.5 cm) and then prepared in the same way as WB, BSG, RB, and DDGS before being used in preparing culture media for cell cultivation or xylanase fermentation. The structural carbohydrates (glucan, xylan, and arabinan) and acid-insoluble solids (Klason lignin) of WP were determined according to the method of the National Renewable Energy Laboratory (DEN, USA) [22]. The structural carbohydrate analysis of WP was carried out in triplicate (Table 1).

Table 1. Relative structural carbohydrate and acid-insoluble solid contents of waste paper. Average values and standard deviations (indicated in parentheses) were calculated from triplicates.

	Percentage of Dry Matter
Glucan	56 (0.7)
Xylan	15 (0.24)
Arabinan	0 (0.0)
Acid-insoluble solid (Klason lignin)	10.5 (0.07)

2.3. Enzyme Production in Shake Flask

C. phragmiteti was grown on culture media containing LB medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) or minimal medium (MM) (1.67 g/L K_2HPO_4, 0.87 g/L KH_2PO_4, 0.05 g/L NaCl, 0.1 g/L $MgSO_4 \times 7H_2O$, 0.04 g/L $CaCl_2$, 0.004 g/L $FeCl_3$, 0.005 g/L $Na_2MoO_4 \times 2H_2O$, 0.01 g/L biotin, 0,02 g/L nicotinic acid, 0.01 g/L pantothenic acid, 1 g/L NH4Cl, 1 g/L yeast extract), all supplemented with 1 *w*/*w*% of different lignocellulosic carbon sources (WP, WB, BSG, RB, RS, and DDGS) which were pretreated according to the method described in Section 2.2. Culture media (LB or MM media with lignocellulosic carbon sources) were sterilized in autoclave at 121 °C for 20 min. Starter cultures were obtained by inoculating colonies (from agar plates) in 10 mL LB medium and incubating them at 30 °C and 220 rpm for 72 h in 100 mL flasks closed by cotton plugs. Cultures for enzyme production (fermentations) were inoculated from the starter cultures to set an initial cell concentration that corresponded to an optical density (OD) of 0.05. Fermentation experiments were performed in 25 mL culture media poured into 100 mL shake flasks at 30 °C and 220 rpm for 72 h. Fermentations were performed in triplicate. After 72 h, the fermentation media were vacuum-filtered to separate the solids (cells and biomass) from the fermentation supernatant, which was stored at −18 °C until further use.

2.4. Enzymatic Activity Measurements

The xylanase and carboxymethyl cellulase (CMC-ase) activities of the fermentation supernatants were assayed in test tubes by using a beechwood xylan (Sigma-Aldrich, St. Louis, MI, USA) suspension (1 *w*/*w*%) or wheat arabinoxylan (arabinoxylan for reducing sugar assays, low-viscosity arabinoxylan, medium-viscosity arabinoxylan, and high-viscosity arabinoxylan (Megazyme, Bray, Ireland)) solutions (1 *w*/*w*%) and CMC solution (2 *w*/*w*%) as substrates, respectively [23]. For these assays, 0.75 mL of appropriately diluted cell-free fermentation broths were added to 0.75 mL of each substrate prepared in citrate buffer (0.1 M, pH 6). The assays were carried out at 45 °C with magnetic stirring for 10 min. Reducing sugars released during the assay were measured by the dinitrosalicylic acid (DNS) method [24] using glucose or xylose standard curves.

The β-glucosidase, cellobiohydrolase, β-xylosidase, and α-arabinofuranosidase activities were assayed using 5 mM p-nitrophenyl-β-D-glucopyranoside (pNPG), p-nitrophenyl-β-D-cellobiosid (pNPC), p-nitrophenyl-β-D-xylopyranoside (pNPX), and p-nitrophenyl-α-L-arabinofuranoside substrates, respectively, according to previously established proto-

cols [25]. Briefly, the reactions were performed in test tubes; 1 mL p-nitrophenyl reagent was added to 0.1 mL appropriately diluted cell-free fermentation broth. The reactions were performed at 45 °C with magnetic stirring for 10 min. The reactions were stopped with 2 mL sodium carbonate (1 M), and absorbance was determined at a wavelength of 400 nm using a spectrophotometer. A p-nitrophenol (pNP) curve was used as a standard.

All enzymatic assays were conducted in triplicate, and control enzymes without a substrate and a substrate without an enzyme were included. In all cases, one international unit (U) was defined as the amount of enzyme required to release 1 µmol of product per minute under the assay conditions.

2.5. Investigation of the Time Dependence of Xylanase Production

Xylanase production by *C. phragmiteti* as a function of the fermentation time was also investigated. Enzyme production was performed on WP as a carbon source as described in Section 2.3. Xylanase activity production was monitored by daily sampling (0, 24, 48, 72, 96 h). Xylanase activity measurements were performed at each sampling time as described in Section 2.4.

2.6. Total Protein Content Assay

The total protein contents of the fermentation supernatants were quantified by the Bradford assay. The Bradford reagent contained 10 mg Coomassie blue, 5 mL 96 *w*/*w*% ethanol, and 10 mL 85 *w*/*w*% phosphoric acid, and the solution was completed to 100 mL with distilled water. The assay was performed in test tubes. Next, 5 mL of reagent was added to a 0.1 mL sample and carefully homogenized. Two minutes later, the absorbance was determined at a wavelength of 595 nm using a spectrophotometer. A bovine serum albumin curve was used as a standard.

2.7. Effect of NaCl Concentration on Xylanase Activity and Xylanase Production

The effect of NaCl concentration on xylanase activity was investigated using cell-free fermentation broth derived from fermentations containing 1 *w*/*w*% WP as a carbon source. Xylanase enzyme activity was measured according to the method previously described in Section 2.4., with small modifications. Different amounts of NaCl (25 g/L, 50 g/L, 100 g/L, 150 g/L, and 200 g/L) were added to the reaction mixtures. The NaCl stock solutions were made using citrate buffer (0.1 M, pH 6). The effect of NaCl concentration on xylanase production by *C. phragmiteti* was investigated by adjusting 100 g/L NaCl concentration during xylanase fermentation on 1 *w*/*w*% WP.

2.8. Determination of the pH and Temperature Optima of the Produced Xylanolytic Supernatant

The pH and temperature optima of the xylanase activity produced by *C. phragmiteti* on WP were investigated. Regarding pH, the experiments were carried out within a pH range of 3–11, at 45 °C. At pH 3, pH 4, pH 5, and pH 6, citrate buffer (0.1 M) was used. At pH 6, pH 7, and pH 8, phosphate buffer (0.1 M) was used, and at pH 8, pH 9, pH 10, and pH 11, carbonate buffer (0.1 M) was used. The substrate suspension (1 *w*/*w*% beechwood xylan) was made using the same buffers as the reaction mixture. Regarding temperature, 25 °C, 35 °C, 45 °C, 55 °C, 65 °C, 75 °C, and 85 °C were applied during the enzyme activity measurements in citrate buffer (0.1 M, pH 6).

2.9. Enzyme Stability Measurement

Enzyme stability tests were carried out at two different temperatures (45 °C and 55 °C) at four different pH values: pH 5, pH 6, pH 7, and pH 8. Enzymatic stability was measured in 0.05 M phosphate buffers. The produced xylanase-containing supernatant was diluted (tenfold) with buffer, and the diluted enzyme solutions were incubated at elevated temperatures (45 °C or 55 °C; 220 rpm for 72 h). During the test, samples were taken at 0, 3, 24, 48, and 72 h to perform xylanase activity measurements.

2.10. Enzymatic Hydrolysis of Xylan

An enzymatic hydrolysis experiment was performed on xylan from birchwood by using the supernatant derived from the fermentation using 1 *w*/*w*% WP as a carbon source. Five *w*/*w*% dry matter of xylan and 10 U/mL of the produced xylanases were set in the enzymatic hydrolysis. The hydrolysis reaction was carried out at 45 °C, pH 6, and 150 rpm for 72 h. Daily sampling was conducted from the reaction mixture. The supernatants were separated by vacuum filtration through a nylon filter (0.2 µm pore size), and they were analysed by high-performance liquid chromatography (HPLC) for xylose. HPLC analysis was performed by using a BioRad (Hercules, CA, USA) Aminex HPX-87H (300 × 7.8 mm) column equipped with a Micro-Guard Cation H+ Refill Cartridge (300 × 4.6 mm) pre-column at 65 °C and a refractive index detector. The eluent was 5 mmol/L sulphuric acid at a flow rate of 0.5 mL/min. The injection volume was 40 µL. To determine the total solubilized xylose content (including the xylose liberated in monomer and oligomer forms), an oligomer hydrolysis step was also carried out with the 72 h samples. They were mixed with sulphuric acid solution (8 *w*/*w*%) at a volume ratio of 1:1 and treated at 120 °C in an autoclave for 15 min to decompose oligosaccharides prior to HPLC analysis.

3. Results and Discussion

3.1. Effect of Different Media and Lignocellulosic Culture Substrates on the Production of a Xylanolytic Enzymatic Supernatant

The use of lignocellulosic residues and by-products as substrates for microbial growth has the advantage of being an inexpensive strategy for inducing the production of enzymes [26]. Thus, *C. phragmiteti* was cultivated on LB and MM media supplemented with different lignocellulosic residues as carbon sources, which were WP, WB, BSG, RB, RS, and DDGS. After 72 h cultivation, the xylanase activities were measured from each cell-free fermentation broth. The results of the xylanase activity measurements are shown in Figure 1.

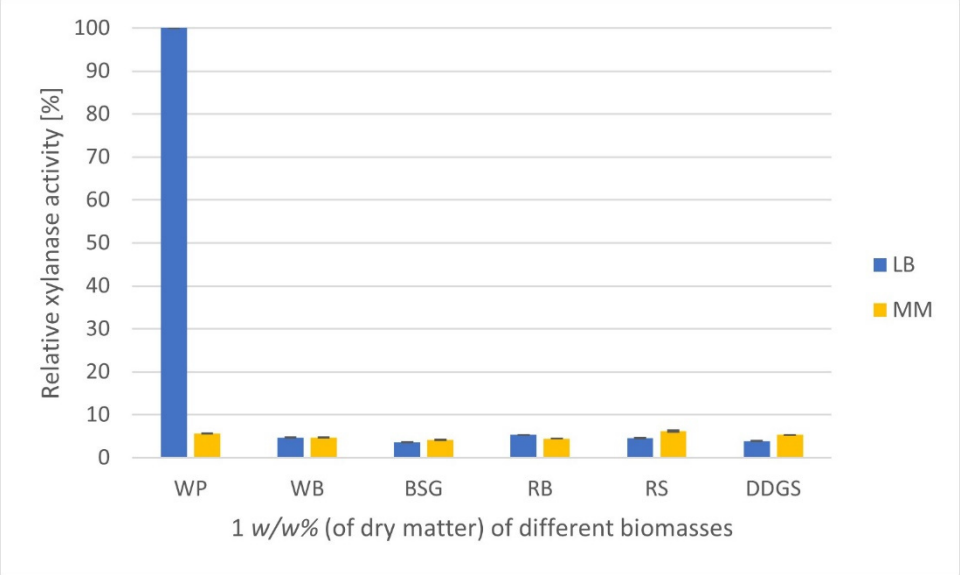

Figure 1. Relative xylanase activities [%] produced by *C. phragmiteti* on different carbon sources (1 *w*/*w*% of dry matter) in LB and MM media. WP—waste paper; WB—wheat bran; BSG—brewer's spent grain; RB—rye bran; RS—rice straw; DDGS—distillers dried grains with solubles. The average values of relative xylanase activity are presented with error bars representing the standard deviations of triplicate cultures in the case of WP and WB and duplicate cultures in the case of RB, RS, BSG, and DDGS.

The highest xylanase activity (referred to as 100% relative xylanase activity) was obtained using WP in LB medium. The relative xylanase activities were between 3.6–5.4% and 4.1–6.2% for the other biomasses (WB, BSG, RB, RS, and DDGS) in LB and MM media, respectively. The xylans of RB, RS, WB, DDGS (corn), and BSG are complex xylans highly decorated with side chains that frequently contain arabinofuranosyl units [27–30]. The complex structure of these xylans hinders the action of xylanases in the absence of accessory side chain-hydrolysing activities [10], which might have contributed to their limited potential for inducing xylanase enzyme production by *C. phragmiteti*. In contrast, the waste paper used in our study contained xylan (15% in terms of dry matter) without arabinose substitutions. In addition, during the process of paper production, the complex structure of its lignocellulose is disintegrated to decrease the lignin content, which could make the remaining cellulose and hemicellulose fractions of paper materials more accessible for hydrolytic enzymes. These factors could play a role in making waste paper an efficient substrate to induce the xylanase production of *C. phragmiteti*. Thus, LB medium containing WP was chosen for the following fermentations for xylanase activity production by *C. phragmiteti*. The use of *Cellulomonas* sp. B6 [31], *C. fimi* [32], *C. flavigena* [14], and *C. biazotea* [33] for the production of xylanases has also been reported. *Cellulomonas* sp. B6 and *C. fimi* showed high xylanase production on wheat bran and pre-treated waste paper, respectively [15], while xylanase production was mainly induced by sugarcane bagasse in the cases of *C. flavigena* and *C. biazotea* [14,33].

3.2. Effect of the Amount of Waste Paper on Xylanase Production

As WP was found to be the most appropriate substrate, the effect of WP concentration on the xylanolytic activity was investigated. The experiments were performed by using 0.5 *w*/*w*%, 1 *w*/*w*%, 1.5 *w*/*w*%, 2 *w*/*w*%, 2.5 *w*/*w*%, and 3 *w*/*w*% WP in LB medium. The highest xylanase activity (15.3 U/mL) was measured when 1 *w*/*w*% dry matter of WP was used for fermentation (Figure 2). Also, high xylanase activities were detected using 0.5 *w*/*w*% and 1.5% *w*/*w*% of WP. Between 1.5 *w*/*w*% and 2 *w*/*w*% of WP, a large decrease was observed in the produced xylanase activity (from 15 U/mL to 2.2 U/mL). Low xylanase activity values were measured in the case of 2 *w*/*w*% (2.2 U/mL), 2.5 *w*/*w*% (1.3 U/mL), and 3 *w*/*w*% (0.3 U/mL) of dry matter of WP (Figure 2). The supernatant derived from the fermentation using 1 *w*/*w*% WP showed the highest xylanase activity, so it was chosen for our further investigations.

C. fimi and *Cellulomonas* sp. B6 were also cultivated on pretreated waste paper (1 *w*/*w*% dry matter) by Ontañon et al. during an investigation of their xylanases enzyme production [15]. The highest xylanase activities achieved were 1.3 U/mL and 1.9 U/mL in the case of *C. fimi* and *Cellulomonas* sp. B6, respectively [15]. Thus, WP seems to be much more suitable for inducing the production of xylanolytic enzymes in *C. phragmiteti* than in the other two strains.

The decrease in the activity of the enzyme extract when the substrate concentration is above the optimal value has been related to the increase in viscosity and nutrient content in the medium. This inhibits microbial growth, lowering enzyme production. Some authors have reported that a lower substrate concentration is more effective for enzyme induction than higher concentrations [34,35]. This could also be an economic advantage in the large-scale production of hemicellulases at low substrate loads.

The xylanase activity of the supernatant derived from fermentation on 1 *w*/*w*% WP was measured by using different substrates, namely xylan from beechwood (BX) and wheat arabinoxylans with different viscosities (WAX-RS—arabinoxylan for reducing sugar assays; WAX-L—low-viscosity arabinoxylan; WAX-M—medium-viscosity arabinoxylan; WAX-H—high-viscosity arabinoxylan) (Figure 3). Relative activity values are displayed compared to that measured with beechwood xylan (100%). Higher xylanase activities were measured using WAX-RS and WAX-L (129% and 131%) compared to BX. Verma et al. indicated that enzymatic activity can vary significantly with the nature of the xylan used [36]. For example, the choice of the type of xylan is critical for obtaining positive clones during a

functional screening. This phenomenon may be related to the good solubility and low viscosity of the listed arabinoxylan substrates, in contrast with xylan from beechwood, which is an insoluble substrate. In addition, BX contains glucuronic acid substitutions instead of arabinose. Regarding WAX-M and WAX-H, slightly lower xylanase activities were detected (102% and 108%) compared to WAX-RS and WAX-L. This is probably because the viscosity of the substrates can affect the enzyme activity [37]. Thus, the higher viscosity values of WAX-M and WAX-H could have caused the lower xylanase activities [35].

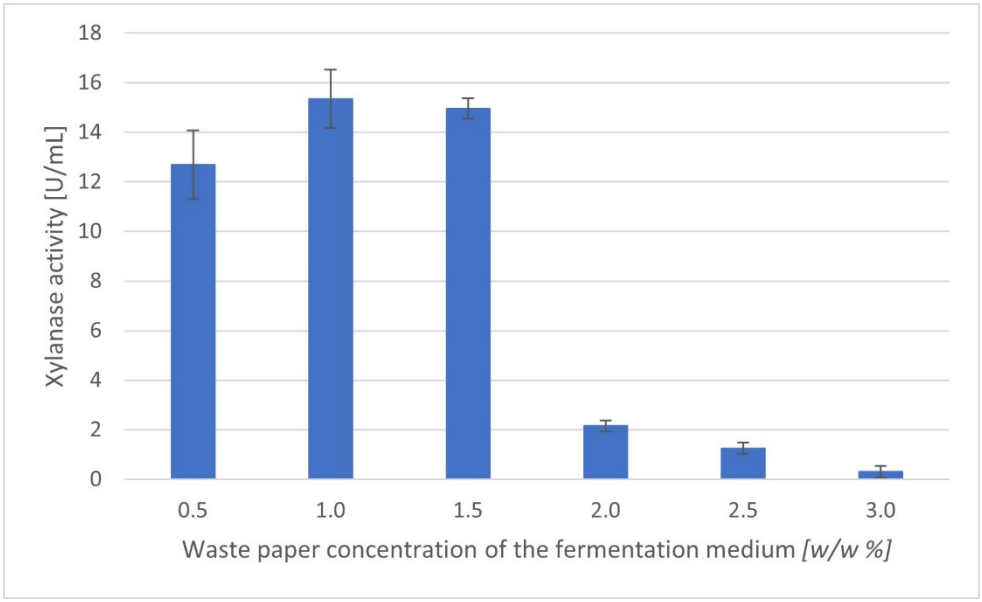

Figure 2. Xylanase activities [U/mL] obtained in LB medium containing different amounts of waste paper (*w*/*w*% of dry matter). The average values of xylanase activity are presented with error bars representing the standard deviations of triplicate cultures.

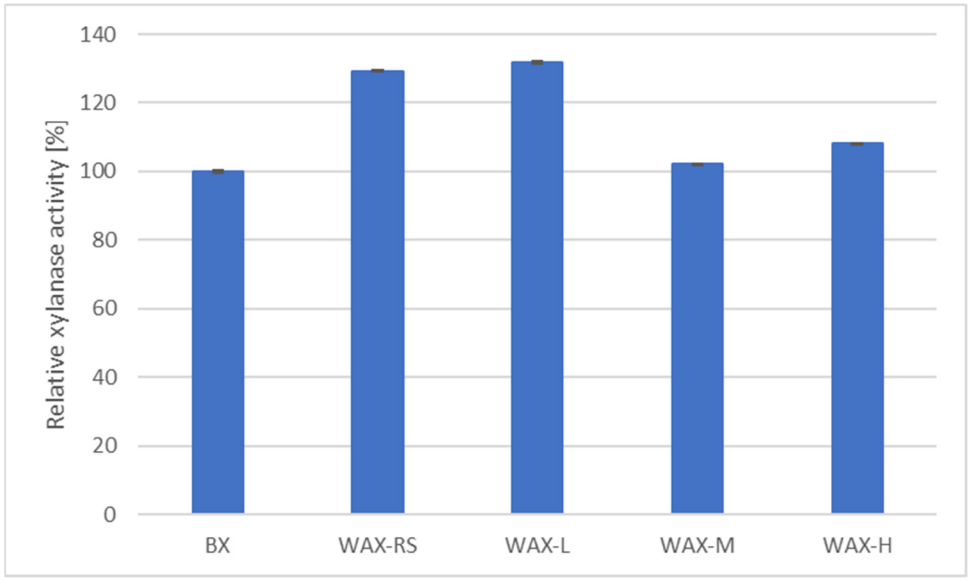

Figure 3. Relative xylanase activities [%] obtained from fermentation by using *C. phragmiteti* in LB medium containing 1 *w*/*w*% of dry matter waste paper. Xylanase activity measurements were performed using different xylan substrates: BX—xylan from beechwood, WAX-RS—wheat arabinoxylan for reducing sugar assays, WAX-L—low-viscosity wheat arabinoxylan, WAX-M—medium-viscosity wheat arabinoxylan, WAX-H—high-viscosity wheat arabinoxylan. Average values of xylanase activity are presented with error bars representing standard deviations of triplicates.

Side enzymatic activities (Table 2) were also measured from cell-free supernatants produced by *C. phragmiteti* on 1 *w*/*w*% WP, such as β-glucosidase, β-xylosidase, cellobiohydrolase, CMC-ase, and α-arabinofuranosidase. β-xylosidase had the highest side activity (0.61 U/mL) (Table 2). β-xylosidase activity was around six times higher compared to that of β-glucosidase (0.11 U/mL), cellobiohydrolase (0.12 U/mL), and CMC-ase (0.15 U/mL). Arabinofuranosidase had the lowest side activity (0.007 U/mL). These results prove that the most dominant enzyme, in terms of activity, in the extracellular extracts of *C. phragmiteti* is xylanase, and other lignocellulolytic enzyme activities are also present. Similarly, Akermann et al., Mayorga-Reyes et al., and Ontañon et al. reported a mainly xylanolytic activity in extracts of *Cellulomonas uda*, *C. flavigena*, *Cellulomonas* sp. B6, and *C. fimi* B-402 obtained by growth on lignocellulosic biomass [14,15,38]. These authors related this activity to the large amount of xylanases secreted by the bacteria under appropriate culture conditions.

Table 2. β-glucosidase, β-xylosidase, cellobiohydrolase, carboxymethyl-cellulase, and α-arabinofuranosidase enzyme activities (U/mL) of a fermentation supernatant obtained by *C. phragmiteti* on 1 *w*/*w*% waste paper in LB. The average values and standard deviations (indicated in parentheses) were calculated from triplicates.

Enzyme Activity	U/mL
β-glucosidase	0.11 (0.010)
β-xylosidase	0.61 (0.013)
Cellobiohydrolase	0.12 (0.005)
Carboxymethyl-cellulase	0.15 (0.032)
α-arabinofuranosidase	0.007 (0.002)

In comparison, *C. biazotea* showed higher cellulolytic activity than xylanolytic activity by growing on CMC and sugarcane bagasse in a study by Saratale et al. [33]. CMC and sugar cane bagasse induced cellulase (endoglucanase, exoglucanase, and cellobiase) production [33]. In our study, *C. phragmiteti* growing on WP showed much lower cellulase (CMC-ase) (Table 2) activity than the reported activity of *C. biazotea* on sugarcane bagasse and CMC. Thus, future investigations could focus on investigating whether other carbon sources would induce preferable cellulase production in *C. phragmiteti*.

3.3. Investigation of the Time Curve of Xylanase Activity Production

An investigation of the produced xylanase activity as a function of fermentation time was carried out for 96 h (Figure S1). Relative activity values at all sampling times are displayed compared to the values measured after 96 h (100%). Xylanase activity was barely observed after one day (1.5%). The production of xylanases started after 24 h. The relative activity of xylanase increased from 1.5% to 67.5% and 91.8% after 48 and 72 h, respectively. The production rate of xylanase activity greatly slowed down between 72 and 96 h; so, 72 h fermentation time was selected. Amaya-Delgado et.al. studied the induction of xylanase activity by sugar cane bagasse in *C. flavigena*, and they found that the maximal xylanase activity was reached after 100 h, but the most significant increase in the xylanase activity occurred between 60 and 82 h [39], which is in line with our results.

3.4. Effect of NaCl Concentration on Xylanase Activity

According to Rusznyák, *C. phragmiteti* is a salt-tolerant bacterium [20], so xylanase activity production might also be affected by the NaCl content of the medium, and the produced xylanolytic supernatants might have a salt-tolerating attribute. In order to investigate these hypotheses, xylanase activity was examined by setting different concentrations of NaCl in the test tubes during the measurement of xylanase enzyme activity, and xylanase enzyme production was measured in the presence of a high concentration of NaCl (100 g/L) in the fermentation medium.

The effect of NaCl concentration on the measured xylanase activity is represented in Figure 4. The xylanase activity slightly decreased by increasing the concentration of

NaCl. Using 25 g/L (0.43 M) NaCl, 13 U/mL xylanase activity was measured, which is slightly lower compared to that measured without NaCl (15.3 U/mL). Similar values were obtained by using 50 g/L (0.86 M) and 100 g/L (1.71 M) of NaCl; these values were 12.4 U/mL and 12.2 U/mL, respectively. The xylanase activity further decreased to 11.6 U/mL and 10.3 U/mL by using 150 g/L (2.56 M) and 200 g/L (3.42 M) of NaCl, respectively. The lowest activity was 10.3 U/mL (achieved by using 200 g/L NaCl), which is approximately 32.7% decrease compared to the highest (15.3 U/mL) activity measured without NaCl. The presence of high salt concentrations can negatively influence xylanase activity; however, our results suggests that the xylanases produced by *C. phragmiteti* are quite resistant against high NaCl concentrations, which might be useful in specific applications. An important challenge of industrial biotechnology is the high consumption of fresh water. A practical solution is the replacement of fresh water with abundant seawater in water-intensive industrial settings such as lignocellulosic biorefineries. In this context, a halophilic xylanase has the potential for application in the enzymatic decomposition of plant biomass in seawater [40].

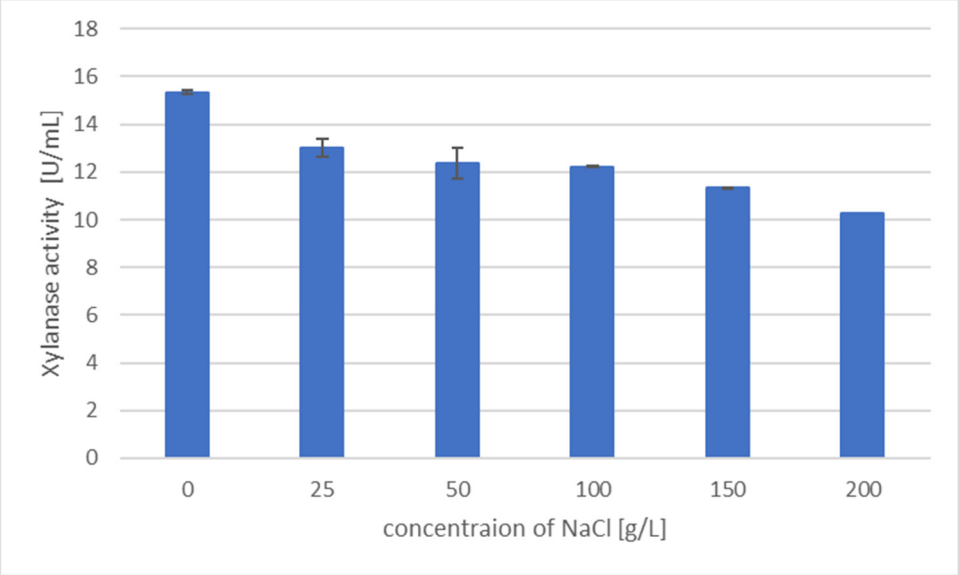

Figure 4. Xylanase activity values (U/mL) using different concentrations of NaCl (25 g/L, 50 g/L, 100 g/L, 150 g/L, and 200 g/L) during the xylanase assay (performed using cell-free fermentation broth of *C. phragmiteti* cultivation in LB medium containing 1 *w*/*w*% of waste paper).

Fermentation was also performed in the presence of high concentrations of NaCl to investigate the influence of salt on the enzyme production of *C. phragmiteti*. The xylanolytic activity dropped to 0.77 U/mL in the medium containing 100 g/L of NaCl despite the fact that *C. phragmiteti* can grow in a salty environment [20]. Rusznyák et al. stated that 5–7 *w*/*v*% NaCl concentration is optimal for its growth. Our measurements prove that xylanase production by *C. phragmiteti* is greatly inhibited by high salt concentrations. However, the xylanolytic supernatant seems to have salt-tolerant attributes. Since, in this study, only the use of 100 g/L NaCl was investigated during enzyme production, further experiments are needed to explore how the concentration of NaCl influences the xylanase production of *C. phragmiteti*.

3.5. Investigation of the pH and Temperature Optima of the Produced Xylanases

The determination of the operating range and optimal conditions of an enzyme of interest is crucial to specify its possible fields of application. Thus, the effect of pH and temperature on the xylanase activity produced by *C. phragmiteti* on 1 *w*/*w*% WP were investigated (Figure 5).

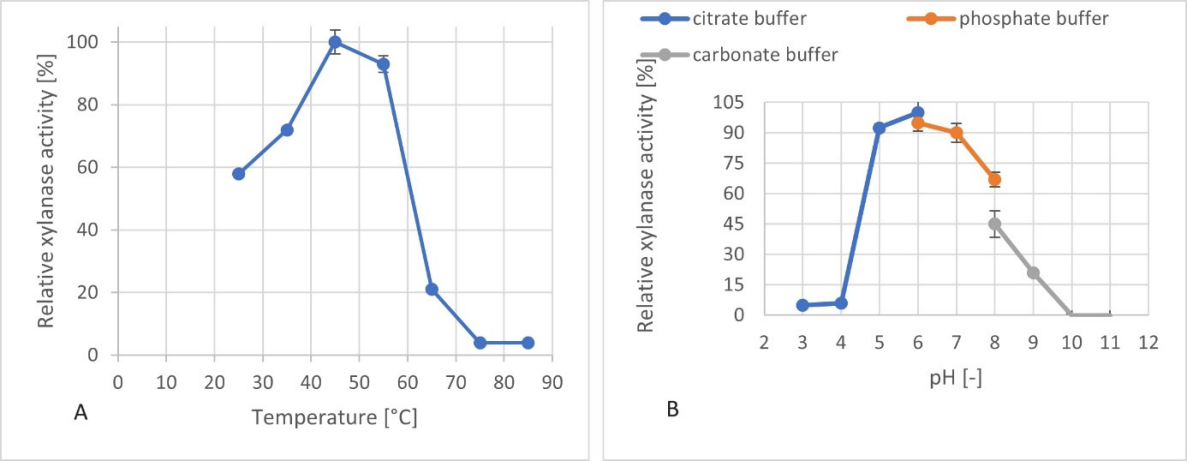

Figure 5. Relative xylanase activities at different temperatures (**A**) and pH values (**B**) measured in fermentation supernatant of *C. phragmiteti* cultivated in LB medium containing 1 *w*/*w*% of waste paper. Average values of relative xylanase activity are presented with error bars representing standard deviations of triplicates.

The results in Figure 5A,B represent the relative xylanase activity compared to the maximal activity, which was reached at 45 °C and pH 6 in citrate buffer. At 55 °C, an activity nearly as high as at 45 °C was detected (93%). At 25 °C, the relative xylanase activity was 58%. The xylanase activity experienced extreme decreases at higher temperatures (65 °C, 75 °C, 85 °C). Almost zero xylanase activity was measured at over 70 °C (Figure 5A).

The effect of pH on the xylanase activity was investigated by changing it between pH 3 and pH 11 and by using three types of buffers: citrate, phosphate, and carbonate (Figure 5B). At the pH values where a buffer change was necessary, xylanase activity was measured by using both buffers in order to monitor the effect of the type of buffer on the measured xylanase activity.

The maximal activity was detected at pH 6 (0.1 M) using citrate buffer (100%). The appropriate pH range for the produced xylanolytic supernatant was found to be pH 5–pH 8, which is a relatively wide range. A considerable effect regarding the type of buffers was observed between the phosphate and carbonate buffers at pH 8; higher xylanase activity was reached using the phosphate buffer (67%) compared to the use of the carbonate buffer (45%). At pH 10 and pH 11, xylanase activity was not measurable. Lisov et al. investigated the temperature and pH optima of xylanase enzymes derived from *C. flavigena* [13]. The optimum activity occurred at pH 7–7.5 and 40–50 °C, which is quite similar to the results observed for the xylanolytic supernatant of *C. phragmiteti*, whose optimal conditions were 45 °C and pH 6 (Figure 5).

3.6. Enzyme Stability Test

The stability of the xylanolytic supernatant of *C. phragmiteti* was evaluated during incubation at elevated temperatures and under different pH conditions. According to the previous results, the highest xylanase activities were detected between pH 5 and pH 8 and at 45 °C and 55 °C. Thus, the supernatant derived from fermentation on WP in LB medium by *C. phragmiteti* was incubated under these conditions for 72 h, and its residual activity was measured at different times. The results obtained at 45 °C are represented in Figure 6.

Relative activity values at all pHs are displayed compared to that of measured at pH 6 (100%) without previous incubation (0 h). The highest xylanase activity was detected at 0 h at each pH value. The most marked loss of activity, between 22 and 26%, was observed for all the evaluated pHs in the first 3 h. Then, the activity remained almost constant. After 72 h of incubation, 57%, 62%, 56%, and 53% of the original xylanase activity were remained at pH 5, pH 6, pH 7, and pH 8, respectively. Thus, all pH values applied in the experiments resulted in similar decreases in xylanase activity at 45 °C.

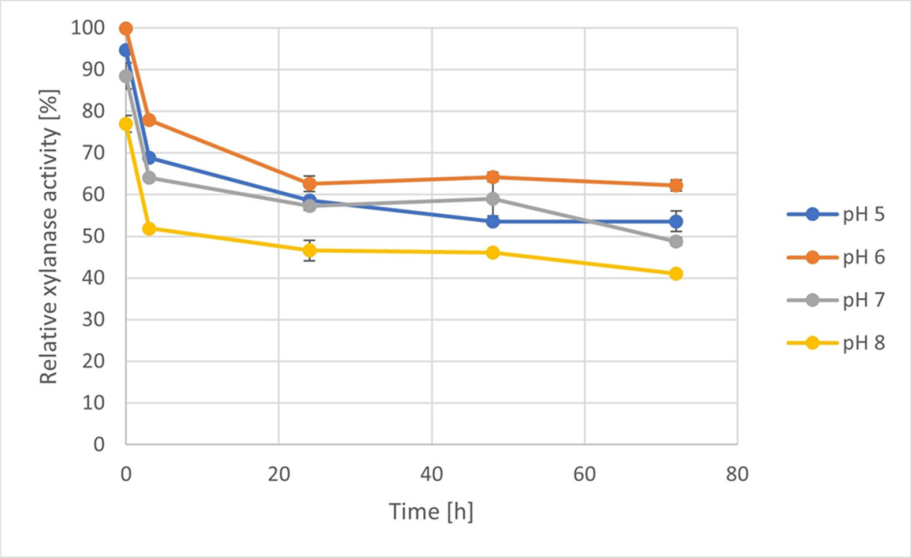

Figure 6. Results of the xylanase enzyme stability test at 45 °C and pH 5, 6, 7, 8 using the fermentation supernatant of *C. phragmiteti* cultivated in LB medium containing 1 *w*/*w*% of waste paper. The average values of relative xylanase activity are presented with error bars representing the standard deviations of triplicates.

The xylanase activity decreased faster and to a greater extent at 55 °C compared to 45 °C (Figure 7). In the first 3 h, the activity decreased by 44%, 35%, 36%, 43%, and 68% at pH 5, pH 6, pH 7, and pH 8, respectively. After 72 h, only 4%, 8%, 15%, and 3% of the original xylanase activities remained at pH 5, pH 6, pH 7, and pH 8, respectively. Thus, xylanases produced by *C. phragmiteti* on 1 *w*/*w*% WP seem to be more stable at 45 °C compared to 55 °C. In comparison, a greater thermostability was reported for the xylanolytic extracts of *Cellulomonas* sp. B6, which retained almost 80% activity after 48 h at 45 °C, although their specific activity was significantly lower than the extract from *C. phragmiteti* [31]. Our results suggest that the extracts of *C. phragmiteti* can be used in long-term reactions at 45 °C with good catalytic efficiency [41].

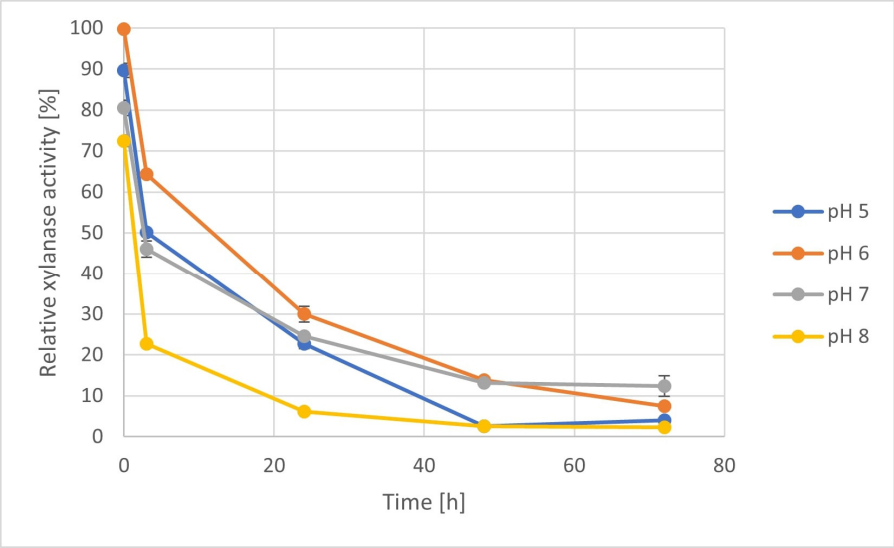

Figure 7. Results of the enzyme stability test at 55 °C and pH 5, 6, 7, and 8 by using the fermentation supernatant of *C. phragmiteti* cultivated in LB medium containing 1 *w*/*w*% of waste paper. The average values of relative xylanase activity are presented with error bars representing the standard deviations of triplicates.

3.7. Enzymatic Hydrolysis of Xylan

The xylanolytic supernatant of *C. phragmiteti* grown on WP was employed to hydrolyse birchwood xylan in a 3-day reaction at 45 °C, taking into account the stability results reported in the previous section. The xylose released from the reaction was quantified daily. Increasing values were measured after each day: 4.7 g/L, 7.2 g/L, and 10.7 g/L xylose was liberated after 24, 48, and 72 h, respectively. The 72 h supernatant was also subjected to sulphuric acid treatment in order to decompose oligosaccharides and determine the amount of total solubilized xylose (monomers and oligomers). The total solubilized xylose content was similar to the released monomer xylose concentration, indicating that the solubilized fraction of the xylan substrate was completely hydrolysed until reaching monomer form by the xyalnolytic supernatant of *C. phragmiteti*.

4. Conclusions

In this study, *C. phragmiteti* was found to be a promising candidate for the production high-value xylanase enzymes from the abundant and cheap lignocellulosic residues of WP. Producing enzymes using low-cost lignocellulosic wastes could reduce the price of the enzymes, which is usually a critical point in biotechnology, and could also contribute to mitigating waste handling issues. The most suitable culture medium and WP concentration to enhance xylanase production by *C. phragmiteti* was determined, and the obtained xylanases were characterized in terms of substrate specificity, pH and temperature optima, thermal stability, and salt tolerance. Water-intensive industrial areas might take advantage of the xylanase produced by *C. phragmiteti* because it can tolerate high salt concentrations. Thus, seawater might be used instead of fresh water in bioprocesses applying this enzyme. In contrast, using high concentrations of salt in the production of xylanases via *C. phragmiteti* should be avoided. Xylanases produced by *C. phragmiteti* show high activity at a wide pH range (from pH 5 to 8) and between 45 and 55 °C, which could provide them with a versatile portfolio of industrial applications, including biofuel, feed, and food production. These xylanases also have great thermal stability, preserving more than half of their activity after 3 days of incubation at 45 °C, making them great candidates for bioprocessing techniques that require long residence times (e.g., second-generation biofuel production) and allowing for them to be recycled multiple times. Thus, cultivating the novel bacterium *C. phragmiteti* on waste paper offers a great alternative for xylanase production, as *C. phragmiteti* shows promising attributes that could be useful in many industrial applications.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pr12020258/s1, Figure S1. Relative xylanase activities [%] obtained from fermentation by using *C. phragmiteti* in LB medium containing 1 *w*/*w*% of dry matter waste paper as a function of fermentation time. Average values of relative xylanase activity are presented with error bars representing the standard deviations of triplicates. Figure S2. Appearance of the waste paper used in the experiments.

Author Contributions: K.B. conducted experiments, analysed data, and wrote the manuscript. T.F. conducted experiments and analysed data. O.M.O. and E.C. analysed data and made corrections to the manuscript. C.F. conceived and designed the study, was responsible for funding acquisition, and corrected the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data presented in this study are available from the corresponding author upon request. The data are not publicly available due to privacy restrictions.

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