Novel Salt-Tolerant Xylanase from a Mangrove-Isolated Fungus Phoma sp. MF13 and Its Application in Chinese Steamed Bread

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ABSTRACT: A novel glycosyl hydrolase family 11 xylanase gene, xynMF13A, was cloned from Phoma sp. MF13, a xylanase-producing fungus isolated from mangrove sediment. xynMF13A was heterologously expressed in Pichia pastoris, and the recombinant XynMF13A (rXynMF13A) was purified by Ni-affinity chromatography. The temperature and pH optima of purified rXynMF13A were 45 °C and pH 5.0, respectively. rXynMF13A showed a high level of salt tolerance, with maximal enzyme activity being seen at 0.5 M NaCl and as much as 53% of maximal activity at 4 M NaCl. The major rXynMF13A hydrolysis products from corn cob xylan were xylobiose, xylotriose, xylotetraose, and xylopentaose, but no xylose was found. These hydrolysis products suggest an important potential for XynMF13A in the production of xylooligosaccharides (XOs). Furthermore, rXynMF13A had beneficial effects on Chinese steamed bread production, by increasing specific volume and elasticity while decreasing hardness and chewiness. These results demonstrate XynMF13A to be a novel xylanase with potentially significant applications in baking, XOs production, and seafood processing.

INTRODUCTION

Mangroves are salt-tolerant forest ecosystems that are situated between terrestrial and marine environments of tropical and subtropical regions.1 These ecosystems are considered to be unique and dynamic environments because their geochemical characteristics such as salinity, soil humidity, and nutrient concentration are cyclically modified by periodic tidal flooding.2 Mangrove forests are characterized by high biological productivity and are the second most productive and rich marine ecosystems after coral reefs.3 In mangrove ecosystems, close relationships in the recycling and conservation of nutrients have been found among soil microorganisms and plants.3,4 Mangroves have very high rates of biomass production because they receive sufficient light and have access to abundant nutrients from mud that is rich in organic matter.5,6 Considerable amounts of leaf litter, twigs, bark, wood, inflorescence material, and other detritus are generated from mangrove forests and decomposed by the productive and diverse microbial community living in mangrove sediments.6,7

Fungi are major components of the microbial community in mangrove forests, playing important roles in the nutritive cycle that supports the mangrove ecosystem.7 Moist conditions, rich organic matter, aeration, and the low pH of mangrove ecosystems favor a very high fungal biodiversity.8,9 Both culture-dependent and culture-independent methods have demonstrated high fungal diversity in mangrove ecosystems.9–12 Mangrove fungi play important roles in the early stages of organic matter decomposition through the efficient production of a wide range of extracellular degradative enzymes.13 Plant cell walls are the major organic matter in mangrove ecosystems; cell wall hydrolysis is a complex process in which hemicellulose digestion is the initial step, prior to cellulose hydrolysis.14,15

Xylan, the predominant component of hemicellulose, is composed of a backbone chain of β-1,4-linked xylopyranose units with substituted side chains at different positions.16 Because of the complex components and structure of xylan, the synergistic action of several xylanolytic enzymes is required to completely hydrolyze xylan. These enzymes include endo-1,4-β-D-xylanase, β-D-xylanase, α-D-glucuronidase, α-1-arabinofuranosidase, and arylesterase.17,18 Among them, the most crucial enzyme is endo-1,4-β-D-xylanase (E.C. 3.2.1.8), which randomly cleaves β-1,4-glycosidic bonds of the xylan backbone and hydrolyzes it into xylooligosaccharide (XO) and xylose. On the basis of sequence similarities of the catalytic domain, 135 glycosyl hydrolase (GH) families have been identified, with xylanases belonging to families 5, 7, 8, 10, 11, and 43.18 The vast majority of xylanases are confined to GH families 10 and 11.

Mangrove fungi are of special interest because they constitute the second largest ecological group of marine fungi and are adapted to extreme conditions, which make them a rich source for novel metabolite and enzyme discovery.19,20 Xylanase activities have been widely reported for fungi isolated from mangroves.21–23 However, few studies of mangrove fungi have focused on novel xylanase gene discovery. In this study, a xylanase-producing fungus, Phoma sp. MF13, was isolated from a mangrove sediment. A novel GH11 xylanase gene

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(xynMF13A) was cloned from it and expressed successfully in Pichia pastoris. Enzyme characterization suggests that the recombinant enzyme, rXynMF13A, is a salt-tolerant xylanase with potential application in baking, XOs production, and seafood processing.

■ RESULTS AND DISCUSSION

Strain Identification. Using xylan as the sole carbon source, a xylanase-producing fungus was isolated from mangrove sediment of the Sankou natural conservation site. The 18S rDNA sequence (948 bp) and internal transcribed spacer (ITS) region sequence (571 bp) of strain MF13 were compared with those in NCBI using BLASTn analysis. The 18S rDNA sequence (948 bp) and internal transcribed spacer (ITS) region sequence (571 bp) of strain MF13 were amplified by using the CODEHOP primers X11-F and X11-R. The 5′ and 3′ flanking sequences amplified by thermal asymmetric interlaced-polymerase chain reaction (TAIL-PCR) were assembled with known fragment sequences to give a full-length xynMF13A of 749 bp. The cDNA of xynMF13A was amplified by xynMF13A-F and xynMF13A-R, and an open reading frame (ORF) of 696 bp starting with ATG and terminating with TAA was identified. On the basis of the alignment of the xynMF13A gene sequence and its cDNA sequence, an intron, 53-bp long, was found that interrupts the coding sequence of xynMF13A. The ORF of xynMF13A encodes a protein of 231 amino acid residues, including a putative signal peptide of 19 residues and a catalytic domain of GH family 11. The theoretical molecular weight and isoelectric point of XynMF13A are 24.42 kDa and 9.17, respectively.

The deduced amino acid sequence of xynMF13A showed highest identity with the following putative fungal xylanases: 91% with Ascochyta pisi (CAA93120), 90% with Epicoccum nigrum (OSS44936), 90% with Ascochyta rabiei (KZM24001), and 82% with Alternaria alternata (XP_018385581). Moreover, XynMF13A showed 65% identity with xylanase XylS from Fusarium oxysporum f. sp. lycopersici (AAK27974) and 63% identity with xylanase XYN-L4 from Pithomyces chartarum (AGV40651) and with XynZG from Plectosphaerella cucumerina (ABA08462).}

Gene Cloning and Sequence Analysis of xynMF13A. A fragment of 213 bp was amplified by using the CODEHOP primers X11-F and X11-R. The 5′ and 3′ flanking sequences amplified by thermal asymmetric interlaced-polymerase chain reaction (TAIL-PCR) were assembled with known fragment sequences to give a full-length xynMF13A of 749 bp. The cDNA of xynMF13A was amplified by xynMF13A-F and xynMF13A-R, and an open reading frame (ORF) of 696 bp starting with ATG and terminating with TAA was identified. On the basis of the alignment of the xynMF13A gene sequence and its cDNA sequence, an intron, 53-bp long, was found that interrupts the coding sequence of xynMF13A. The ORF of xynMF13A encodes a protein of 231 amino acid residues, including a putative signal peptide of 19 residues and a catalytic domain of GH family 11. The theoretical molecular weight and isoelectric point of XynMF13A are 24.42 kDa and 9.17, respectively.

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Expression and Purification of rXynMF13A. The mature protein coding gene of XynMF13A was cloned into the pPICZα-C vector and then transformed into P. pastoris X-33 competent cells. After induction with methanol (0.5% every 12 h) for 120 h, significant xylanase activity (about 20.1 U mL⁻¹) was detected in the culture supernatant of the positive transformants, demonstrating successful expression of xynM-
F13A in *P. pastoris*. After purification by Ni-affinity chromatography, the recombinant xylanase (rXynMF13A) migrated as a single band of approximately 27 kDa in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 2).

**Enzyme Characterization.** Fungal xylanases are generally neutral or acidic,

and rXynMF13A has a pH optimum of 5.0. It retained more than 80% of its maximum activity between pH 4.0 and 7.0 (Figure 3a), with good stability. The pH stability profile showed that purified rXynMF13A was highly stable from pH 5.0 to 10.0 and retained more than 70% of its maximum activity throughout this range (Figure 3b). The majority of known fungal xylanases are optimally active at mesophilic temperatures (from 40 to 50 °C). For rXynMF13A, the optimal temperature was 45 °C when assayed at pH 5.0 (Figure 3c), and the enzyme retained 50% of maximum activity when assayed at 30–50 °C. However, there was a sharp decline at temperatures above 50 °C, with only 40% of maximum activity being observed at 55 °C. Purified rXynMF13A was stable at 40 °C and retained 89% of its initial activity after incubation for 60 min (Figure 3d); however, at 50 and 55 °C, the half-lives of the enzyme were approximately 5 and 2 min, respectively. Using beechwood xylan as the substrate, the \( k_{\text{cat}} \), \( V_{\text{max}} \), and \( k_{\text{cat}} / K_{\text{m}} \) values for rXynMF13A were 3.16 ± 0.33 mg mL\(^{-1}\) min\(^{-1}\), 2688.17 ± 1.98 μmol mg\(^{-1}\) min\(^{-1}\), and 1075.27 ± 0.75 s\(^{-1}\), respectively. The specific activity of rXynMF13A for beechwood xylan was 1322.82 ± 4.86 U mg\(^{-1}\).

The effects of different metal ions and chemical reagents on purified rXynMF13A activity are shown in Table 1. Slightly enhanced enzyme activity was observed in the presence of 5 mM Ni\(^{2+}\), Co\(^{2+}\), Cu\(^{2+}\), or Fe\(^{3+}\), the activity of rXynMF13A decreased to 60–80% of its initial activity. Mn\(^{2+}\) or Hg\(^{2+}\) (5 mM) significantly inhibited the activity of rXynMF13A. The remaining metal ions had little or no effect. Unlike most xylanases, whose activity is usually enhanced by β-mercaptoethanol, the activity of rXynMF13A was completely inhibited by 5 mM β-mercaptoethanol. SDS is an anionic detergent and a strong denaturant that inhibits most xylanases at a low concentration.

In contrast, rXynMF13A showed good resistance to SDS.

**Effect of NaCl on the Activity and Stability of rXynMF13A.** Salt-tolerant xylanases are usually isolated from microorganisms found in marine or saline environments, and such xylanases have potential applications in seafood processing. One geochemical characteristic of mangroves is high salinity. The effect of salt on the activity and stability of XynMF13A was determined given that it contains a putative signal peptide, which suggests its potential segregation. Purified rXynMF13A showed excellent tolerance to various concentrations of NaCl and was very stable at high NaCl concentrations. It exhibited maximal activity (105%) in 0.5 M NaCl, which is approximately equivalent to the salinity of seawater (Figure 4a), and it retained approximately 54% of its activity in 4 M NaCl. Moreover, purified rXynMF13A showed strong tolerance to sustained exposure to these high concentrations of NaCl, retaining more than 92 and 72% xylanase activity after incubation with 3 or 4 M NaCl, respectively, at 37 °C for 1 h (Figure 4b). Salt-tolerant proteins have been shown previously to have a high ratio of acidic to basic amino acids, with a deficiency of hydrophobic amino acids. Amino acid sequence analysis of XynMF13A revealed a low ratio of acidic to basic amino acids (4.3% vs 6.5%). However, there was a high ratio of polar (70.1%) to nonpolar amino acids (29.9%). These results were similar to those for the GH11 xylanase from the marine isolate, *Bacillus subtilis* cho40. Further, the ratio of acidic amino acids of the two salt-tolerant GH11 xylanases was much lower than that for the salt-tolerant GH10 xylanases (Table 2). The reason for salt tolerance by xylanases is unknown and requires more investigation at the structural level.

**Hydrolysis Product Analysis.** One of the most important xylanase applications is the production of XOs from agricultural wastes (e.g., corncobs, rice hulls, straw, bran, and bagasse), which have a high content of xylan. 39,40 XOs are particularly important as food additives, in feed formulations, and in pharmaceuticals. 39 XOs are not carcinogenic, are low in caloric content, have good thermal properties, and are pH-stable. 41 Hence, rXynMF13A was assessed for its ability to produce XOs by hydrolysis of corncob xylan. Hydrolytic products were separated and detected by thin-layer chromatography (TLC) (Figure 5). The main hydrolytic products were xylobiose (X2), xylotriose (X3), xylotetraose (X4), and xylopentaose (X5) with a small amount of other XOs that had a low degree of polymerization (DP). No xylene was found. With increased time of hydrolysis, the amount of each type of XOs increased but their relative amounts remained essentially unchanged. These results demonstrate rXynMF13A to be a strict *endo-*1,4-xylanase. It is important to note that hundreds of xylanases have been characterized, but only a few hydrolyze xylan into XOs with low DP and without producing xylene. The rXynMF13A hydrolysis products from corncob xylan are mainly XOs with a DP from two to five, demonstrating its
potential application for the production of XOs from corncob xylan.

Effect of rXynMF13A on Chinese Steamed Bread Quality. Another important food-industry application for xylanase is baking.16,18 Xylanase is added to bread formulas to increase bread volume and thus improve bread quality.43,44 In China, about 40% wheat flour is used to make Chinese steamed bread (CSB), a traditional food with a history of hundreds of years.45 However, only a few studies have focused on the use of xylanases for CSB. For example, XynA from Streptomyces sp. FA146 and a thermostable xylanase from Chaetomium sp.47 have positive effects on CSB, increasing specific volume and decreasing firmness. In this study, three concentrations of rXynMF13A were assessed for their effect on the quality of CSB and compared to a control condition in which no xylanase was added. As shown in Table 3, with increasing enzyme concentration, rXynMF13A improved CSB specific volume and elasticity while decreasing hardness and chewiness of CSB. The specific volume of CSB, with different concentrations of rXynMF13A, was higher than that of the control. When 0.75 U g⁻¹ rXynMF13A was used, the specific volume was 0.109 higher than that of the control, that is, 4.45% higher. With the addition of 0.75 U g⁻¹ xylanase, CSB hardness and chewiness decreased by 25.7 and 25.2%, respectively, when

Table 1. Effect of 5 mM Metal Ions and Chemical Reagents on the Xylanase Activity of Purified rXynMF13A

| reagent | relative activity (%) | reagent | relative activity (%) |
|---------|----------------------|---------|----------------------|
| CK      | 100 ± 0.4            | Cr²⁺    | 93.7 ± 0.9           |
| Ag⁺     | 102.8 ± 0.1          | Ni⁺     | 88.9 ± 2.4           |
| Zn²⁺    | 100.6 ± 1.8          | Co²⁺    | 78.1 ± 2.0           |
| Fe³⁺    | 100.5 ± 0.3          | Cu²⁺    | 68.8 ± 1.5           |
| Li⁺     | 100.2 ± 1.3          | Fe³⁺    | 67.0 ± 0.9           |
| Pb²⁺    | 99.2 ± 0.6           | Mn²⁺    | 40.8 ± 1.8           |
| K⁺      | 98.9 ± 0.3           | Hg²⁺    | 0.9 ± 0.7            |
| Na⁺     | 97.9 ± 1.6           | EDTA    | 98.7 ± 2.4           |
| Ca²⁺    | 97.0 ± 0             | SDS     | 30.8 ± 0.2           |
| Mg²⁺    | 96.3 ± 1.5           | β-mercaptoethanol | 0 |

*Values represent mean ± SD (n = 3) relative to the untreated control samples.

Figure 3. Enzymatic properties of purified rXynMF13A. (a) Effect of pH on XynMF3 activity. Activities at various pHs were assayed at 37 °C for 10 min. (b) pH stability of rXynMF13A. Residual activities after incubation of the purified enzyme at various pHs for different periods of time at 37 °C and assayed at pH 5.0 and 45 °C for 10 min. (c) Effect of temperature on rXynMF13A activity in McIlvaine buffer (pH 5.0). (d) Thermostability of rXynMF13A. Residual activity was assayed at pH 5.0 and 45 °C for 10 min after preincubation at 40, 45, 50, and 55 °C for different periods of time. Each value in the panel represents mean ± SD (n = 3).

Figure 4. Effect of NaCl on rXynMF13A activity and stability. (a) Effect of different concentrations of NaCl on the activity of rXynMF13A. (b) Effect of 3 and 4 M NaCl on the stability of rXynMF13A. The error bars represent mean ± SD (n = 3).
Table 2. Amino Acid Composition Comparison of Salt-Tolerant Xylanases

| parameters                              | XynMF13A | Xyl40 | XynAHJ14 | XynSL4 | Xyn10A | XynA | XynA | XynRBM26 |
|-----------------------------------------|----------|-------|----------|--------|--------|------|------|----------|
| GH family                               | 11       | 11    | 10       | 10     | 10     | 10   | 10   | 10       |
| acidic amino acids (%)                  | 4.30     | 4.46  | 14.91    | 17.37  | 15.68  | 12.88| 12.06| 12.01    |
| hydrophobic amino acids (%)              | 29.87    | 28.71 | 34.72    | 36.32  | 33.14  | 33.10| 37.59| 39.95    |
| highest activity with NaCl (%)          | 105      | 140   | 100      | 100    | 100    | 134  | 190  | 120      |
| concentration of NaCl (M)               | 0.5      | 0.5   | 0        | 0      | 0.5    | 0.4  | 0.5  | 0        |
| references                              | this study| 37    | 54       | 55     | 36     | 56   | 35   | 34       |

*Including amino acids: A I L F W V. *The highest activity of the enzyme when NaCl was added to the reaction system.

compared to the control. These results suggest that rXynMF13A has beneficial effects on the quality of CSB. Hence, this xylanase could be combined effectively with other enzymes such as α-amylase, cellulase, and glucose oxidase to improve CSB.49

CONCLUSIONS

In this study, a novel GH11 xylanase gene (xynMF13A) was cloned from a mangrove fungus and expressed in *P. pastoris*, and the resultant expressed protein was purified and characterized. The results demonstrate XynMF13A to be a salt-tolerant enzyme, with a high degree of salt resistance up to 4 M NaCl. Hydrolysis products generated from corncob xylan by this xylanase are mainly xylobiose, xylotriose, xylotetraose, and xylpentaoase, though no xylose was found. Furthermore, XynMF13A had a beneficial effect on CSB by improving specific volume and elasticity while at the same time decreasing hardness and chewiness. Hence, XynMF13A is a novel xylanase with excellent potential for both basic research and industrial applications.

MATERIALS AND METHODS

Strains, Vectors, and Chemicals. *Escherichia coli* Top10 and the pMD18-T vector used for gene cloning and sequencing were obtained from Takara (Otsu, Japan). *P. pastoris* X-33 and vector pPICZα-C were used for gene expression and were purchased from Invitrogen (Carlsbad, CA, USA). Kits for fungal DNA extraction, DNA purification, and plasmid isolation were purchased from Omega (Norcross, GA, USA). Restriction endonucleases, T4 DNA ligase, DNA polymerase, dNTPs, and zeocin were purchased from Thermo Fisher Scientific (Ipswich, MA, USA). Nickel-NTA agarose (Qiagen, Valencia, CA, USA) was used to purify the His6-tagged protein. An RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) was used to extract total RNA. A Transcript One-Step gDNA Removal and cDNA Synthesis SuperMix kit (TransGen Biotech, Beijing, China) was used for first-strand cDNA synthesis. Beechwood xylan substrate, yeast nitrogen base (YNB) medium, and biotin were purchased from Sigma (St. Louis, MO, USA). Corncob xylan was purchased from Yuanye Biotech (Shanghai, China). All other chemicals were of analytical grade and commercially available.

Xylanase-Producing Fungi Isolation. Mangrove sediments were sampled from Shankou Mangrove National Nature Reserve in Beihai city, Guangxi province, China (29° 50′ 32″ N, 109° 45′ 36″ E). One gram of sediment was suspended in sterilized water and diluted stepwise to concentrations of 10⁻¹, 10⁻², 10⁻³, and 10⁻⁴. Subsequently, 0.1 mL of each diluted soil suspension was spread onto screening agar plates containing 0.5% (w/v) beechwood xylan, 0.5% (w/v) peptone, and metal salts (0.1% KNO₃, 0.05% KH₂PO₄, 0.05% MgSO₄, 0.05% NaCl, and 0.05% FeSO₄). After incubation at 25 °C for 2–3 d, one of the fast-growing fungal colonies (identified as MF13) was separately transferred to fresh screening agar plates for further purification. The strain MF13 was identified by PCR amplification and sequencing of the 18S rDNA and intergenic region sequence (ITS) of ribosomal DNA, as described elsewhere.49

Full-Length Xylanase Gene Cloning. The genomic DNA of strain MF13 was extracted and purified with a fungal DNA

Table 3. Effects of Recombinant Xylanase on the Quality of CSB

| U g⁻¹  | specific volume | hardness | elasticity | chewiness     |
|-------|-----------------|----------|------------|--------------|
| control | 2.454 ± 0.007   | 1018.350 ± 3.342 | 0.916 ± 0.001 | 788.858 ± 0.487 |
| 0.25   | 2.546 ± 0.008   | 924.61 ± 9.132   | 0.923 ± 0.010 | 717.576 ± 7.415 |
| 0.50   | 2.558 ± 0.009   | 765.802 ± 8.348   | 0.933 ± 0.011 | 613.759 ± 6.977 |
| 0.75   | 2.563 ± 0.006   | 756.137 ± 7.929   | 0.940 ± 0.005 | 590.387 ± 3.107 |
kit (Norcross, GA, USA) following the manufacturer’s instructions. Purified genomic DNA was used as a template, and a degenerate primer set specific for GH11 xylanases (X11-F: 5′-AACTGCTAACCTGCTGGAGTTGCGAGTCCAGGGGG-3′; X11-R: 5′-CGGCAGGGACCAGCTAATGTTG-3′) was used for xylanase gene fragment PCR amplification. The PCR products were excised, purified, and ligated into vector pMD18-T, transformed into *E. coli* Top10, and sequenced by Invitrogen (Carlsbad, CA, USA). TAIL-PCR with three nested specific primers (Table 4) was used to obtain the flanking regions of the gene. Third-round PCR products of the expected sizes were excised, purified, cloned into the pMD18-T vector, and then sequenced. The complete sequences were assembled from the known fragment sequences using Vector NTI 10.3 (InforMax, USA).

### Total RNA Extraction and First-Strand cDNA Synthesis

To obtain the cDNA sequence of xynMF13A, total RNA was extracted and purified from the mycelia of MF13 grown in potato dextrose liquid medium 0.5% xylose by using an RNasy Plant Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions. First-strand cDNA was synthesized using a TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix kit (TransGen Biotech, Beijing, China).

### Cloning of xynMF13A from cDNA and Sequence Analysis

The full-length cDNA of xynMF13A was amplified by PCR using the specific primers xynMF13A-F and xynMF13A-R (Table 4) and sequenced in Invitrogen. Identification of the ORF was performed using Vector NTI 10.3 (InforMax, MD, USA), and the putative signal peptide was predicted using SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/). The DNA and protein sequence similarities were assessed using BLASTn and BLASTp programs (http://www.ncbi.nlm.nih.gov/BLAST/).

### Expression and Purification of XynMF13A in *P. pastoris*

The XynMF13A coding sequence without the predicted signal peptide was amplified by using primers xynMF13A-m-F and xynMF13A-m-R (Table 4). The PCR product was digested with EcoR I and Xba I and inserted into vector pPICZα-C with in-frame fusion of the α-factor from *Saccharomyces cerevisiae* to construct the recombinant plasmids. The recombinant clones were selected on low-salt lysisogeny broth agar plates containing 100 μg mL⁻¹ zeocin and confirmed by PCR and DNA sequencing. The recombinant plasmid, pPIC-xynMF13A, was linearized with *Pme I* and transformed into *P. pastoris* X-33 competent cells by electroporation using a Gene Pulser (Bio-Rad, Hercules, CA, USA). Positive transformants were screened on yeast extract peptone dextrose sorbitol plates (1% yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol, and 1.5% agar) containing 100 μg mL⁻¹ zeocin. Positive colonies with strong resistance to zeocin were transferred to buffered glycerol complex medium (BMGY: 2% peptone, 1% yeast extract, 100 mM pH 6.0 potassium phosphate, 1.34% YNB, 4 × 10⁻¹⁰% biotin, and 1% glycerol) and grown at 30 °C for 48 h. The cells were harvested by centrifugation, suspended in buffered methanol complex medium (BMMY: 2% peptone, 1% yeast extract, 100 mM pH 6.0 potassium phosphate, 1.34% YNB, 4 × 10⁻¹⁰% biotin, and 0.5% methanol), and cultured at 30 °C for 120 h. Methanol (0.5%) was added to cultures every 24 h for the purpose of induction. Samples were withdrawn at intervals, and supernatant xylanase activity was measured, as described below.

To purify the His-tagged recombinant protein (rXynMF13A), culture supernatants were collected after centrifugation (13 000g, 4 °C for 5 min) and concentrated by using an ultrafiltration membrane (PES5000, Sartorius Stedim Biotech, Göttingen, Germany). The concentrate containing rXynMF13A was loaded onto a Ni²⁺-NTA agarose gel column and washed with a linear imidazole gradient of 20–200 mM in Tris-HCl buffer (20 mM Tris-HCl, 500 mM NaCl, 10% glycerol, pH 7.6). The purity and apparent molecular mass of the purified rXynMF13A were estimated by SDS-PAGE (12.0% running gel). The protein concentration of the purified rXynMF13A was determined using an EasyII-BCA Protein Quantitative Kit (TransGen Biotech, Beijing, China), using bovine serum albumin as the standard.

### Xylanase Activity Assay

Xylanase activity was determined by measuring the release of reducing sugar from substrates, using the 3,5-dinitrosalicylic acid (DNS) method. The reaction mixture contained 0.1 mL of appropriately diluted enzyme and 0.9 mL of McIlvaine buffer (pH 5.0) containing 1% (w/v) beechnought xylan as the substrate. After incubation at 45 °C for 10 min, 1.5 mL of DNS reagent was immediately added to stop the reaction and the mixture was boiled for 5 min. When the reaction mixture had cooled to room temperature, the presence of reducing sugars was determined spectrophotometrically at 540 nm. The amount of reducing sugars was estimated based on the linear portion of an equivalent xylose standard curve. One unit (U) of xylanase activity was defined as the amount of enzyme that released 1 μmol of reducing sugar equivalent to xylose per min. Enzyme activity was measured by this procedure unless otherwise noted. All experiments were performed in triplicate.

### Biochemical Characterization

Beechwood xylan was used as the substrate for enzyme characterization. The optimal pH of purified rXynMF13A activity was determined at 37 °C in

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Table 4. Primers Used in This Study

| Primers | Sequences (5′ → 3′) | Size (bp) |
|---------|---------------------|----------|
| Usp1    | CGCAACCCCTCTCAGTTGACTACGG | 25       |
| usp2    | GAGAACCTCGACCTAGACCACCACCACT | 26       |
| usp3    | GTCTTACAGATTCGGCGAGCCAGCCAGC | 26       |
| dsp1    | GCTGAGTTGGCCCAATGCTGTAGGAC | 26       |
| dsp2    | GGTGACGGAGCCCTTGACGCCTAG | 23       |
| dsp3    | GTATGACTCGAGCGAGAGGGTTCGCC | 25       |
| xynMF13A-F | ATGTCGCTTCTTCTCTACATCTATCATCCAGG | 28       |
| xynMF13A-R | TTAAGGGCAGTTGACATGATGGAAAGC | 27       |
| xynMF13A-m-F | CAATGAATTGACGCCATGTGAGTTCAGCCAGCGC | 36       |
| xynMF13A-m-R | CACTCTAGAAGAGCGACATTGACAGTGATGGAAAGCGCTG | 39       |

"Restriction sites are bold and underlined."
buffers of pH range 3.0–11.0. McIlvaine buffer (0.2 M Na₂HPO₄/0.1 M citric acid) was used for pH 3.0–7.0, 0.1 M Tris-HCl for pH 7.0–9.0, and glycine-NaOH for pH 9.0–11.0. For the pH stability assay, the appropriate dilution of purified rXynMF13A was incubated in buffer at pH 3.0–11.0 at 37 °C for 1 h, and the residual enzyme activity was measured at pH 5.0 and 45 °C. The initial activity of rXynSL3 was set as 100%.

The optimal temperature of purified rXynMF13A was determined over temperature ranges of 10–60 °C in McIlvaine buffer (pH 5.0). The thermostability of rXynMF13A was determined by measuring the residual activity after preincubation of the enzyme in McIlvaine buffer (pH 6.0) at 40, 45, 50, and 55 °C without substrate for various time periods.

The Kₘ, Vₘₐₓ, and kₘₐₓ values for XynMF13A were determined in McIlvaine buffer (pH 5.0) containing 1–10 mg mL⁻¹ beechwood xylan and 0.1 mL of enzyme solution (2.56 U mL⁻¹) at 45 °C. Kₘ and Vₘₐₓ were determined by using the nonlinear regression computer program GraFit (Erithacus, Horley, Surrey, UK).

The effects of different metal ions and chemical reagents on purified rXynSL3 activity were measured at 37 °C in McIlvaine buffer (pH 5.0) containing, separately, 5 mM (final concentration) LiCl, NaCl, KCl, AgCl, CaCl₂, CoCl₂, HgCl₂, FeCl₃, CrCl₃, MnSO₄, ZnSO₄, NiSO₄, CuSO₄, FeSO₄, Pb(CH₃COO)₂, EDTA, SDS, and β-mercaptoethanol. Purified rXynSL3, without any additive, was used as a control, and its activity was set as 100%.

The effect of NaCl on purified rXynMF13A was determined at 45 °C in McIlvaine buffer (pH 5.0) containing 0.25–4.0 M NaCl. To examine its resistance to salt, rXynMF13A was incubated with 3 or 4 M NaCl at 37 °C for 1 h, and the residual enzyme activity was measured in McIlvaine buffer (pH 5.0) at 45 °C.

Hydrolysis Products Generated from Corn cob Xylan by rXynMF13A. Purified rXynMF13A was mixed with 1% (w/v) of corn cob xylan in McIlvaine buffer (pH 5.0) and incubated at 45 °C for 8 h. Hydrolysis products were analyzed by TLC using silica gel G-60 and developed in a solvent system consisting of butanol, ethanol, and water (5:3:2, v/v/v). Carbohydrate products were visualized by spraying with a mixture of 1% (v/v) aniline, 1% (w/v) diphenylamine, and 5% (v/v) phosphoric acid in acetone. Xylose, xylobiose, xylotriose, xylotetraose, and xylopentaose were used as standards.

Application of rXynMF13A for CSB Making. The recipe for CSB is 100 g of wheat flour, 0.8 g of dehydrated yeast, 0.8 g of NaCl, 10 g of sugar, and 48 mL of water. A mixture of dehydrated yeast and rXynMF13A was dissolved in 37 °C water before making the dough. The effect of xylanase was studied by adding rXynMF13A in dosages of 250, 500, and 750 U kg⁻¹ flour. After mixing and kneading, the dough was sheeted 30 times. The dough was then rounded, placed in a mold, and proved for 1 h at 37 °C and 80% relative humidity. The proved dough was steamed for 20 min using a steam tray and boiling water. The control sample was prepared as described above, but without adding xylanase.

The quality of CSB was evaluated by determining its weight, volume, hardness, elasticity, and chewiness. loaf volume measurements were taken using the rapeseed displacement method after 2 h of steaming. Specific volume (the volume to the weight, mL g⁻¹) was calculated as the ratio of volume to weight of the loaf. The hardness, elasticity, and chewiness were determined using a texture analyzer (TA-XT plus) and Texture Expert software. The TPA mode was used, and the test conditions were as follows: test speed, 1 mm/s; trigger type, auto; tare mode, auto; trigger force, 5 g; distance, 10.0 mm; and time, 10.00 s.

Nucleotide Sequence Accession Numbers. The nucleotide sequences of the Phoma sp. MF13 18S rDNA and ITS gene were deposited in the GenBank database under accession numbers MG925673 and MG925674, respectively. The nucleotide sequence of the GH11 xylanase gene (xynMF13A) was deposited in the GenBank database under accession number MG925675.

Author Contributions
G.W. and X.Y. conceived and designed the experiments. J.W., C.Q., and Y.R. performed the experiments. G.W. and J.W. analyzed the data. All authors contributed to the writing of the manuscript. All authors have given approval to the final version of the manuscript.

Notes
The authors declare no competing financial interest.

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