Identification of a Novel Fungus, *Leptosphaerulina chartarum* SJTU59 and Characterization of Its Xylanolytic Enzymes

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Abstract

Xylanolytic enzymes are widely used in processing industries, e.g., pulp and paper, food, livestock feeds, and textile. Furthermore, certain xylanolytic enzymes have demonstrated the capability to improve the resistance and immunity of plants. Screening of high-yield microbial xylanolytic enzyme producers is significant for improving large-scale cost-effective xylanolytic enzyme production. This study provided new evidence of high-level xylanolytic enzyme production by a novel fungus, designated *Leptosphaerulina chartarum* SJTU59. Under laboratory conditions, *L. chartarum* SJTU59 produced xylanolytic enzymes of up to 17,566 U/mL (i.e., 878.307 U/g substrate). The enzyme solution was relatively stable over a wide range of pH (pH 3.0 to pH 9.0) and temperature (40°C to 65°C) while showing high resistance to the majority of metal ions tested. Composition analysis of the hydrolytic products of xylan showed sufficient degradation by xylanolytic enzymes from *L. chartarum* SJTU59, mainly the monosaccharide xylose, and a small amount of xylobiose were enzymatically produced; whereas in the presence of sufficient xylan substrates, mainly xylooligosaccharides, an emerging prebiotic used in food industry, were produced. In addition, the xylanolytic enzyme preparation from *L. chartarum* SJTU59 could initiate tissue necrosis and oxidative burst in tobacco leaves, which may be related to enhanced plant defense to adversity and disease. *L. chartarum* SJTU59 possessed a complex xylanolytic enzyme system, from which two novel endo-β-1,4-xylanases of the glycoside hydrolase (GH) family 10, one novel endo-β-1,4-xylanase of the GH family 11, and one novel β-xyllosidase of the GH family 43 were obtained via rapid amplification of complementary DNA ends. Given the high yield and stable properties of xylanolytic enzymes produced by *L. chartarum* SJTU59, future studies will be conducted to characterize the properties of individual xylanolytic enzymes from *L. chartarum* SJTU59. xylanolytic enzymes-encoding gene(s) of potential use for industrial and agricultural applications will be screened to construct genetically engineered strains.

Introduction

Xylanolytic enzymes are an extensive group of enzymes (EC 3.2.1.x) [1]. An abundance of diverse xylanolytic enzymes are currently known to catalyze the hydrolysis of xylan. These xylanolytic enzymes are generally classified using standard means based on primary structure comparisons of catalytic domains and enzymes in families of related sequences [2]. At least a total of 131 glycoside hydrolase (GH) families exist in the carbohydrate-active enzyme (CAZy) classification system [3]. However, only members of GH families 5, 7, 8, 10, 11, and 43 contain truly distinct catalytic domains with a demonstrated xylanolytic enzyme activity. Among these, endo-β-1,4-xylanases (EC 3.2.1.8) are normally found in GH families 10 and 11, whereas β-xyllosidases (EC 3.2.1.37) in the GH family 43. By contrast, xylanolytic enzymes belonging to GH families 5, 7, and 8 have been studied to a lesser extent. Endo-β-1,4-xylanase, which catalyzes the hydrolysis of β-1,4-xyllosidic linkages of xylan backbone, and β-xyllosidase, which catalyzes the hydrolysis of nonreducing end-xyllose residues from xylooligosaccharides, both belong to the group of xylanolytic enzymes [1].

Applications of xylanolytic enzymes can be found in feed, food, pulp/paper, textile, deinking, metal-polluted sewage treatment and so on. In grain processing for feeds or energy sources, xylanases and β-xyllosidase are commonly used in solid-state fermentation of agro-industrial wastes such as rice straw [4], wheat bran [5], and sugarcane bagasse [6]. As for the food industry, xylanases are beneficial enzymes in hydrolysis of xylan and production of xylooligosaccharides, an emerging prebiotic beneficial for human intestinal health [7]. In bleaching and pulp/paper industries, xylanase pretreatment is considered efficient for improving the product quality [8]. For plant disease management, the xylanase II gene has been shown to induce ethylene biosynthesis, which potentially improves the resistance and immunity of host plants to specific diseases [9].

Isolation and characterization of high-efficiency xylanolytic enzymes have become an important focus of research for decades, given their excellent industrial and agricultural values [7,9]. To achieve high-yield, cost-effective production of xylanolytic enzymes, substantial efforts have been continuously made to screen for microbial xylanolytic enzyme producers from soil [10], composting materials [11], and agricultural and industrial wastes.
Materials and Methods

Fungal Cultivation and Isolation

The novel fungal strain, designated L. chartarum SJTU59, was isolated from a contaminated broth in the Department of Resource and Environmental Science, School of Agriculture and Biology, Shanghai Jiao Tong University, Shanghai, China. In another experiment involving enzyme activity assay of a transgenic Streptomyces strain with the xylanase gene, a semisynthetic xylanolytic enzyme-inductive fermentation broth (2% corn cob meal, 0.4% NH4NO3, 0.05% K2HPO4, 0.05% MgSO4·7H2O, 0.05% KCl, and 0.001% FeSO4·7H2O) was contaminated by L. chartarum SJTU59, which produced large amounts of xylanolytic enzymes. Then, L. chartarum SJTU59 mycelia were harvested, washed in distilled water, and cultured on a potato-dextrose agar (PDA) plate in a thermostatic incubator (25°C, 7 d). Afterward, one of the outermost and youngest mycelia was harvested and cultured on a PDA plate in a thermostatic incubator (25°C, 1 d). For morphological observation, mycelia with spores of L. chartarum SJTU59 were harvested with an inoculating loop and examined under a DM 2500 M microscope (Leica, Germany) at 400× magnification.

For molecular analysis, L. chartarum SJTU59 was grown in a potato-dextrose (PD) broth on a 180 rpm rotary shaker (28°C, 3 d). After the removal of supernatant with multiple layers of gauze, total genomic DNA (gDNA) was extracted from the precipitate using hexadecyl trimethyl ammonium bromide (CTAB) method [22]. The gDNA extract was electrophoresed on 1% agarose gel and subsequently served as the template for PCR amplification of ITS and EF-1α sequences with specific primer sets ITS1-F/ITS4 [23] and EF1-728F/EF1-968R [24] (Table 1). For ITS and EF-1α amplification, the 25 μL PCR reaction contained 9.5 μL of ddH2O, 12.5 μL of premix TaqTM (Takara, Japan), 1 μL of primer-F (ITS1-F or EF1-728F, 10 μM), 1 μL of primer-R (ITS4 or EF1-968R, 10 μM), and 1 μL of gDNA. PCR amplification was conducted on a thermal cycler (Eppendorf, Germany) under the following conditions: pre-heating at 95°C for 5 min, followed by 33 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, and final extension at 72°C for 10 min. PCR products were electrophoresed on 1% agarose gel, stained with ethidium bromide dye (200 ng/mL), and then ligated into the pMD18-T Easy vector (Takara, Japan) for DNA sequencing (Biosune, China).

The obtained ITS and EF-1α sequences of L. chartarum SJTU59 were compared with the GenBank database using the NCBI Web BLAST service [http://www.ncbi.nlm.nih.gov/BLAST/]. Nucleotide alignments were done via DNAStar software.

For phylogenetic tree construction of rDNA ITS (incl. ITS1, 5.8S, and ITS2) sequences, were downloaded from the GenBank database and then, together with the sequence of L. chartarum SJTU59, 13 representative sequences of known strains (incl. 16S rDNA sequences of Streptomyces and Bacillus, two representative bacteria with high production of xylanolytic enzymes), were analyzed using the neighbor-joining method with MEGA 4.0 software. The tree was constructed using the Kimura two-parameter model with 1000 bootstrap replications. We included the type strain L. chartarum NRRL 30422 in the analysis. The tree was visualized using FigTree v1.3.1 software.
imported into the MEGA 4.0 software [25]. For phylogenetic tree construction of EF-1α gene, sequences of the closely related species and outgroup were downloaded from the related literature [24] and then, together with the sequence of *L. chartarum* SJTU59, 12 representative sequences of known strains, were imported into the MEGA 4.0 software [25]. Multiple sequence alignment was performed using the Clustal W method and phylogenetic trees were re-constructed using the neighbor-joining method. Confidence for tree topology was estimated based on 1,000 bootstrap replicates.

**Xylanolytic Enzyme Activity Assay**

The productivity of xylanolytic enzyme by *L. chartarum* SJTU59 was tested with two broth media, i.e., the PD seed broth and the semisynthetic xylanolytic enzyme -inductive fermentation broth (2% carbon source, 0.4% NH₄NO₃, 0.05% K₂HPO₄, 0.05% MgSO₄·7H₂O, 0.05% KCl, and 0.001% FeSO₄·7H₂O). The latter was prepared with agricultural waste as carbon source, e.g., 80-mesh sieve powders of corncob meal, wheat bran, sugarcane bagasse, wood chips, or cottonseed hulls.

Mycelia of *L. chartarum* SJTU59 were inoculated into the seed broth at 1:500 (w/v) and then incubated on a 180 rpm rotary
shaker at 28°C for 3 d. Then, the precipitate was collected by filtration with multiple layers of gauze. One gram of the precipitated seed was added to 50 mL of fermentation broth and then incubated on a 180 rpm rotary shaker at 28°C for 4 d. Xylanolytic enzyme activity of the fermentation supernatant was assayed by determination of enzymatically produced reducing-sugar content (i.e., xylose) using the 3,5-dinitrosalicylic acid (DNS) method [26]. The 2 mL reaction mixture contained 1.8 mL of 1% beechwood xylan (Serva, Germany) in 0.1 M sodium acetate buffer (pH 5.0) and 0.2 mL of diluted crude enzyme extract (30 μL crude enzyme extract in 170 μL of sodium acetate buffer). After gentle vortexing, the mixture was incubated in a 50°C water bath for 10 min. Reaction was terminated by adding 3 mL of DNS reagent and heating at 100°C for 10 min. Prior to enzyme activity assay, the mixture was diluted thrice with 0.1 M sodium acetate buffer (pH 5.0). Absorption of the appropriately diluted sample was measured at 540 nm (A540) using a UV-1800 spectrophotometer (Mapada, China). Uninoculated fermentation broth and xylose were used as blank control and standard, respectively [26]. Each measurement was performed in triplicate. One unit (U) of xylanolytic enzyme activity was defined as the amount of enzyme required to release 1 μmol xylose per minute under the standard conditions described above. The values were displayed with two kinds of units (U/mL and U/g substrate) to compare with existing data. Enzyme activity was statistically analyzed via SPSS software and the graph was prepared with Origin 8.0 software.

Figure 2. Phylogeny analysis of *L. chartarum* SJTU59. (A) Phylogenetic tree based on nrDNA ITS sequence of *L. chartarum* SJTU59 (bold and marked with an asterisk) and some related sequences of representative strains from the GenBank database. (B) Phylogenetic tree based on EF-1α sequences of *L. chartarum* SJTU59 (bold and marked with an asterisk) and its closely related species and outgroup retrieved from the literature. doi:10.1371/journal.pone.0073729.g002
Evaluation of the Effects of pH, Temperature, and Chemicals on Xylanolytic Enzyme Activity

Optimum pH for xylanolytic enzyme activity from *L. chartarum* SJTU59 was determined at 50°C in three buffer systems containing 1% beechwood xylan, i.e., McIlvaine buffer (pH 3.0 to pH 7.0), Tris-HCl (pH 7.0 to pH 9.0), and 0.1 M glycine-NaOH (pH 9.0 to pH 11.0) [27]. Stability of xylanolytic enzymes at different pH levels was evaluated by residual enzyme assay under standard conditions (pH 5.0, 50°C, 10 min) after pre-incubation without substrate in the above mentioned buffer solutions (pH 3.0 to pH 11.0, 4°C, 12 h).

Optimum temperature for xylanolytic enzyme activity was determined over the range of 10°C to 100°C in 0.1 M sodium acetate buffer (pH 5.0) containing 1% beechwood xylan. Thermostability of xylanolytic enzymes was determined by residual activity assay under standard conditions (pH 5.0, 50°C, 10 min) after pre-incubation without substrate in 0.1 M sodium acetate buffer (pH 5.0) at 40, 50, or 60°C for various periods [27,28].

To determine the effects of metal ions, chemical modifiers and detergent on xylanolytic enzyme activity, 1 or 10 mM of Li⁺, Na⁺, K⁺, Ca²⁺, Mg²⁺, Cu²⁺, Zn²⁺, Ni²⁺, Fe³⁺, Cr³⁺, Co²⁺, Al³⁺, Ba²⁺, ethylene diamine tetracetic acid (EDTA), or sodium dodecyl sulfonate (SDS) was added to the enzyme solution, respectively. After gentle vortexing, residual xylanolytic enzyme activity was assayed under standard conditions (pH 5.0, 50°C, 10 min). Control experiments were carried out in the absence of each compound under the same conditions [27,28]. Each measurement was performed in triplicate. Enzyme activity was statistically analyzed via SPSS software and the graph was prepared with Origin 8.0 software.

Composition Analysis of Hydrolytic Products

The composition of hydrolytic products of beechwood xylan by xylanolytic enzymes from *L. chartarum* SJTU59 was analyzed on silica gel GF254 plates (Xinchu, China) by TLC. The 1 mL reaction mixtures were prepared with an increasing volume of crude enzyme extract and a decreasing volume of 1% beechwood xylan (i.e., 20 μL, 500 μL; 50 μL, 400 μL; 100 μL, 300 μL; 150 μL, 200 μL; 200 μL, 300 μL; 300 μL, 400 μL; 200 μL, 300 μL; 300 μL, 400 μL; 100 μL, 200 μL; 50 μL, 100 μL; 200 μL, 50 μL; 100 μL, 200 μL; and 50 μL, 100 μL) in 0.1 M sodium acetate buffer (pH 5.0). The mixtures were incubated at 50°C for 24 h. Then, 25 μL aliquots of the hydrolytic products were spotted onto TLC plates. The plates were developed with butanol-acetic acid-water (2:1:1, v/v), sprayed with a staining solution of 1% (w/v) diphenylamine and 1% (w/v) aniline in acetone, and then heated at 120°C in an oven for 10 min [27]. A mixture of xylose and xylooligosaccharides (X₁–X₄, 5 mg/mL each) was used as standard.

Plant Inoculations and Assays of Tissue Necrosis and Oxidative Burst

*Tobacco (Nicotiana benthamiana)* plants were grown to the flowering phase in soil under 10 h light/14 h darkness in a 24°C phytotron. A total of 30 μL of filtered xylanolytic enzyme preparation from *L. chartarum* SJTU59, blank broth (control), and distilled water (control) were injected into tobacco leaves using 1 ml syringes, respectively. Injected leaves in each group were of the similar size and position in the same tobacco plant. The inoculated plant was placed in darkness in a 24°C phytotron, and tissue necrosis was observed every 12 h. For oxidative burst assay, injected leaves were stained in DAB solution (pH 3.8) for 8 h in darkness, boiled in a solution of ethanol, glycerin and acetic acid (1:1:1, v/v) for 10 min to 15 min, and washed twice with 60% ethanol [29]. Each measurement was performed in triplicate.

Conserved Region Amplification of Novel Xylanolytic Enzyme Genes and Sequence Analysis

Conserved regions of novel xylanase and xylosidase genes of GH families 10, 11, and 43 were PCR-amplified using three sets of degenerate primers (Table 1), namely, X10-F/X10-R [30], X11-F/X11-R [30], and XyIDF/XyIDR [31], respectively. The 25 μL PCR reactions contained 7.5 μL of ddH₂O, 12.5 μL of premix Taq™ (Takara, Japan), 1 μL of primer-F (10 μM), 1 μL of...
primer-R (10 μM), and 3 μL of gDNA. The PCR conditions were as follows: pre-heating at 95°C for 10 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 50°C for GH 10 (52°C for GH 11 and 55°C for GH 43) for 1 min, and extension at 72°C for 1 min, and final extension at 72°C for 10 min. PCR products were electrophoresed on 1% agarose gel and visualized by staining with ethidium bromide dyestuff D1210 (Applygen, China). Visible bands of interests were excised. DNA samples were purified and ligated into the pMD18-T Easy vector (Takara, Japan) for DNA sequencing (Biosune, China). Conserved genomic DNA regions of xylanase and xylosidase genes were analyzed and identified using the NCBI Web BLAST service (tblastx; http://

Figure 4. Effects of pH and temperature on the activity and stability of xylanolytic enzymes from L. chartarum SJTU59. (A) pH effect on xylanolytic enzyme activity determined in the McIlvaine buffer (pH 3.0 to pH 7.0), Tris-HCl (pH 7.0 to pH 9.0), and Glycine-NaOH solution (pH 9.0 to pH 11.0) at 50°C. (B) pH stability of xylanolytic enzyme determined at 50°C after 12 h of pre-incubation in the McIlvaine buffer (pH 3.0 to pH 7.0), Tris-HCl (pH 7.0 to pH 9.0) or Glycine-NaOH solution (pH 9.0 to pH 11.0) at 4°C. (C) Temperature effect on xylanolytic enzyme activity determined in sodium acetate buffer (0.1 M, pH 5.0, 10°C to 100°C). (D) Thermostability of xylanolytic enzymes determined at 50°C after pre-incubation in sodium acetate buffer (pH 5.0) at 40, 50, or 60°C without substrate.
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www.ncbi.nlm.nih.gov/BLAST/). Introns were predicted using the ExPASy Web service (http://web.expasy.org/translate/) and the GT-AG rule.

Full-length Amplification of Novel Xylanolytic Enzyme Genes and Sequence Analysis

Mycelia of *L. chartarum* SJTU59, cultured in semisynthetic xylanase-inductive fermentation broth on a 180 rpm rotary shaker for 2 d, were collected by filtration with multiple layers of gauze and then washed twice with distilled water. Total RNA was prepared using a TRIzol kit (Tiangen, China). Total RNA was reverse-transcribed using SuperScript trademark II Reverse Transcriptase (Invitrogen, America). For 5’ RACE, the RACE PCRs were performed by following the manufacturer’s instructions of 5’ RACE System for Rapid Amplification of cDNA Ends kit (Invitrogen, America). Resultant PCR products were cloned into pMD19-T Easy vector (Takara, Japan) and then sequenced (Majorbio, China). For 3’ RACE, the RACE PCRs were performed by following the manufacturer’s instructions of 3’ RACE System for Rapid Amplification of cDNA Ends (Invitrogen, America). Resultant PCR products were cloned into

Table 2. Effects of metal ions and chemical compounds on xylanolytic enzyme activity.

| Metal ions and chemical compounds | Relative activity of xylanolytic enzyme (%)<sup>a</sup> |
|----------------------------------|---------------------------------------------------|
| 1 mM                             | 10 mM                                             |
| Control                          | 100.000                                           |
| Li<sup>+</sup>                   | 97.813                                            |
| Na<sup>+</sup>                   | 97.998                                            |
| K<sup>+</sup>                    | 104.097                                           |
| Ca<sup>2+</sup>                  | 105.752                                           |
| Mg<sup>2+</sup>                  | 101.174                                           |
| Ca<sup>2+</sup>                  | 101.74                                            |
| Zn<sup>2+</sup>                  | 96.774                                            |
| Ni<sup>2+</sup>                  | 96.188                                            |
| Fe<sup>3+</sup>                  | 109.497                                           |
| Cr<sup>3+</sup>                  | 124.776                                           |
| Co<sup>2+</sup>                  | 100.010                                           |
| Al<sup>3+</sup>                  | 100.004                                           |
| EDTA                             | 8.241                                             |
| SDS                              | 5.692                                             |
| **Control**                      | **100.000**                                       |
| Li<sup>+</sup>                   | 105.243                                           |
| Na<sup>+</sup>                   | 101.221                                           |
| K<sup>+</sup>                    | 101.676                                           |
| Ca<sup>2+</sup>                  | 100.485                                           |
| Mg<sup>2+</sup>                  | 130.262                                           |
| Ca<sup>2+</sup>                  | 45.751                                            |
| Zn<sup>2+</sup>                  | 91.729                                            |
| Ni<sup>2+</sup>                  | 92.818                                            |
| Fe<sup>3+</sup>                  | 94.227                                            |
| Cr<sup>3+</sup>                  | 108.752                                           |
| Co<sup>2+</sup>                  | 154.135                                           |
| Al<sup>3+</sup>                  | 111.729                                           |
| Ba<sup>2+</sup>                  | 100.903                                           |
| EDTA                             | 2.779                                             |
| SDS                              | 1.566                                             |

<sup>a</sup>The data represent the mean to untreated control samples from triplicate measurements (SD ±5%).

![Figure 5. TLC analysis of the hydrolytic products of xylan and xylooligosaccharides by xylanolytic enzymes of *L. chartarum* SJTU59.](image)

Lane 1, marker of xylose and xylooligosaccharides; Lanes 2 to 8, crude enzyme solution +1% beechwood xylan substrate in 0.1M sodium acetate buffer (pH 5.0) at 50°C for 24 h (Lane 2, 20 μL +980 μL; Lane 3, 30 μL +970 μL; Lane 4, 50 μL +950 μL; Lane 5, 200 μL +800 μL; Lane 6, 300 μL +700 μL; Lane 7, 500 μL +500 μL; Lane 8, 800 μL +200 μL). The composition of hydrolytic products gradually changed with the proportions of enzymes and substrate.

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pMD19-T Easy vector (Takara, Japan) and then sequenced (Majorbio, China). Full-length sequences were spliced with DNAStar software. The specific primer sets (F and R, Table 1) were designed for identification of novel xylanolytic enzyme genes (xyn-I, xyn-2, xyn-4, and xyn-5) with PCR amplification. The 25 μL PCR reaction contained 9.5 μL of ddH2O, 12.5 μL of premix Taq™ (Takara, Japan), 1 μL of primer-F (10 μM), 1 μL of primer-R (10 μM), and 1 μL of cDNA. PCR amplification was conducted on a thermal cycler (Eppendorf, Germany) under the following conditions: pre-heating at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min 30 s, and final extension at 72°C for 10 min. PCR products were electrophoresed on 1% agarose gel and stained with ethidium bromide dyestuff D1210 (Applygen, China). PCR amplicons of appropriate sizes were ligated into the pMD19-T Easy vector (Takara, Japan) for sequencing (Majorbio, China). Gene accession numbers were acquired using the NCBI Web BLAST service (http://www.ncbi.nlm.nih.gov/WebSub/?tool=genbank).

Homology and Phylogeny Analyses of Novel Xylanolytic Enzymes

Homology between novel xylanolytic enzymes of L. chartarum SJTU59 and known xylanolytic enzymes in the NCBI GenBank database were analyzed using the NCBI Web BLAST service (protein blast; http://www.ncbi.nlm.nih.gov/BLAST/), GeneDoc software, and DNAStar software.

Phylogenetic tree was constructed based on the amino acid sequences of the four novel xylanolytic enzymes from L. chartarum SJTU59 and five representative industrial strains from the

Figure 6. Tissue necrosis in tobacco plant and DAB staining analyses. (A) Photographs of tissue necrosis in tobacco leaves after inoculation for 24 h; a1, tobacco leaf injected with xylanolytic enzyme preparation of L. chartarum SJTU59; a2, tobacco leaf injected with supernatant of blank broth (control); a3, tobacco leaf injected with distilled water (control). (B) Photographs of DAB staining in tobacco leaves after inoculation for 24 h; b1, tobacco leaf injected with xylanolytic enzyme preparation of L. chartarum SJTU59; b2, tobacco leaf injected with supernatant of blank broth (control); b3, tobacco leaf injected with distilled water (control). doi:10.1371/journal.pone.0073729.g006
GenBank database using MEGA 4.0 software [25]. Amino acid alignments were done with DNAStar software.

Results and Discussion

Strain Identification with Morphology and Phylogeny

Morphological observation showed that, cultured 7 d later, the front side of the velutinate colony of _L. chartarum_ SJTU59 was gray brown, and the reversed side of it was tan. The colony with moderate thickness was 5.8 cm to 6.1 cm in diameter (Figure 1A). The transparent mycelium, with branch and septa, was smooth or slightly rough and the brown colored or colorless. Vegetative and spore hypha were 2.0 μm to 7.5 μm and 2.0 μm to 3.0 μm in diameter, respectively. Single spore cell, terminal or laterally born in spore hypha, was denteate or short cylindrical and measured (2.7 μm to 13.0 μm) × (2.0 μm to 3.0 μm). The dark brown conidium, which carried some parts of the spore mother cell at its base, was elliptic and obtuse on the apex. Three transverse septum and 0 to 1 longitudinal septa were usually observed in the conidium, and measured (10.0 μm to 19.0 μm) × (3.0 μm to 12.5 μm) (Figure 1B).

Electrophoretic gel images showed that the conserved ITS (652 bp, KC879283) and EF-1α (373 bp) sequences of _L. chartarum_ SJTU59 were successfully amplified using specific primer sets (Figure S1). Nucleotide alignment showed that nrDNA ITS of _L. chartarum_ SJTU59 shared 100% similarity with that of _L. chartarum_ CY233 (HQ608046) (Figure S2A). The EF-1α gene of _L. chartarum_ SJTU59 had 100% similarity to that of _L. chartarum_ CY233 (100% bootstrap support), _L. chartarum_ L119 (clade 2). However, the closely related species in clade 1 were grouped out from _L. chartarum_ SJTU59 and L119 with 100% bootstrap support, which suggested that _L. chartarum_ was distinct from the closely related species. _Cochliobolus heterostrophus_ B1 (anamorph: _Bipolaris maydis_) shared the same family (Pleosporaceae) with _Leptosphaerulina_ (Figure 2B).

Xylanolytic Enzyme Activities with Different Carbon Sources

Phylogenetic analysis based on nrDNA ITS (incl. two 16S rDNA sequences) consisted of five distinct clades with high bootstrap support. As bacteria, _Streptomyces_ sp. S27 and _Bacillus subtilis_ N7 in clade 5 were grouped out from fungi (kingdom) in clade 1 to clade 4 with 100% bootstrap support. With the strong bootstrap supports (91% and 98%), fungi belonging to the Ascomycota (phylum) in clade 1 and clade 2, were separated from that of other phyla in clade 3 and clade 4. And with 100% bootstrap support, fungi belonging to the Dothideomycetes (class) in clade 1 were grouped out from that of different classes in clade 2. Within clade 1, _Jahnula aquatica_ R68-1 and _L. chartarum_ SJTU59 belonged to the same class (Dothideomycetes) but to different order. Identical with _L. chartarum_ CY233 (100% bootstrap support), _L. chartarum_ SJTU59 shared the same family (Didymellaceae) but different genus with _Cochliobolus heterostrophus_ 6028, and was in the same order (Pleosporales) but different family with _Lophiostoma cynaroidis_ FREIIIED2 (Figure 2A). In conclusion, the novel strain could be identified as a fungus belonging to Ascomycota, Dothideomycetes, Pleosporales, Didymellaceae, Leptosphaerulina and _Chartarum_, and was named as SJTU59.

Furthermore, phylogenetic analysis of the EF-1α sequence showed that _L. chartarum_ SJTU59 was identical with _L. chartarum_ L119 (clade 2). However, the closely related species in clade 1 were grouped out from _L. chartarum_ SJTU59 and L119 with 100% bootstrap support, which suggested that _L. chartarum_ was distinct from the closely related species. _Cochliobolus heterostrophus_ B1 (anamorph: _Bipolaris maydis_) shared the same family (Pleosporaceae) with _Leptosphaerulina_ (Figure 2B).

Table 3. Amino acid sequence homologies among novel xylanases in GH family 10 from _L. chartarum_ SJTU59 and known xylanases from five representative industrial strains.

| Number allocated to strain (GH 10, endo-β-1,4-xylanase) | 1 | 2 | 3 | 4 | 5 | 6 | 7 | Number | Strain |
|----------------------------------------------------------|---|---|---|---|---|---|---|--------|-------|
| -                                                       | 32.7 | 32.4 | 42.1 | 35.5 | 32.9 | 27.4 | 1 | XYN-L1 (KF367459) |
| -                                                       | 55.1 | 43.5 | 61.4 | 43.6 | 31.7 | 2 | XYN-L2 (KF305938) |
| -                                                       | 39.2 | 60.6 | 41.4 | 30.0 | 3 | Trichoderma pseudokoningii (ABY71931) |
| -                                                       | 44.9 | 34.3 | 32.9 | 4 | Aspergillus fumigatus AF293 (XP_751237) |
| -                                                       | 44.5 | 33.4 | 5 | Penicillium oxalicum (ADM34973) |
| -                                                       | 35.3 | 6 | Streptomyces griseoflavus Tu4000 (ZP_07314547) |
| -                                                       | 7 | Bacillus sp. HJ2 (A082288) |

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containing fermentation broth because it demonstrated high yield xylanolytic enzyme producers, thus contributing to industrial applications in pulp/paper leaching at high temperatures (55°C) and alkaline pH (pH 7.0 to pH 9.0), or acid pre-treatment in bioconversion processes or detergent application at high temperatures [1]. The xylanolytic enzyme solution with high resistance to a variety of metal ions can be used in deinking and metal-polluted sewage treatment [32]. Given the initially high production and stable properties of xylanolytic enzymes under various adverse conditions, further improvement of xylanolytic enzyme production by L. chartarum SJTU59 can be done via optimization of fermentation conditions [14,19]. Alternatively, site-directed and random mutagenesis can be used to modify the enzymes using protein engineering techniques [33].

Hydrolytic Product Compositions of Xylanolytic Enzymes
TLC analysis showed that in the presence of sufficient substrate, hydrolytic products of beechwood xylan degraded by xylanolytic enzymes mainly contained xylooligosaccharides (Figure 5), an emerging prebiotic in applications of food industry [7]. Thus, L. chartarum SJTU59 can be modified and enhanced for use in industrial applications in pulp/paper leaching at high temperatures (55°C to 70°C) and alkaline pH (pH 7.0 to pH 9.0), or acid pre-treatment in bioconversion processes or detergent application at high temperatures [1]. The xylanolytic enzyme solution with high resistance to a variety of metal ions can be used in deinking and metal-polluted sewage treatment [32]. Given the initially high production and stable properties of xylanolytic enzymes under various adverse conditions, further improvement of xylanolytic enzyme production by L. chartarum SJTU59 can be done via optimization of fermentation conditions [14,19]. Alternatively, site-directed and random mutagenesis can be used to modify the enzymes using protein engineering techniques [33].

### Table 4. Amino acid sequence homologies among novel xylanase in GH family 11 from L. chartarum SJTU59 and known xylanases from five representative industrial strains.

| Number allocated to strain (GH 11, endo-β-1,4-xylanase) | Strain |
|---|---|
| 1 2 3 4 5 6 | |
| 54.5 54.8 53.9 55.6 51.5 | XYN-L4 (KF305939) |
| 55.9 63.1 54.9 47.3 | Trichoderma harzianum (ACF40831) |
| 59.0 63.1 51.5 | Aspergillus oryzae RIB40 (XP_001818666) |
| 61.1 50.5 | Penicillium citrinum (BAE71133) |
| 61.8 | Streptomyces sp. e14 (ZP_06710529) |
| 6 | Bacillus subtilis BS5 (YP_004203820) |

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Table 5. Amino acid sequence homologies among novel xylosidase in GH family 43 from L. chartarum SJTU59 and known xylosidases from five representative industrial strains.

| Number allocated to strain (GH 43, β-xylosidase) | Strain |
|---|---|
| 1 2 3 4 5 6 | |
| 25.4 70.3 68.4 23.0 21.3 | XYS-L5 (KF305940) |
| 22.5 21.4 59.6 14.2 | Trichoderma atroviride IMI206040 (EHK39865) |
| 75.5 20.4 22.3 | Aspergillus terreus NIH2624 (XP_001210179) |
| 20.2 19.6 | Penicillium herquei (BAC75546) |
| 14.2 | Streptomyces gancidicus BSK13-15 (ZP_22995124) |
| 6 | Bacillus cellulosilyticus DSM522 (YP_004093398) |

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**chartarum** SJTU59 has great potential for large-scale production of xylooligosaccharides by optimization of the reaction system and fermentation conditions, including the reaction time, temperature, and/or pH. With an increase in volume of enzyme solution and a decrease in volume of xylan substrate, the degree of xylan hydrolysis gradually increased, and the main hydrolytic products changed to xylose with a small amount of xylobiose (Figure 5).

In summary, the results indicate that the xylanolytic enzyme system of *L. chartarum* SJTU59 contains at least two types of xylanolytic enzymes, i.e., endo-xylanase that cleaves internal xylosidic linkages on the backbone of xylan, and xylosidase that cleaves glycoside linkage of xylooligosaccharides (or exo-xylanase that cleaves external xylosidic linkages on the backbone of xylan).

**Assays of Tissue Necrosis and Oxidative Burst**

At 12 h after inoculation, necrotic lesions were seen on tobacco leaves injected with xylanolytic enzyme preparation from *L. chartarum* SJTU59, but no necrotic lesions were seen on the leaves treated with blank broth and distilled water. This contrasting result was most apparent at 24 h (Figure 6 A). In addition, inoculated sites and certain distant sites of tobacco leaves injected with xylanolytic enzyme preparation from *L. chartarum* SJTU59 could be stained strongly by DAB, whereas those in tobacco leaves injected with blank broth and distilled water showed nearly no staining (Figure 6 B).

As DAB staining reflects H$_2$O$_2$ accumulation, results illustrate that oxidative burst in tobacco leaves is induced by xylanolytic enzyme preparation from *L. chartarum* SJTU59. H$_2$O$_2$ and O$_2^-$ from oxidative burst are correlated with hypersensitive response (HR) and cause general cell death [34,35]. In addition, as a typical second messenger, H$_2$O$_2$ directly and indirectly (such as via HR) regulates the innate immune system and systemic acquired resistance (SAR) of plants, to enhance plant defense against adversity and disease [36,37].

For industrial and agricultural uses, further study at the molecular level on the xylanolytic enzyme genes of *L. chartarum* SJTU59 is important.

**Sequence Analysis of Novel Xylanolytic Enzyme Genes**

PCR assay with GH 10-targeting degenerate primers yielded four bands on the electrophoretic gel image (Figure S3A). DNA sequencing (data not shown) and sequence alignment on NCBI website showed that the two bands may represent the conserved regions (322 bp and 262 bp) of two endo-β-1,4-xylanase genes in the GH family 10, designated *xyn-l1* (intron: 57 bp) and *xyn-l2* (non-intron), respectively. PCR assay with GH 11-targeting degenerate primers yielded one band on the electrophoretic gel image (Figure S3B). However, DNA sequencing and sequence alignment on NCBI website showed that the PCR amplicons may contain conserved regions (273 bp and 266 bp) of two endo-β-1,4-xylanase genes in the GH family 11, designated *xyn-l3* (intron: 57 bp) and *xyn-l4* (non-intron), respectively. PCR assay with GH43-targeting degenerate primers yielded one band on the electrophoretic gel image (Figure S3C). DNA sequencing (data not shown) and sequence alignment on NCBI website showed that the band may represent the conserved region (613 bp) in a β-xylosidase gene in the GH family 43, designated *xys-l5* (non-intron).

PCR identification of four novel full-length genes of xylanolytic enzymes, including *xyn-l1*, *xyn-l2*, *xyn-l4*, and *xys-l5* acquired via RACE, all yielded one specific band with the appropriate size on electrophoretic gel images (Figure S4). DNA sequences (data not shown) and sequence alignments with RACE results showed that the sequences of *xyn-l1* (KF367459, 1,062 bp), *xyn-l2* (KF305938, 1,152 bp), and *xyn-l4* (KF305939, 693 bp), and *xys-l5* (KF305940, 1,020 bp) were all correct. However, *xyn-l3* was inferred to be a pseudogene or low-expression abundance gene, as at RNA level its conserved, 5' RACE and 3' RACE sequences all could not be obtained.

Results prove that *L. chartarum* SJTU59 has a complex xylanolytic enzyme system, thus ensuring high-efficiency hydrolysis.
sis of xylan. Considering the potential limitation of selected primers [30,31], we believe that undetected gene(s) are possibly present in the xylanolytic enzyme system of *L. chartarum* SJTU59. To identify other novel xylanolytic enzyme genes, future studies will be conducted on primer design in consideration of other less-known GH families, e.g., GH 5, 7, and 8.

**Homology and Function Analyses of Novel Xylanolytic Enzymes**

Amino acid homology analyses showed that XYN-L1 shared 41.7% homology with that of the endo-β-1,4-xylanase (ELA24762, GH family 10) from *Colletotrichum gloeosporioides* Nara gc3, and XYN-L2 shared 63.8% homology with that of the endo-β-1,4-xylanase (AFD63136, GH family 10) from *Aspergillus terreus* (Figure 7A).

XYN-L4 shared 78.9% homology with that of the endo-β-1,4-xylanase (ADW78230, GH family 11) from *Chaetomium sp.* CQ31, 76.5% homology with that of the endo-β-1,4-xylanase (XP_003006739, GH family 11) from *Verticillium albo-atrum* VaMs.102 and 73.9% homology with that of the endo-β-1,4-xylanase (EMT73821, GH family 11) from *Fusarium oxysporum* f. sp. cibdlea race 4 (Figure 7B).

XYS-L5 shared 75.7% homology with that of the β-xylosidase (XP_001929295, GH family 43) from *Pyrenophora tritici-repentis* Pt-1C-BFP, 68.1% homology with that of the β-xylosidase (EKD19100, GH family 43) from *Marssonina brunnea* f. sp. malaglutinula MB_m1, and 69.2% homology with that of the β-D-xylosidase (AFX58964, GH family 43) from *Talaromyces purpuragenus* (Figure 7C).

In accordance with the CAZy classification system, members of the same GH family share similar structural and functional roles [3]. Previously, the majority of (hyper) thermophilic, psychrophilic, alkaliphilic, and/or acidophilic xylanases were identified in GH families 10 and 11 [1]. A GH family 10-related xylanase, Xylk, is activated by the presence of a certain amount of Ca$^{2+}$, Co$^{2+}$, and Fe$^{3+}$ [38]. We propose that the xylanase stability and resistance of *L. chartarum* SJTU59 in various temperatures, pH, or chemical conditions (Figure 4 and Table 2) are related to the existence of the three endo-β-1,4-xylanases of GH families 10 and 11. In addition, xylanase II from GH family 11 induces necrotic lesions and ethylene biosynthesis in tobacco, thereby potentially improving the resistance and immunity of plants to diseases [39]. We infer that necrotic lesions and oxidative burst (Figure 6) of tobacco may be related to endo-β-1,4-xylanase of GH families 11 from *L. chartarum* SJTU59. However, little is known regarding the structural or functional roles of GH family 43 members. We are uncertain about the properties of β-xylosidase detected in *L. chartarum* SJTU59.

**Phylogeny Analysis of Novel Xylanolytic Enzymes**

The amino acid sequence homologies among novel xylanolytic enzymes detected in *L. chartarum* SJTU59 and known xylanolytic enzymes in five representative industrial strains of xylanolytic enzyme producer were shown in Table 3 to Table 5. Overall, the homologies of deduced amino acid sequences were 27.4% to 61.4% between XYN-L1 to XYN-L2 and known endo-β-1,4-xylanases from the GH family 10, 47.3% to 63.1% between XYN-L4 and known endo-β-1,4-xylanases from the GH family 11, and 14.2% to 70.3% between XYS-L5 and known β-xylosidases from the GH family 43 (Figure 8). Hence, the amino acid sequence homologies and phylogenetic tree results indicate that *L. chartarum* SJTU59 contains at least four novel genes that encode three novel xylanases and one novel xylosidase, including two endo-β-1,4-xylanases of the GH family 10, one endo-β-1,4-xylanase of the GH family 11, and one β-xylosidase of the GH family 43.

In summary, for industrial and agricultural uses, cloning, expression, and characterization of high-yield xylanolytic enzymes from *L. chartarum* SJTU59 are critical to improve current understanding of their enzyme activities and functions [28]. Cloned and expressed xylanolytic enzymes can be compared with known xylanolytic enzymes from recognized microbial producers to identify the advantage of novel xylanolytic enzymes detected in *L. chartarum* SJTU59. Based on the present study, novel xylanolytic enzyme-producing engineered strain(s) can be developed via transgenic approach or overexpression in yeast [40].

**Conclusion**

This study was the first demonstration of high-level xylanolytic enzyme production by a novel fungus, designated *L. chartarum* SJTU59. The enzyme solution was relatively stable over a wide range of pH (pH 3.0 to pH 9.0) and temperature (40°C to 65°C) while showing high resistance to the majority of metal ions tested. These characters of xylanolytic enzymes are suitable for pulp/paper leaching, deinking and metal-polluted sewage treatment in industrial applications. Analysis of the hydrolytic products of xylan showed that in the presence of sufficient xylan substrate, xylkoosaccharides, an emerging prebiotic in applications of food industry, were mainly produced. Excluding industrial applications, the xylanolytic enzyme fermentation supernatant of *L. chartarum* SJTU59 may be related to enhanced plant defense against adversity and disease. *L. chartarum* SJTU59 possessed a complex xylanolytic enzyme system: two endo-β-1,4-xylanases of the GH family 10, one endo-β-1,4-xylanase of the GH family 11, and one β-xylosidase of the GH family 43, respectively. Future studies will be conducted to characterize the properties of individual xylanolytic enzymes from *L. chartarum* SJTU59 via expression them in *Escherichia coli*, respectively, and construct genetically engineered strains with the xylanase-encoding gene(s) for potential use in industrial and agricultural applications.

**Supporting Information**

Figure S1 Electrophoretic gel images of PCR amplification of ITS and EF-1α sequences from *L. chartarum* SJTU59. (A) ITS amplification; M, Marker DL2000 (Takara, Japan); 1, band of ITS. (B) EF-1α amplification; M, Marker DL2000 (Takara, Japan); 1, band of EF-1α.

Figure S2 Molecular identification of *L. chartarum* SJTU59 with nrDNA ITS and EF-1α sequences. (A) Alignment of nrDNA ITS sequences from *L. chartarum* SJTU59 and CY233; 1, nrDNA ITS sequence of *L. chartarum* SJTU59; 2, nrDNA ITS sequence of *L. chartarum* CY233. (B) Alignment of EF-1α sequences from *L. chartarum* SJTU59 and L119; 1, EF-1α sequence of *L. chartarum* SJTU59; 2, EF-1α sequence of *L. chartarum* L119.

Figure S3 Electrophoretic gel images of PCR amplification of conserved regions from five novel xylanolytic enzyme genes. (A) PCR amplification of the conserved regions of two novel xylanase genes (syn-11 and syn-12) belonging to the GH family 10; M, DL2000 (Takara, Japan); GH 10, bands of amplification; 1 and 2 are the bands of syn-11 and syn-12. (B) PCR amplification of the conserved regions of two novel xylanase genes (syn-13 and syn-14) of the GH family 11; M, DL2000 (Takara, Japan); GH11, bands of amplification; 3 and 4 are the bands of syn-13 and syn-14. (C) PCR amplification of the conserved region of one novel xylanase genes (syn-15) of the GH family 43; M,


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