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Discovery of a Novel β-xylosidase with Xylanase Activity and Its Application in the Production of Xylitol from Corncob Xylan

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Abstract: Although β-xylosidases with xylanase activity are preferential for the hydrolysis of xylan and production of xylitol, reports on their use are scarce. In this study, a multifunctional β-xylosidase (XYL4) was identified. In addition to β-xylosidase activity, XYL4 also exhibited xylanase and low α-arabinosidase activity. The enzyme was able to hydrolyze bagasse xylan, oat spelt xylan, birchwood xylan, beechwood xylan, and corncob xylan, and showed the highest hydrolysis activity for corncob xylan. Structural modeling analysis indicated that XYL4 had an additional PA14 domain, which may play a key role in binding xylan substrates. Moreover, XYL4 was used to hydrolyze corncob xylan to produce xylose. When enzymatic hydrolysis and whole-cell catalysis were used to hydrolyze 100 g/L of corncob xylan, the xylose yields were 60.26% and 35.85%, respectively. Then, the *Candida tropicalis* was inoculated with the above hydrolysates for fermentation to produce xylitol. Using enzymatic hydrolysis and whole-cell catalysis, xylitol yields of 77.56% and 73.67% were obtained by *C. tropicalis* after the optimization of fermentation, respectively.

Keywords: β-xylosidase; corncob xylan; synergistic fermentation; xylitol production

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1. Introduction

With the excessive consumption of fossil fuels, the consequent energy shortage and environmental pollution have become the focus of increasing concern [1]. To alleviate this problem, the biorefinery of abundantly available and renewable lignocellulosic biomass is a feasible solution [2]. Corncob is one of the most abundant lignocellulosic wastes and contains cellulose (30–40%, *w*/*w*), xylan-type hemicellulose (30–35%, *w*/*w*), and lignin (10–20%, *w*/*w*), which are considered to be satisfactory sources for the production of bioproducts, chemicals and solvents [3]. Xylans consist of a β-1,4-linked xylose backbone, which may be branched with different substituents in the side chain, such as L-arabinofuranose, ferulic acid, 4-O-methylglucuronic acid, and acetyl groups [4]. In the biorefining process, xylans in biomass are initially degraded into monosaccharides via chemical, physical or enzymatic methods [5]. Among these methods, enzymatic degradation is considered a potential strategy due to its environmental friendliness [6]. The complete degradation of xylan depends on the synergistic action of multiple xylanolytic enzymes, of which endo-1,4-β-xylanase (EC 3.2.1.8) and β-xylosidase (EC 3.2.1.37) are the key enzymes. These enzymes are commonly found in bacteria and filamentous fungi such as *Geobacillus stearothermophilus*, *Clostridium* spp., *Trichoderma reesei*, and *Aspergillus* spp. Generally, endo-xylanase acts randomly on the internal xylosidic linkages of xylan to produce short-chain xylo-oligosaccharides, and β-xylosidase subsequently hydrolyses the soluble xylo-oligosaccharides to release xylose [7]. To date, there have been many reports on the synergistic hydrolysis of xylan by xylanase

and β-xylosidase [8,9]. In addition to the high cost of using two kinds of enzyme, the xylose yields still remain low [10–12], and the efficient production of xylose remains challenging. Thus, it is important to explore novel xylanolytic enzymes for the high-efficiency and low-cost production of xylose from xylan. Xylose can also be converted into a series of industrially important chemicals such as xylitol, ethanol, furfural, lactic acid, and butyric acid, through either chemical or biotechnological routes [13].

Xylitol is a five-carbon sugar alcohol which is marketed as a sucrose substitute for obese and diabetic patients because it has similar sweetness but fewer calories [14,15]. In addition, xylitol can be used to prevent many bone-related diseases like osteoporosis [16]. Given its outstanding properties, xylitol has been widely used in the food, beverage, and pharmaceutical industries [17,18]. The traditional method of xylitol production is mainly through the chemical hydrogenation of purified xylose, using Raney nickel as a catalyst. This process is costly, energy-intensive, and environmentally unfriendly, thus hindering the development and use of xylitol in the food and pharmaceutical industries [19]. In contrast, xylitol production via microbial pathways is relatively simple and environmentally friendly. However, most of the industrial microbes currently in use lack an efficient metabolic pathway that utilizes xylose. The yeast *Candida tropicalis* can efficiently metabolize xylose to produce xylitol, and it has been considered as a desirable microorganism for converting xylose to xylitol due to its high xylitol production capacity [20].

In this work, to demonstrate that a single xylosidase has the ability to hydrolyze xylans to produce xylose for further use, a multifunctional β-xylosidase XYL4 from *Sphingomona elodea* ATCC 31461 was characterized and was used to prepare corncob xylan hydrolysate via enzymatic hydrolysis and whole-cell catalysis. Then, xylitol fermentation from the hydrolysate by *C. tropicalis* was evaluated. Therefore, an environmentally friendly bioprocess for producing xylitol from enzymatic corncob xylan hydrolysate was developed.

2. Results and Discussion

2.1. Enzymatic Properties of Recombinant Protein

The gene *xyl4* encoding a hypothetical GH3 hydrolase from *S. elodea* ATCC 31461 was cloned and predicted to encode 852 amino acids. The *xyl4* was ligated with the expression vector pSE380, and the recombinant plasmid was then introduced into *Escherichia coli* XL1-Blue for expression. After purification via nickel nitrilotriacetic acid (Ni-NTA) chromatography, recombinant XYL4 migrated as a single band of approximately 90 kDa in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Figure S1), which was in accordance with the theoretical molecular weight.

The purified XYL4 showed its maximum activity at 50 °C on *p*NPX (Figure 1A). The enzyme was stable, retaining at least 85.72% of its original activity after incubation for 60 h at 40, 45, or 50 °C (Figure 1B). At 55 °C, the half-life of XYL4 was about 12 h. XYL4 was more thermostable than some reported β-xylosidases [21,22], which had half-lives of <4 h at 50 °C. The purified XYL4 showed optimal activity at pH 5.5 and retained >90.74% of its maximum activity at pH 5.0–6.0 (Figure 1C). Furthermore, XYL4 was stable over a broad pH range (3.0–9.5), retaining >84.38% of its original activity after incubation in specified buffers for 24 h at 4 °C (Figure 1D).

The effects of various organic solvents on XYL4 activity are shown in Figure S2. The relative activity of XYL4 was maintained at 116.2% in 15% (*v*/*v*) ethanol. Similar to ethanol, the enzyme retained at least 100% of its initial activity in the presence of 15% (*v*/*v*) 1,2-propanediol, 1,3-propanediol, dimethyl sulfoxide (DMSO), and dimethylformamide (DMF). Furthermore, the effects of several saccharides, metal ions, and chemical solvents on the activity of XYL4 are described in Table S1. The results indicated that the activity of XYL4 was virtually insensitive to most of the carbohydrates, metal ions, and chemicals listed in Table S1, even at a concentration of 20 mM. In addition, the enzyme activity was moderately activated by K^+, Ca^{2+}, Fe^{2+}, Zn^{2+}, and cetyltrimethyl ammonium bromide (CTAB) at concentrations of 5 mM and 20 mM. However, the activity of XYL4 was completely inhibited by SDS, which was similar to β-xylosidase from *Thermobifida halotolerans* YIM 90462 [23].

The above results indicated that XYL4 was stable in most organic solvents, metal ions, and chemical solvents.

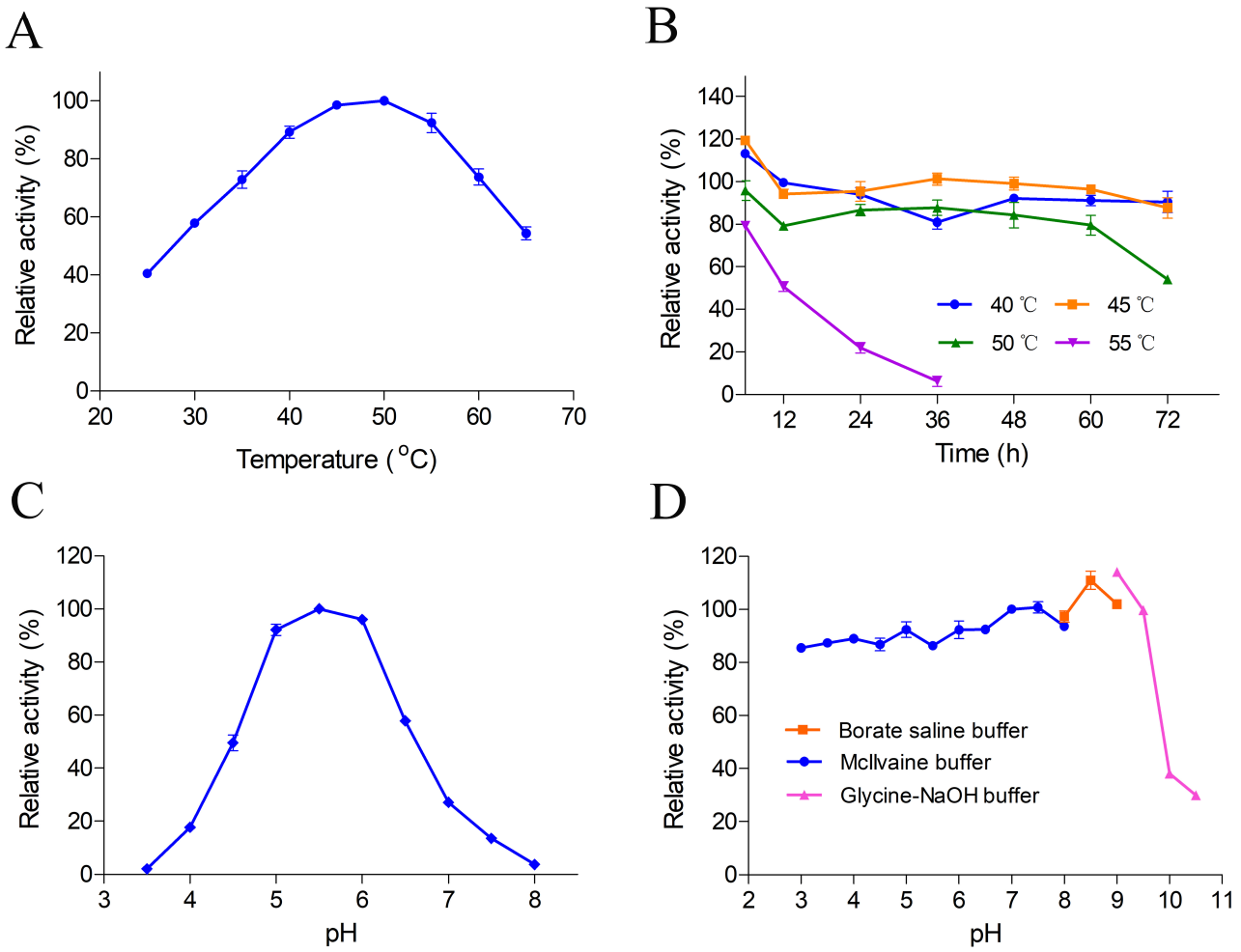

Figure 1. Effect of temperature and pH on the activity and stability of XYL4. (**A**) Effect of temperature on the enzyme activity. (**B**) Effect of temperature on stability. (**C**) Effect of pH on the enzyme activity. (**D**) Effect of pH on stability.

XYL4 could hydrolyze *p*NPX and *p*NPA, and the corresponding β-xylosidase and α-L-arabinosidase activities were 2.0 U/mg and 0.4 U/mg, respectively. The enzyme also had the ability to attack xylo-oligosaccharides with a degree of polymerization of 2–6 units. Activity assays conducted with xylo-oligosaccharides indicated that the hydrolysis rate increased with the chain length (Figure S3), and the specific order was xylohexaose > xylopentaose > xylotetraose > xylotriose > xylobiose. The results indicate that XYL4 tends to hydrolyze longer xylo-oligosaccharides, and a similar observation has been reported previously [24].

2.2. Xylan Substrate Specificity

XYL4 exhibited a broad substrate specificity for xylans from different sources. It was able to hydrolyze bagasse xylan, oat spelt xylan, birchwood xylan, beechwood xylan, and corncob xylan, and showed its highest hydrolysis activity of 8.16 U/mg for corncob xylan. This finding was significantly different from those reported for β-xylosidases, which exhibited no activity toward xylan substrates [25]. Several β-xylosidases have been found to have additional α-arabinosidase activity [26,27], but β-xylosidases with xylanase activity have rarely been reported and, in such reports, the xylanase activity is very low. As shown in Table 1, β-xylosidases from *Phanerochaete chrysosporium* and *Colletotrichum*

graminicola had the ability to hydrolyze birchwood xylan and beechwood xylan, but their activities were very low. β-xylosidase from *Aspergillus niger* had relatively high hydrolytic activity for birchwood xylan; however, it could only hydrolyze a small number of xylans. In contrast, XYL4 was not only able to hydrolyze a variety of xylans, it also had the highest hydrolyzing activity for corncob xylan. Although xylanases with high activity have been identified [28], the hydrolysis of xylan is still inseparable from the synergy of β-xylosidase [8]. Therefore, β-xylosidase XYL4 with xylanase activity shows a broad potential application in the saccharification of hemicellulose xylan.

Table 1. Xylan substrate specificities of some β-xylosidases with xylanase activity.

Enzyme	Organism	Family	Specific Activity (U/mg)						Ref.
			*p*NPX	Bagasse Xylan	Oat Spelt Xylan	Birchwood Xylan	Beechwood Xylan	Corncob Xylan	
XYL4	*Sphingomona elodea*	GH3	2	0.73	0.97	1.99	2.85	8.16	This study
rPcXyl	*Phanerochaete chrysosporium*	GH43	1797	—	—	<0.03	<0.1	—	[27]
Bxcg	*Colletotrichum graminicola*	GH3	284	—	—	0.63	0.60	—	[29]
Xylosidase	*Aspergillus niger*	GH3	61	—	—	5.4	—	—	[30]

—: not reported.

2.3. Comparative Modeling and Structural Analysis

Protein blast (BLASTp) analysis indicated that XYL4 shared the highest identity (42.36%) with the GH3 β-xylosidase from *Aspergillus flavus* NRRL3357 (GenBank: B8NYD8.1). However, in addition to β-xylosidase activity, XYL4 also exhibited xylanase activity. Therefore, this study constructed a protein model of XYL4 and attempted to analyze the cause from a structural perspective. First, the XYL4 model was compared with the protein structures of β-xylosidases that ranked high in sequence similarity to XYL4 in the protein data bank. As shown in Figure 2, XYL4 shared obvious structural similarities with these characterized β-xylosidases as a whole, but XYL4 had an additional PA14 domain that was mainly composed of β-strands and was combined with the catalytic domain via a loop, including an α-helix. Despite having the same catalytic nucleophile and acid-base residue (Figure S4), these characterized β-xylosidases could only catalyze small molecules, including *p*NPX and xylo-oligosaccharides [31,32], while XYL4, with its additional PA14 domain, showed good hydrolytic activity toward small molecules and a variety of xylans. In addition, studies have shown that the PA14 domain has the function of binding to carbohydrates [33,34]. Therefore, this study attempted to compare the PA14 domain of XYL4 with xylanase-derived carbohydrate-binding modules (CBMs). As shown in Figure 3, the PA14 domain of XYL4 was slightly similar to the CBM from CBM9-2 and CBM35, which had the ability to bind to monosaccharides, xylo-oligomers, or an unknown component of xylan [35,36]. Currently, all CBMs with known structures are composed mainly of β-strands, and some of these CBMs have the ability to bind to cellulose, glucan, galactan, starch, and xylan. The PA14 β-strand domain, which has a carbohydrate-binding function, is regarded as a novel CBM [33]. Therefore, the above results indicate that the PA14 domain of XYL4 might be a novel CBM with the ability to bind xylans.

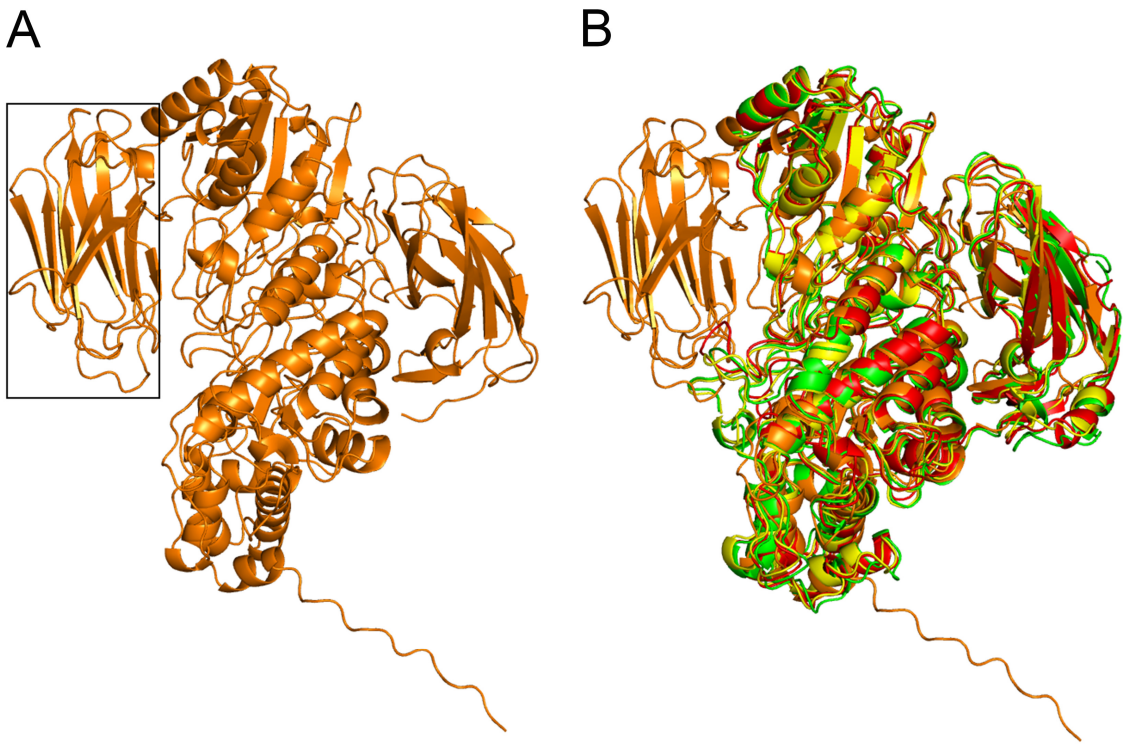

Figure 2. Comparison of protein structures between XYL4 (orange) and other GH3 β-xylosidases from *Phanerochaete chrysosporium* (red, PDB: 7VC6), *Hypocrea jecorina* (yellow, PDB: 5A7M), and *Aspergillus niger* (green, PDB: 6Q7I). (**A**) The protein structure of XYL4. The black box highlights the PA14 domain for XYL4. (**B**) Structural overlap of XYL4 and other GH3 β-xylosidases.

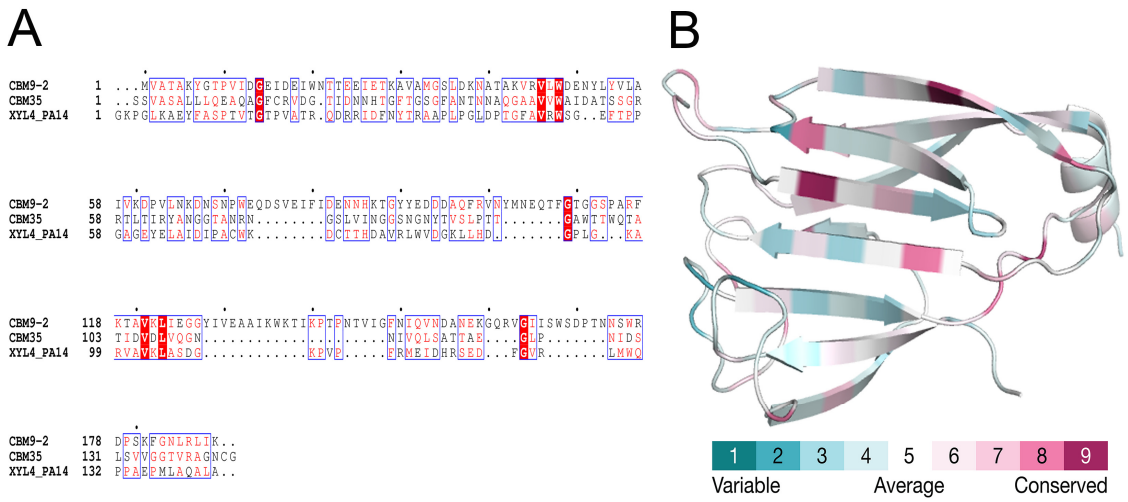

Figure 3. Bioinformatic analysis of XYL4. (**A**) Amino acid sequence alignment of the PA14 domain of XYL4 with xylanase-derived carbohydrate-binding modules (CBMs). CBM9-2 and CBM35 were from *Thermotoga maritima* xylanase Xyn10A (PDB: 1I82) and *Cellvibrio japonicus* xylanase Xyn10B (PDB: 2W46), respectively. Conserved residues were shadowed in red. (**B**) Three-dimensional evolutionary model of the PA14 domain of XYL4. The model was prepared based on the similarity between the PA14 domain and CBMs (CBM9-2 and CBM35) and was colored according to ConSurf conservation scores.

2.4. Optimization of Hydrolysis Conditions for Corncob Xylan

XYL4 was used to hydrolyze corncob xylan to produce xylose for xylitol production, and different factors affecting the hydrolysis were investigated. First, the effect of tempera-

ture was tested. As shown in Figure 4A, the highest amount of xylose was obtained at 45 °C, and the concentration of xylose decreased significantly as the temperature changed. pH is another factor that can significantly affect hydrolysis; as shown in Figure 4B, the optimum pH for the hydrolysis of corncob xylan was 5.0. Enzyme dosage had a significant effect on the production of xylose: the concentration of xylose increased with the increasing enzyme dosage from 2 to 8 U/g, but a further increase in enzyme dosage had little promotive effect on hydrolysis (Figure 4C). Therefore, the optimum dosage for corncob xylan hydrolysis was 8 U/g.

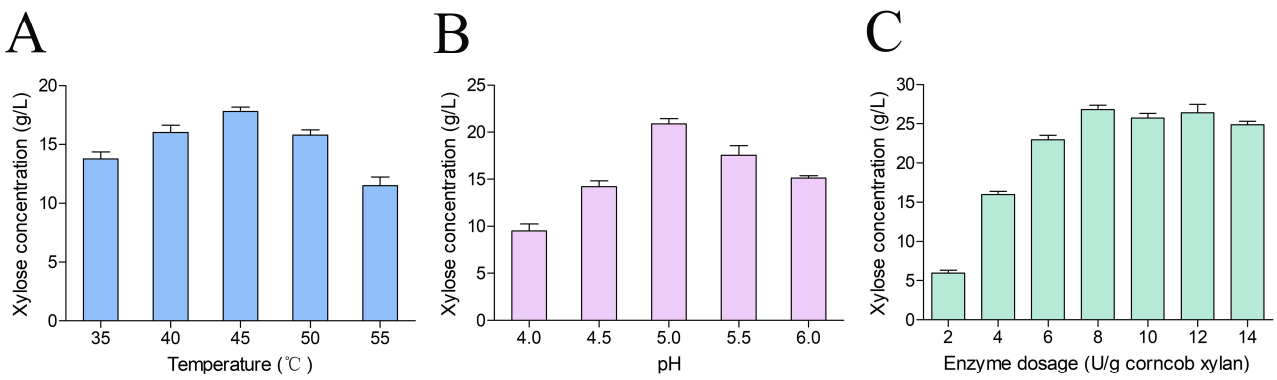

Figure 4. Effects of temperature (**A**), pH (**B**), and enzyme dosage (**C**) on the hydrolysis of corncob xylan by XYL4.

The common methods used for the hydrolysis of hemicellulose materials include steam explosion, alkali, and dilute acid treatment [37]. The steam explosion process requires no chemicals, but it is performed at high temperature and pressure, and the major liquor components produced are xylo-oligosaccharides, which are not suitable for fermentation by microorganisms [38]. The effect of alkali pretreatment is mediocre, and the xylose production is relatively low [39]. Compared with other hydrolysis methods, dilute acid pretreatment is particularly useful for the conversion of xylans to xylose. However, the process of acid hydrolysis always produces several inhibitors such as furan derivatives, weak acids, and phenolic compounds, which severely affect the subsequent bioconversion through fermentation. In contrast, enzymatic pretreatment has a lower environmental impact, moderate process conditions, and produces fewer inhibitors. Currently, the enzymatic hydrolysis of hemicellulose xylans is mainly accomplished through the synergistic action of a variety of xylanolytic enzymes, especially xylanase and β-xylosidase [9]. There are few reports on the hydrolysis of hemicellulose xylans by multifunctional xylanolytic enzymes [40]. Therefore, in this study, enzymatic hydrolysis of xylans by XYL4 is a novel and environmentally friendly process.

2.5. Production of Xylitol from Enzymatic Corncob Xylan Hydrolysate

XYL4 was used to hydrolyze corncob xylan to prepare corncob hydrolysate for xylitol production. As shown in Figure 5A, with the increase in the corncob xylan concentration from 40 to 180 g/L, the xylose concentration in the hydrolysate increased from 28.88 to 84.71 g/L. Then, the hydrolysate containing different xylose concentrations was used for xylitol production by *C. tropicalis*. After 48 h of fermentation, the maximum xylitol concentration of 29.67 g/L was obtained, and the corresponding xylitol yield was 49.24% when the initial xylose concentration in the hydrolysate was 60.26 g/L (Figure 5B). However, the concentration of xylitol in the fermentation broth, as well as the xylitol yield, decreased with higher initial concentrations of xylose, possibly because the growth of *C. tropicalis* was inhibited by the increasing osmotic pressure and fermentation inhibitors [41].

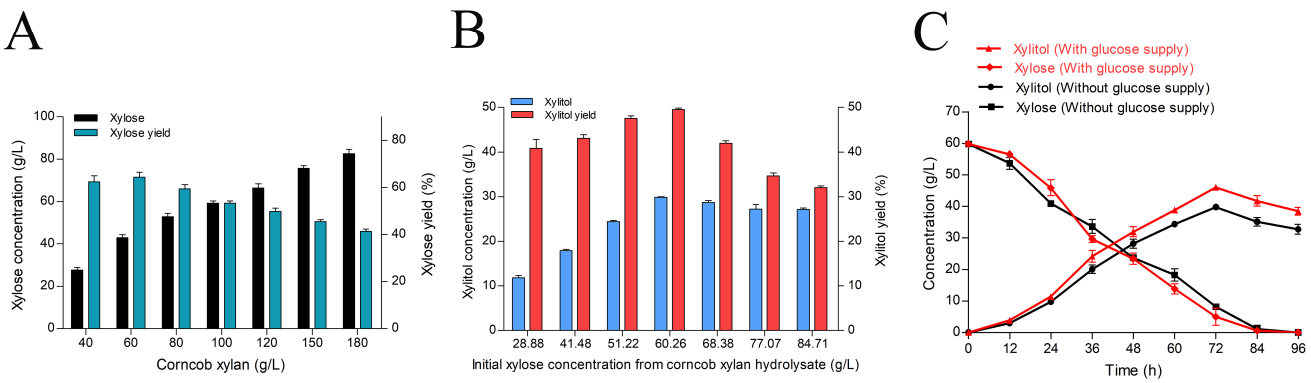

Figure 5. Hydrolysis of corncob xylan and production of xylitol. (**A**) Results of hydrolysis of different concentrations of corncob xylan by XYL4. (**B**) Effect of initial xylose concentration from corncob xylan hydrolysate on xylitol yield. The hydrolysate containing different xylose concentrations (28.88–84.71 g/L) was fermented by *C. tropicalis* for 48 h. (**C**) Time course of xylitol production by *C. tropicalis* using corncob xylan hydrolysate supplemented with or without 20/L of glucose.

To obtain a higher xylitol yield, the initial concentration of xylose and the fermentation time should be controlled. Therefore, corncob xylan hydrolysate, including 60.26 g/L of xylose, was selected for follow-up fermentations, and the concentrations of xylitol and xylose in the fermentation broth were recorded at 12, 24, 36, 48, 60, 72, 84, and 96 h. As shown in Figure 5C, the xylitol concentration increased to 40.12 g/L at 72 h, and the corresponding yield increased to 66.58%. Additional glucose added to the hydrolysate can significantly increase the yield of xylitol [6]. As shown in Figure 5C, with glucose addition, the xylitol concentration was 46.74 g/L and the yield was 77.56%, which was an increase of 11.0% compared to that without glucose addition.

Table 2 compares the xylitol production in this study with that in other reports using pure xylose and hemicellulose with/without co-substrate for xylitol production. The range of xylitol concentration (1.94–200 g/L) and yields (32–98%) obtained in these studies varied broadly, depending on the feedstock/carbon source, inhibitor composition, and/or pretreatment method. With the pretreatment of XYL4, the yield of xylitol in this study reached 77.56%, which was comparable to or even better than the currently reported state-of-the-art data, making this process competitive. Furthermore, in comparison with these previously reported methods, the xylitol production in this study was simple and no chemicals were needed in the fermentation pretreatment. Therefore, this xylitol production method was environmentally friendly, economical, and efficient, demonstrating a wide potential for application in xylitol bioconversion.

2.6. Production of Xylitol via Whole-Cell Catalysis

On the basis of the above xylitol production, in order to avoid the complex process of XYL4 production and improve the production efficiency of xylitol, the whole-cell catalysis of recombinant *E. coli* for corncob xylan hydrolysis was examined. First, the optimal *E. coli* cell concentration during the hydrolysis of corncob xylan was investigated. As shown in Figure 6A, the concentration of xylose increased with the increase in *E. coli* cell concentration from 15 to 30 g/L. Further increasing the cell concentration did not improve the hydrolysis of the corncob xylan, and therefore, the optimal cell concentration for hydrolysis was determined to be 30 g/L. Furthermore, the optimal time of hydrolysis was tested. The results showed that the optimal time for the hydrolysis of corncob xylan by 30 g/L *E. coli* cells was 12 h (Figure 6B). Under optimal conditions, 100 g/L of corncob xylan could be converted to 35.85 g/L of xylose, and the corresponding xylose yield was 35.85%. Finally, *C. tropicalis* cells collected from 50 mL YPD medium were added to the reaction mixture for 96 h to produce xylitol. As shown in Figure 6C, the xylitol concentration reached a maximum of 26.41 g/L at 80 h, and the xylitol yield was 73.67%.

Table 2. Comparison of xylitol production by different microorganisms.

Microorganisms	Feedstock	Pretreatment	Xylitol Production		Ref.
			Concentration (g/L)	Xylitol Yield (%)	
Yarrowia lipolytica	Xylose and glycerol	—	53.2	97	[42]
Candida tropicalis BSXDH-3	Xylose, glycerol and glucose	—	48.6	98	[43]
Escherichia coli	Xylose	—	200	—	[44]
Pichia fermentans	Xylose	—	98.9	67	[45]
Candida tropicalis W103	Corncob	0.5% sulfuric acid and 1.5% phosphoric acid	17.1	32	[46]
Candida tropicalis MTCC 6192	Corncob	1% sulfuric acid	21.98	37	[47]
Candida tropicalis	Corncob	0.5% nitric acid	56.5	62	[48]
Candida tropicalis 31949	Sugarcane bagasse	Ultrasonic-Assisted NaOH and xylanase	62.98	62.98	[41]
Candida tropicalis	Corncob	Xylanase and β-xylosidase	8.18	45.37	[6]
Candida tropicalis IT-Xol-1	Xylan	Xylanase AtN and β-xylosidase AtL	3.78	77.1	[49]
Saccharomyces cerevisiae	Xylan	Xylanase and β-xylosidase	1.94	71	[50]
Candida tropicalis	Corncob xylan	XYL4	46.74	77.56	This study

—: not reported.

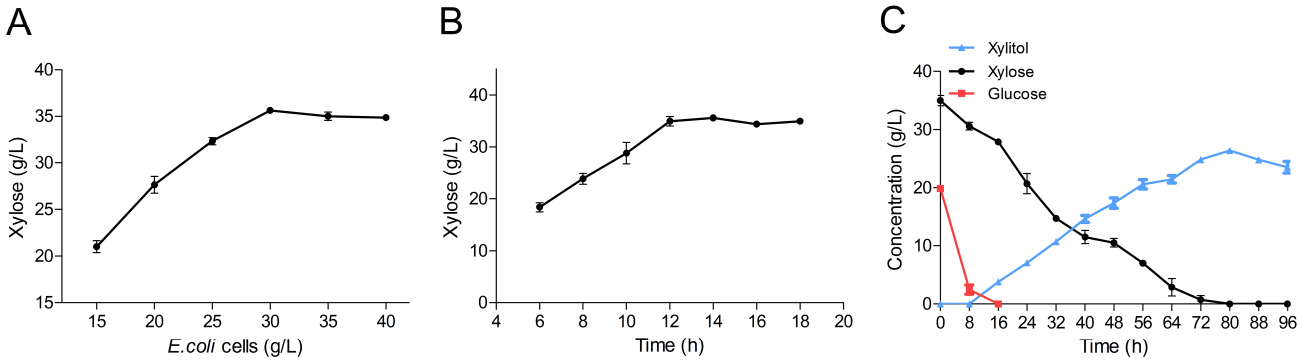

Figure 6. Xylitol production by the whole-cell catalysis. (**A**) The optimal concentration of recombinant *E. coli* cells for the hydrolysis of 100 g/L of corncob xylan. (**B**) The optimal time of recombinant *E. coli* cells for the hydrolysis of 100 g/L of corncob xylan. (**C**) Xylitol production from corncob xylan hydrolysate supplemented with 20 g/L of glucose by the whole-cell catalysis of *C. tropicalis*.

Comparing the above two methods of xylitol production revealed that less xylitol was produced from corncob xylan hydrolysate by whole-cell catalysis. With 100 g/L of corncob xylan as a substrate, 60.26 g/L of xylose was produced by enzymatic hydrolysis, while only 35.85 g/L of xylose was produced by whole-cell catalysis. The higher xylose concentration in the hydrolysate was beneficial to xylitol conversion [51]. The activity of corncob xylan hydrolysis under whole-cell catalysis was weak, which might be related to the permeability of *E. coli* cell membranes to substrates and enzymes. Previous research has shown that recombinant yeast strains are well suited for xylan hydrolysis and xylitol fermentation [50]. Therefore, introducing the multifunctional β-xylosidase XYL4 into yeast cells may improve the production efficiency of xylitol.

3. Materials and Methods

3.1. Strains, Plasmids and Chemicals

The vector pSE380 (Invitrogen, Carlsbad, CA, USA) was used as the expression vector, and *E. coli* XL1-Blue was employed as the host strain for expression. *P*-nitrophenyl derivatives, beechwood xylan, birchwood xylan and oat spelt xylan were purchased from Sigma-Aldrich (St. Louis, MO, USA). Corncob xylan was purchased from Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China). Bagasse xylan and xylo-oligosaccharides were purchased from Shanghai Yuanye Biological Technology Co., Ltd. (Shanghai, China).

3.2. Production of Recombinant Protein

The XYL4 gene was amplified from the genomic DNA of *Sphingomona elodea* ATCC 31461 using polymerase chain reaction (PCR) with the primers sGH3-1-F (5′-ATTACCATGG ATGCCGCTTCTCCCCCTGCTCCTC-3′) and sGH3-1-R (5′-CCTAAGCTTTCAATGGTGGT GGTGGTGGTGTTTCGGCAACTCCTGCGCGGTGCC-3′); the underlined sequences represent restriction sites for *Nco*I and *Hin*dIII, respectively. The amplified product was digested with these restriction enzymes and then ligated into pSE380. After verification, the recombinant plasmid pSE-*xyl4* was introduced into *E. coli* XL1-Blue for protein expression. The constructed strain was cultured in Terrific Broth (TB) medium containing 100 μg/mL ampicillin at 37 °C until the cell density (OD_{600}) reached 0.6. Recombinant protein expression was induced by the addition of 0.5 mM isopropyl β-D-1-thiogalactopyranoside; the cells were then further cultured at 30 °C for 12 h with shaking at 180 rpm and collected by centrifugation at 4 °C (8000× *g*, 10 min). Then, the cells were resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) and were then disrupted by ultrasonication. The supernatant was collected and used as crude enzyme after centrifugation at 12,000× *g* for 30 min at 4 °C. The crude enzyme was mixed with pre-equilibrated Ni-NTA agarose resin for 1 h at 4 °C with slow shaking, and then the mixture was loaded into a prechilled column. After washing with 4 mL wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0), the recombinant enzyme was eluted in elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0). The fraction containing the pure XYL4 was concentrated and desalted against 100 mM NaH_2PO_4–Na_2HPO_4 buffer (pH 7.0) using a PD MiniTrap G-25 column (GE Healthcare, Freiburg, Germany). The purity and molecular mass of the recombinant protein were determined using 10% SDS-PAGE and visualized via staining with Coomassie blue R-250.

The protein sequence accession number of XYL4 in GenBank is WP_010546304.1.

3.3. Enzyme Activity Assays and Biochemical Properties

The β-xylosidase activity of XYL4 was assessed by measuring the release of *p*-nitrophenol (*p*NP) from *p*NP-β-D-xylopyranoside (*p*NPX). The standard reaction (200 μL) was performed in McIlvaine buffer (100 mM citric acid–200 mM Na_2HPO_4, pH 5.5) containing 2 mM *p*NPX and dilute enzyme solution at 50 °C for 20 min. After preheating for 2 min, the reaction was initiated by adding enzyme solution, and the reaction was terminated by adding 50 μL of 2 M Na_2CO_3 to the reaction mixture. The amount of *p*NP released was detected by measuring the absorbance at 405 nm using a microplate reader (BioTek, Winooski, VT, USA). A standard curve of *p*NP was prepared and used to calculate the amount of product. All enzyme activities were determined in three independent experiments, and the reaction without enzyme was used as a blank control. One unit (U) of β-xylosidase activity was defined as the amount of enzyme (in milligrams) that released 1 μmol of *p*NP per minute under the experimental conditions described above. The specific activity was defined in U per milligram of total protein.

The optimum pH for the activity of XYL4 was determined by incubating the reactions in McIlvaine buffer (pH 3.5–8.0) supplemented with 2 mM *p*NPX at 37 °C for 20 min. The pH stability of the enzyme was determined by measuring the residual activity under the optimum reaction conditions after incubating purified XYL4 for 24 h at 4 °C without substrate in the following buffer systems: McIlvaine buffer (pH 3.0–8.0), $Na_2B_4O_7$–H_3BO_3

buffer (100 mM, pH 8.0–9.0), and glycine–NaOH buffer (100 mM, pH 9.0–10.5). The optimum temperature for the activity of XYL4 was determined by conducting reactions in McIlvaine buffer (pH 5.5) containing 2 mM *p*NPX at 25–65 °C for 20 min. Thermostability was characterized by analyzing the residual enzyme activity under the optimum reaction conditions after incubating the purified enzyme without substrate for 6, 12, 24, 36, 48, 60, or 72 h at 40–55 °C.

The effects of organic solvents containing ethanol, 1,2-propanediol, 1,3-propanediol, DMSO, and DMF on the activity of XYL4 were determined with the final concentration of 5–30% (*v*/*v*). The effects of carbohydrates, metal ions, and chemical compounds including xylose, glucose, fructose, arabinose, maltose, galactose, KCl, $CaCl_2$, $CdCl_2$, $CoCl_2$, $FeCl_2$, $ZnCl_2$, $MnCl_2$, $MgCl_2$, $CuCl_2$, urea, SDS, DTT, CTAB, and ethylenediamine tetraacetic acid (EDTA) on the activity of XYL4 were determined with the final concentrations of 5 mM and 20 mM. All enzyme activities were investigated under the standard reaction method described above.

3.4. Substrate Specificity Assays

The substrate specificities of XYL4 were determined using 2 mM *p*NP-α-L-arabinofuranoside (*p*NPA), *p*NP-β-D-glucopyranoside (*p*NPG), and *p*NP-β-D-cellobioside (*p*NPC), as well as 5 g/L xylo-oligosaccharides, birchwood xylan, beechwood xylan, oat spelt xylan, and corncob xylan. The hydrolysis reactions of *p*NPA, *p*NPG, and *p*NPC were measured in the same manner as that used in the *p*NPX assay. The sugars released by the hydrolysis of xylo-oligosaccharides, oat spelt xylan, bagasse xylan, birchwood xylan, beechwood xylan and corncob xylan were determined using the 3,5-dinitrosalicylic acid method [52]. One U of xylanase activity was defined as the amount of enzyme (in milligrams) that produced 1 μmol of reducing sugar (xylose) from the xylan substrate per minute under the optimum reaction conditions.

3.5. Bioinformatic Analysis

The XYL4 gene was analyzed and aligned using the Vector NTI software (version Vector NTI Advance 11.5, Invitrogen, Carlsbad, CA, USA), and further aligned using BLASTx and BLASTp (http://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 10 January 2023). The signal peptide and domain information were predicted using the SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP-4.1/, accessed on 12 January 2023) and SMART (http://smart.embl-heidelberg.de/, accessed on 12 January 2023), respectively. Multiple sequence alignment of proteins was conducted using ClustalW (http://www.ebi.ac.uk/clustalW, accessed on 5 March 2023). Three-dimensional protein modeling of XYL4 was performed by using AlphaFold2 [53] and further analyzed using PyMOL (version PyMOL 2.2.3, DeLano Scientific LLC, San Carlos, CA, USA). A three-dimensional model of evolutionary conservation was prepared using ConSurf [54].

3.6. Optimization of Hydrolysis Conditions for Corncob Xylan

XYL4 was used to hydrolyze corncob xylan and the basic hydrolysis conditions were as follows: enzyme dosage of 6 U/g corncob xylan, initial concentration of corncob xylan 100 g/L, temperature 45 °C, pH 5.5, reaction time 6 h. Then, different temperatures (35–55 °C), pH values (4.0–6.0), and enzyme dosages (2–16 U/g corncob xylan) were investigated to determine the most suitable hydrolysis conditions for corncob xylan.

3.7. Production of Xylitol from Enzymatic Corncob Xylan Hydrolysate

A single colony of *C. tropicalis* CICC 31949 was inoculated into 5 mL of YPD medium (10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose), and the culture was grown for 12 h at 30 °C with shaking at 220 rpm to obtain seed liquid. Then, the seed liquid was inoculated (10% *v*/*v*) in 250 mL Erlenmeyer flasks containing 50 mL of corncob hydrolysate (with or without 20 g/L glucose) and incubated at 30 °C and 170 rpm for 96 h. The culture was sampled every 12 h and the concentrations of xylitol and xylose in the

centrifuged supernatant were determined using high-performance anionic exchange liquid chromatography (HPAEC, Thermo Scientific, Waltham, MA, USA).

3.8. Production of Xylitol by Whole-Cell Catalysis

The recombinant *E. coli* harboring the plasmid pSE-*xyl4* was induced, collected via centrifugation, and washed with distilled water three times. Individual clones of *C. tropicalis* were inoculated in 50 mL of YPD medium and cultured at 30 °C and 220 rpm for 28 h, and cells were then collected via centrifugation and washed with distilled water three times. A reaction mixture containing 100 g/L of corncob xylan and an appropriate concentration of *E. coli* cells was incubated at 45 °C and 220 rpm. After 12 h, *C. tropicalis* cells and 20 g/L glucose were added to the reaction mixture and incubated at 30 °C and 170 rpm for 96 h. The products of the reactions were analyzed using HPAEC.

3.9. Analytical Methods

The concentrations of xylose and xylitol in the fermentation medium were determined via HPAEC using a Dionex electrochemical detector and a CarboPac PA100 Analytical Column (4 mm × 250 mm). The sample was eluted with a gradient, as follows: 15% B and 2% C (0–10 min), 15–50% B and 2–20% C (10–20 min), 50–15% B and 20–2% C (20–25 min), and 15% B and 2% C (25–30 min). The flow rate was 1 mL/min and the analysis was conducted at 30 °C.

The xylose yield was determined by the ratio of the final xylose concentration to the initial corncob xylan concentration. The xylitol yield was determined by the ratio of the final xylitol concentration to the initial xylose concentration in the corncob xylan hydrolysate.

4. Conclusions

In this study, a novel β-xylosidase XYL4 from *S. elodea* ATCC 31461 with xylanase and α-arabinosidase activity was identified. XYL4 was able to hydrolyze bagasse xylan, oat spelt xylan, birchwood xylan, beechwood xylan, and corncob xylan, and showed the highest hydrolysis activity of 8.16 U/mg for corncob xylan. Enzymatic hydrolysis and whole-cell catalysis were used to hydrolyze 100 g/L of corncob xylan, and the corresponding xylose yields were 60.26% and 35.85%, respectively. The above hydrolysates were used for xylitol production, and xylitol yields of 77.56% and 73.67% were obtained by *C. tropicalis* using enzymatic hydrolysis and whole-cell catalysis after the optimization of fermentation, respectively. This study is the first report of the conversion of corncob xylan into xylitol by a multifunctional β-xylosidase in collaboration with *C. tropicalis*, which provides a new method for the utilization of hemicellulose xylan and the production of xylitol.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9070606/s1. Figure S1: Analysis of the purified XYL4 by SDS-PAGE; Figure S2: The effect of organic solvents on the activity of XYL4; Figure S3: Hydrolytic activity of XYL4 toward xylo-oligosaccharides; Figure S4: Amino acid sequence alignment of XYL4 with other GH3 β-xylosidases from *Hypocrea jecorina* (PDB: 5A7M), *Aspergillus niger* (6Q7I) and *Phanerochaete chrysosporium* (7VC6); Table S1: The effect of carbohydrates, metal ions and chemical solvents on the activity of XYL4.

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