


## Research Article

# Cloning, Expression, and Characterization of Xylanase G2 from *Aspergillus oryzae* VTCC-F187 in *Aspergillus niger* VTCC-F017

Do Thi Tuyen <sup>1,2</sup>, Nguyen Tien Cuong,<sup>1</sup> Nguyen Sy le Thanh,<sup>1</sup> Nguyen Thi Thao,<sup>1</sup>  
Le Thanh Hoang,<sup>1</sup> Nguyen Thi Hien Trang,<sup>1</sup> Nguyen Thi Trung,<sup>2</sup> and Dao Thi Mai Anh<sup>3</sup>

<sup>1</sup>Institute of Biotechnology, Vietnam Academy of Science and Technology, Vietnam

<sup>2</sup>Graduate University of Science and Technology, Vietnam Academy of Science and Technology, Vietnam

<sup>3</sup>Department of Biochemistry, Hanoi University of Pharmacy, Vietnam

Correspondence should be addressed to Do Thi Tuyen; [dtuyen@ibt.ac.vn](mailto:dtuyen@ibt.ac.vn)

Received 14 September 2020; Revised 26 February 2021; Accepted 5 March 2021; Published 12 March 2021

Academic Editor: Gustavo Graciano Fonseca

Copyright © 2021 Do Thi Tuyen et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

The study focuses on engineering of recombinant *Aspergillus niger* to produce highly active xylanase. The xylanase G2 encoding gene originating from *Aspergillus oryzae* VTCC-F187 was cloned, amplified, and inserted into the pAN7.1GluA vector with specific primers possessing *Bam*HI. The recombinant plasmid was introduced into *Aspergillus niger* VTCC-F017 by chemical methods. The recombinant strain was checked by polymerase chain reaction method and Southern blot. Next, the recombinant protein was expressed and purified by His-tag column. The molecular mass of the purified xylanase G2, as determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), was 21 kDa with a specific activity of 1025 IU/mg towards 0.5% (w/v) of birchwood xylan. The optimal temperature and pH were 55°C and pH 6.5, respectively. The enzyme was stable in a temperature ranges 25–40°C and a pH ranges 5–7. The presence of Tween 80 enhanced xylanase activity. Triton X-100, however, had no impact on the function of the enzyme. The xylanase activity was reduced by Tween 20, SDS, and organic solvents. The enzyme was completely inhibited by Hg<sup>2+</sup> and partially by Zn<sup>2+</sup>, Fe<sup>2+</sup>, and Ag<sup>+</sup>, while it was slightly stimulated by K<sup>+</sup> and EDTA.

## 1. Introduction

Xylanase is a class of enzymes produced by microorganisms to breakdown xylans, the most abundant hemicellulose-polysaccharides found in plant cell walls. The complete hydrolysis of xylan requires the synergistic action of several xylanases of different functions including endo- $\beta$ -1,4-D-xylanase (EC 3.2.1.8). Endoxylanases have a wide variety of biotechnological applications. In the pulp industry, they are used as a pre-bleaching agent which facilitates subsequent chemical bleaching, reduces the toxic chemical demand (especially organochlorine compounds), and improves the brightness of the pulp [1]. Xylanases are also used in the clarification of fruit juice, wine, beer, and forming xylitol in the confectionery industry [2]. Besides, the enzymes hydrolyze food containing xylan and, at the same time, help to reduce the viscosity in the digestive system followed by many posi-

tive effects such as improved food absorption, improved microorganism populations of the intestine in the advantageous direction, and reduced digestion disorder [3, 4].

On another hand, arabinoxylan oligosaccharides, the main products of xylanase-catalyzed hydrolysis reaction, are widely known as prebiotic carbohydrates with promising health-promoting properties by improving the gut microbes [5] and enhancing the quality of immunotherapy of cancer patients [6]. An alternative form of arabinoxylan with ferulic acid has been drawn interests in the pharmaceutical industry which is presented as antioxidant and anticancer components [7].

Various microorganisms such as bacteria, yeast, and filamentous fungi have been reported with the ability to produce xylanase. Among them, fungi are capable of synthesizing highly active xylanases [8]. Owing to different applications, the purifications and biochemical characterizations of xylanase from various *Aspergillus* strains such as *A. awamori*,

*A. ficuum*, *A. giganteus*, *A. niger*, and *A. sydowii* have been described in previous lectures [2, 9, 10].

In order to produce highly active enzymes, many recombinant microorganisms were created. The recombinant xylanase from *E. coli*, *Bacillus subtilis*, and *Pichia pastoris* were purified and characterized. The gene encoding xylanase from *B. pumilus* was overexpressed in *E. coli* and *B. subtilis*. These *B. subtilis* clones produced 2.7-3.0 folds higher than those of *B. pumilus* [11]. *Aureobasidium pullulans* Y-2311-1 *xynA* had an open reading frame encoding a polypeptide of 221 amino acids containing a putative 34 amino acids. Purified xylanase had the highest activity at pH 4.8 and 54°C. This enzyme was stable at 55°C and pH 4.5 [12]. Two family xylanases *xynG2* and *xynF3* were overexpressed and purified from *A. oryzae* [13, 14]. The structural part of *xynG2* was found to be 767 bp. The nucleotide sequence of cDNA amplified by reverse transcription polymerase chain reaction (RT-PCR) showed that the open reading frame of *xynG2* was interrupted by a single intron which was 71 bp and encoded 232 amino acids. The purified *xynG2* showed optimal activity at pH 6.0 and 58°C. It had a  $K_m$  of 5.1 mg/mL and a  $V_{max}$  of 123  $\mu\text{mol}/\text{min}/\text{mg}$  when birchwood xylan was used as a substrate. The structural part of *xynF3* was found to be 1468 bp. The nucleotide sequence of cDNA amplified by RT-PCR showed that the open reading frame of *xynF3* was interrupted by ten short introns, and encoded 323 amino acids. The purified *xynF3* showed an optimal activity at pH 5.0 and 58°C [14]. The *xlnR* gene encoded a polypeptide of 875 amino acids was cloned and overexpressed in *A. niger* with *goxC* promoter [15]. Xylanase B from *Aspergillus cf. niger* BCC14405 was purified and characterized. It was found to have a molecular mass of 21 kDa, an optimal pH of 5.0, and a temperature of 55°C. When xylanase was tested using xylan from birchwood, it showed  $K_m$  and  $V_{max}$  values of 8.9 mg/mL and 11,100 U/mg, respectively [9].

In our previous study, a GH 10 enzyme, xylanase A, was expressed in *Pichia pastoris* GS115. The gene encoding this xylanase A has been cloned from *A. niger* DSM1957. The maximal activity of this recombinant xylanase (808.5 U/mg protein) was reached after 144 h of fermentation with shaking (200 rpm) at 30°C in YP medium (induced by methanol 0.5%) [16]. In this study, the gene encoding xylanase G2 from *A. oryzae* VTCC-F187 has been cloned and analyzed in pAn-GluA vector. This gene was used to engineer recombinant protein in *A. niger* with the aim of producing highly active xylanase to produce arabinoxylan as functional foods. The enzyme was purified and characterized to determine its potential in this regard.

## 2. Materials and Methods

**2.1. Chemicals and Reagents.** Restriction enzymes, Taq DNA polymerase, and T4 ligase were purchased from Fermentas, Thermo Fisher Scientific Inc. (Waltham, USA). Kit Pro-Bond™ Nickel-chelating resin was from Invitrogen Corp. (Carlsbad, CA, USA). 3,5,5-Dinitrosalicylic acid (DNS), birchwood xylan, and SDS were from Sigma-Aldrich Co. (St. Louis, USA). Peptone, yeast extract, Tween 80, and Tween 20 were from BioBasic Inc. (Ontario, Canada). Triton X-100

was from Merck (Darmstadt, Germany). All other chemicals were of analytical grade unless otherwise stated.

**2.2. Vectors, Strains, and Culture Conditions.** *Aspergillus oryzae* VTCC-F187 and *A. niger* VTCC-017 strains were provided by Vietnam Type Culture Collection (VTCC). They are used as the source for the xylanase gene (*xlnG2*).

*Escherichia coli* DH5 $\alpha$  (F<sup>-</sup>,  $\phi$ 80dlacZDM15, D(lacZYA-argF)U169, deoR, recA1, endA1, hsdR17(rk<sup>-</sup>, mk<sup>+</sup>), phoA, supE44, k<sup>-</sup>, thi-1, gyrA96, and relA1) and pJET1.2/blunt vector (Fermentas, Thermo Fisher Scientific Inc., Waltham, USA) were used for DNA manipulations and amplification. pAN7.1GluA vector was from Department of Genetic and Plant Pathology, University of Hamburg, Germany.

The recombinant *E. coli* was inoculated in Luria-Bertani medium (LB) containing (g/L) bacto tryptone 10, yeast extract 5, NaCl 10, and at a pH of 7–7.5. Next, LB agar contained additional 20 agar and 100  $\mu\text{g}$  ampicillin.

Yeast peptone glucose medium (YPG) contains (g/L) yeast extract 10, and peptone 20 supplemented with glucose 10. Yeast peptone dextrose (YPD) medium (g/L) contains yeast extract 10, peptone 20, dextrose 20, and agar 20 for plates only. Minimal medium (AMM) (g/L) contains NaNO<sub>3</sub> 0.6, KCl 0.52, KH<sub>2</sub>PO<sub>4</sub> 1.52, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.52, sucrose 2, and 1 ml MNS. Complete media (CM) contain 10 mL solution A (Ca(NO<sub>3</sub>)<sub>2</sub>·4H<sub>2</sub>O 10%), 10 mL solution B (KH<sub>2</sub>PO<sub>4</sub> 2 g/L, MgSO<sub>4</sub>·7H<sub>2</sub>O 2.5 g/L, NaCl 1.5 g/L, and pH 5.3), yeast-casein 0.2 g/L, glucose 1 g/L, 1 mL MNS, and 900 mL H<sub>2</sub>O. TB3 medium (g/L) contains yeast extract 3, casamino-acid 3, glucose 10, and sucrose 20. All medium was from Merck (Darmstadt, Germany).

**2.3. Total RNA Extraction.** Total RNA from *A. oryzae* VTCC-F187 was extracted from 2 g of mycelia using Trizol Reagent Kit (Invitrogen Corp., Carlsbad, CA, USA). The protocol was previously described in detail [17].

**2.4. PCR Amplification.** Based on the nucleotide sequence of *xlnG2* available in GenBank, two oligonucleotides *xlnG2F* (5' -CG GGATCCATGGTGTCCTTCTCTCCATCC-3') and *xlnG2R* (5' -CG GGATCCTCAGTGGTGGTGGTGGTGGT GATAAACAGTGATAGCAGA-3') were designed as primers to amplify the *xlnG2* from *A. oryzae* VTCC-F187 with the introduction of the underlined *EcoRI* and *XbaI* restriction sites at 5' of forward and reverse primer, respectively. The first-strand cDNA product was used to amplify *xlnG2* using two primers *xlnG2F* and *xlnG2R*.

The PCR mixture contained 2.5  $\mu\text{L}$  of 10 times PCR buffer, 2  $\mu\text{L}$  of 2.5 mM dNTP, 1.5  $\mu\text{L}$  of 25 mM MgCl<sub>2</sub>, 2  $\mu\text{L}$  of cDNA (50 ng) from control RT reaction, 0.5  $\mu\text{L}$  of 5 unit Taq polymerase, and 1  $\mu\text{L}$  of each primer (10 pmol), supplemented with 14.5  $\mu\text{L}$  of distilled water to a final volume of 25  $\mu\text{L}$ . The thermocycler conditions were as follows: 94°C/3 min; 35 cycles of 95°C/45 s, 54°C/1 min, and 72°C/1 min; 72°C/10 min. PCR products were inserted into the pJET1.2/blunt vector resulting in pJxlnG2 and transformed into the *E. coli* DH5 $\alpha$ . The recombinant plasmid was confirmed by restriction enzyme analysis and DNA sequencing.

**2.5. Analysis of Genomic DNA.** The nucleotide sequence was determined by the Sanger method, on the ABI PRISM 3100 Avant Genetic Analyzer. Nucleotide sequence analysis was performed by the DNASTAR software.

**2.6. Transformation of Xylanase Gene in *A. niger*.** Approximately 30000 spores were bred in 50 mL YPD medium at 150 rpm, 28°C overnight; then, the whole culture was ground with 150 mL YPD medium (3 times, 10 s/time). Next, 50 mL of the above mixture was added to 150 mL YPD medium and shaken overnight at 150 rpm, 28°C in a 500 mL-flask bottle. The entire pre-culture was filtered by a Wilson-sieve filter funnel (100 µm) and washed several times with deionized water. The weight of the hyphae mixture was determined after drying with sterilized blotting paper. The enzyme solution was mixed with the above hyphae mixture (20 mL with 1-1.5 g), in a 100 mL-flask bottle at 75 rpm, 28°C. Next, the collected cells were filtered through the 100 µm and 40 µm filter funnel Wilson-sieve, respectively. The dregs of the cell were dissolved carefully in 12 mL 0.7 M NaCl pH 5.6, then centrifuged it again and reconstituted. The number of cells was checked with a microscope. 50 µg plasmid-DNA and 1 mL of precooled PTC (40% PEG 4000 and 60% STC (20% sucrose, 10 mM Tris-HCl, pH 8.0, and CaCl<sub>2</sub> 50 mM)) were added and mixed well. The mixture was kept at 4°C for 25 min and shaken every 2 min and incubated at 80 rpm, 28°C overnight. Later, the cells were mixed with AMM medium added 50 µL hygromycin, then poured into 2 plates, and kept at 28°C overnight. Each plate was coated with 10 mL of 1.2% agar containing 50 µL of hygromycin. The plates were checked every day until colonies became visible. The colonies were picked and transferred to a selective agar plate of CM containing 900 µg/L hygromycin (w/v).

**2.7. DNA Isolation from *A. niger*.** A square of 0.5 cm<sup>2</sup> agar plates were cut and cultured in 50 mL of YPG liquid medium, at 200 rpm, 28°C for 3-4 days. Hyphae were collected by filtering through a filter funnel and rushed in porcelain pestle with liquid nitrogen, then transferred into 2 mL eppendorf. 600 µL lysis buffer was added to each eppendorf tube and placed them at 65°C from 40 min to 1 h. After that, all eppendorf tubes above were centrifuged 13000 rpm, 10 min at 4°C. The upper phase was transferred to another 1.5 mL eppendorf tube. Chloroform: isoamyl alcohol solution (24:1; v/v) was added with the equivalent volume and shaken well, then centrifuged the mixture 13000 rpm at 4°C for 15 min. The upper phase was transferred to a new 1.5 mL eppendorf tube, and the DNA was precipitated by adding 750 µL of isopropanol, shaking well, leaving it in the refrigerator for 30 min at 4°C and centrifuging 13000 rpm at 4°C for 20 min. Then the residue was collected and washed with 500 µL cold alcohol (70%), dried, and precipitated in TE.

**2.8. Southern Blot.** After treatment with restricted enzymes, the DNA samples isolated from *A. niger* were checked by electrophoresis on an agarose gel. Next, the gel was placed in a plastic box and washed 2 times with distilled water, then soaked in denaturation solution, and shaken for 30 min at room temperature (25°C). After that, the gel was washed with

distilled water and soaked in neutralization solution, shaken for 30 min at room temperature (25°C), and then balanced in 20 × SSC for at least 10 min. After had being removed from the balanced solution, the gel was placed on top of a sheet of Whatman 3MM blotting paper which had been already pre-wet with 20 × SSC solution. Air bubbles between the two layers should be avoided. The nylon membrane was placed on top of the gel, and 4-5 sheets of Whatman 3MM blotting paper were put on the nylon membrane. A 10 cm stack of absorbent paper and a heavy object weighing about 200-500 g were placed on top, then leaving them overnight in order to transfer DNA onto the nylon membrane. Before fixing, the film was washed by 2 × SSC buffer, then placed on the 3MM Whatman plate (placing the face containing DNA on top). The membrane was laid under UV light for 1-3 min, washed with 2 × SSC, then put it back on the 3MM Whatman plate, and allow it to dry, shaken overnight at 68°C. Next, the solution covering the membrane was removed, then poured the probe mixture, and incubated overnight at 68°C. Accordingly, the membranes were washed in three steps: (1) 2 times, 5 min each time at room temperature (25°C) with 20 mL of W1 solution; (2) 2 times, 15 min each time at 68°C with 20 mL of W2 solution; (3) 30 min in WP solution and B2 solution at room temperature (25°C). The antibody was mounted on the membrane for 30 min (5 µL antibody was mixed in 50 mL B2 solution). The membrane was washed 3 times, 20 min each time with WP solution at room temperature (25°C), then washed for 5 min in B3 solution. 500 µL substrate was spread onto the nylon plastic sheet, then covered the membrane with DNA sample on the substrate for 5 min at room temperature (25°C); the results were visualized by fluography.

**2.9. Gene Expression.** For the expression of *xylanase G2* in recombinant *A. niger* VTCC-F017, 0.5 mL of an overnight culture was inoculated into 50 mL YP medium supplemented with 1% (w/v) glucose in a 1 L-flask bottle and grown at 30°C with agitation at 200 rpm. The culture supernatant was collected periodically to detect xlnG2 activity during 120 h incubation.

**2.10. Purification of Recombinant xlnG2.** The culture supernatant containing xlnG2 was harvested from 50 mL culture by centrifugation at 5000 rpm and 4°C for 10 min. After that, 8 mL of culture supernatant was applied to a ProBond™ Ni<sup>2+</sup> affinity chromatography column containing 2 mL resin, which had been equilibrated with native binding buffer (250 mM NaH<sub>2</sub>PO<sub>4</sub>, 2.5 M NaCl, and pH 8.0) and incubated for 60 min at room temperature (25°C) with gentle hand-shaking for several times. The column was washed 4 times with 8 mL of native wash buffer (250 mM NaH<sub>2</sub>PO<sub>4</sub>, 2.5 M NaCl, 20 mM imidazole, and pH 8.0). The binding protein was eluted with 1 mL of a native elution buffer containing 250 mM imidazole (250 mM NaH<sub>2</sub>PO<sub>4</sub>, 2.5 M NaCl, 300 mM imidazole, and pH 8.0). The purified enzyme was used for characterization.

**2.11. Determination of Xylanase Activity.** Xylanase activity was determined by measuring the concentration of reducing



sugars produced by enzymatic hydrolysis of birchwood xylan. A reaction mixture of 100  $\mu$ L of the crude or purified xylanase containing 11  $\mu$ g for each reaction was incubated with 400  $\mu$ L of 0.5% (w/v) birchwood xylan in 20 mM potassium phosphate buffer pH 6.5 at 55°C for 5 min. To arrest the reducing sugar released in the reaction mixture, 1.25 mL of DNS was added. The reducing sugars were determined by measuring the absorbance at 540 nm [18]. D-xylose was used as a standard. One unit (U) of xylanase activity was defined as the amount of enzyme that released 1 mol of xylose per minute under the standard assay conditions.

**2.12. SDS-PAGE Electrophoresis and Protein Concentrations.** The relative molecular weight and purity of the xylanase G2 enzyme were analyzed through 12.5% (w/v) SDS polyacrylamide gel electrophoresis [19]. Proteins were visualized by staining with 0.1% (w/v) Coomassie Brilliant Blue R-250. Protein concentrations were estimated by the method of Bradford with bovine serum albumin as a standard [20].

**2.13. Optimal Temperature and Thermostability.** We determined the optimal temperatures by carrying out the experiments at different temperatures at 37, 40, 45, 50, 55, 60, 65, and 70°C. To determine the thermostability, the purified enzyme was incubated 0-24 h at different temperatures from 25 to 50°C. The residual activity of the enzyme was observed by standard assay procedure.

**2.14. Optimal pH and pH Stability.** We determined the optimal pH by carrying out experiments at different pH 3.5-8 (acetate: 3.5-5; phosphate: 6-8). To determine the pH stability, the enzyme was incubated in 10 mM buffers with different pH 3.5-8 (acetate: 3.5-5; phosphate: 6-8) at 4°C for 2, 8, and 24 h. The residual activity of the enzyme was observed by standard assay procedure.

**2.15. Effect of Metal Ions.** We assessed the influences of metal ions on xylanase activity by incubating enzyme with 10 mM metal ions ( $Mg^{2+}$ ,  $Fe^{2+}$ ,  $Cu^{2+}$ ,  $Ca^{2+}$ ,  $Ag^+$ ,  $Ni^{2+}$ ,  $K^+$ ,  $Hg^{2+}$ , and  $Zn^{2+}$ ) and ethylenediaminetetraacetic acid (EDTA) at room temperature (25°C) for 2 h. The residual activity of the enzyme was observed by standard assay procedure.

**2.16. Organic Solvent Tolerant.** We assessed the effects of organic solvents on xylanase activity by incubating the enzyme with organic solvents (methanol, ethanol, isopropanol, acetone, and n-butanol) at the final concentration 30% (v/v) at 30°C, for 1 h. The residual activity of the enzyme was observed by standard assay procedure.

**2.17. Effect of Detergents.** The enzyme was incubated with 2% Tween 20, Tween 80, Triton X-100, and SDS (v/v) at 30°C, for 1 h. The residual activity of the enzyme was observed by standard assay procedure. All measurements were carried out in triplicate with the resulting values being the mean of the cumulative data obtained.

### 3. Results and Discussion

**3.1. Cloning Xylanase Gene.** The mRNA total from *A. oryzae* VTCC-F187 was extracted and purified to be used as a

template to create cDNA with Fementas kit (Supplement 1A). The xylanase genes were then amplified with *xlnG2F/xlnG2R* primer pairs. PCR product of size 699 bp was observed (Supplement 1B).

These PCR amplification products were inserted to the vector pJET1.2 blunt to be transformed into *E. coli* DH5 $\alpha$ . The recombinant colonies were collected; recombinant plasmid was extracted and then identified by electrophoresis. The plasmid contained in the lower band was selected to be used as the template for PCR reaction using primer *xlnG2F/xlnG2R*. The PCR product had a size of 699 bp, which corresponded to the size of the *xlnG2* (Supplement 1C). These results suggested that the *xlnG2* was successfully cloned in to pJET1.2 and transformed into *E. coli* DH5 $\alpha$ . Recombinant plasmids carrying the gene encoding *xlnG2* were denoted by pJxlnG2.

**3.2. Xylanase Gene Sequence Analysis.** After nucleotide sequencing, the putative sequence of xylanase G2 gene in GeneBank and amino acid sequences were compared using the DNASTar software. The gene sequence of xylanase G2 of *A. oryzae* VTCC-F187 (Ao\_Xln\_07) had a high similarity to the sequences of xylanase genes of *A. oryzae* Ao\_Xln\_41 gene (99.3%) in GenBank (Figure 1(a)). Accordingly, the predicted amino acid sequence of *A. oryzae* VTCC-F187 xylanase had a high homology to Ao\_Xln\_41 (99.9%) (Figure 1(b)). The *xlnG2* (699 bp, 232 aa) from *A. oryzae* VTCC-F187 belongs to the glycosyl hydrolase family 11, which was completely similar to the *xlnG2* (767 bp, 232 aa) (glycosyl hydrolase family 11) [14], *A. oryzae xlnG1* (725 bp, 189 aa) [21], *xlnC* (636 bp, 211 aa) [22], *xlnA* (glycosyl hydrolase family 10) [16], and *xlnD* (957 bp, 318 aa) [23] from *A. niger* strains (from GenBank). The phylogenetic tree of nucleotide and amino acids are not much different; this can be explained by a multitude of codons encoding for an amino acid. The nucleotide sequence of gene xylanase G2 of *Aspergillus oryzae* VTCC-F187 had been registered on GenBank with the code of EU848307.

**3.3. Design of Plasmid to Express the Xylanase Gene in *Aspergillus* sp.** The xylanase gene was amplified from pJxlnG2 by PCR reaction with specific primers containing *Bam*HI site and His coding sequence (Supplement 2A). PCR product was attached to the pGEMT vector. The adhesive product is transformed on a medium containing X-gal and ampicillin. White colonies were selected to separate plasmid and examine the presence of the xylanase gene (Supplement 2B).

To design the vector expressing xylanase gene, the plasmid pAN7.1GluA with a strong promotor GPDA from *A. nidulan* and a *Bam*HI site was used. Plasmids containing the xylanase gene were cut by *Bam*HI (Supplement 3A), purified, and tested by electrophoresis on agarose gel (Supplement 3B). Vector and the xylanase genes were ligated together by the T4-ligate. The adhesive product was transferred into *E. coli* DH5 $\alpha$ .

The colonies were randomly selected, separated plasmid, and examined the presence and direction of the gene by primers in which the forward primer was designed in the

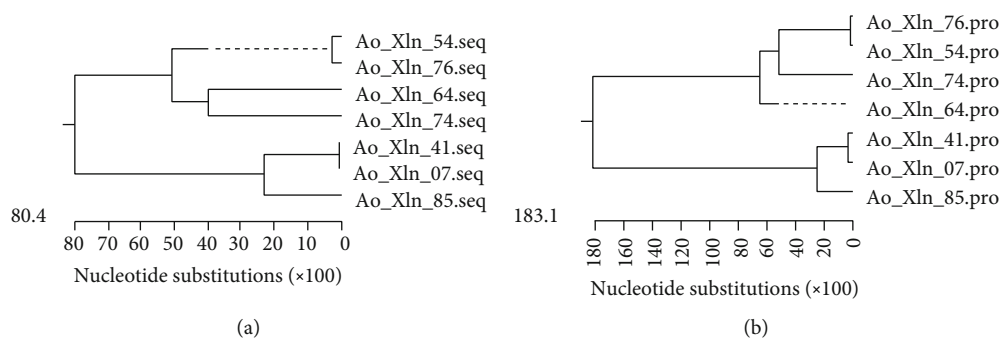


FIGURE 1: Phylogenetic tree of the (a) xylanase G2 gene family and (b) xylanase G2 protein family. Ao\_Xln\_41: *Aspergillus oryzae* AB044941; Ao\_Xln\_54: *Aspergillus oryzae* AP007154; Ao\_Xln\_64: *Aspergillus oryzae* AP007164; Ao\_Xln\_74: *Aspergillus oryzae* AP007174; Ao\_Xln\_76: *Aspergillus oryzae* AB066176; Ao\_Xln\_85: *Aspergillus oryzae* AB003085.

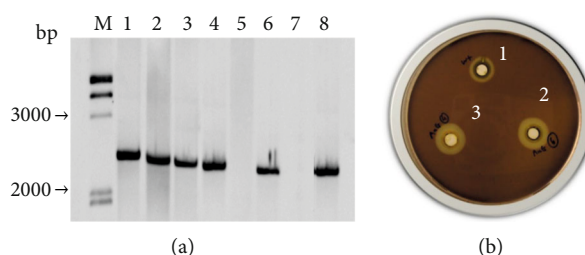


FIGURE 2: (a) Southern blot analysis of the recombinant *xlnG2* in *A. niger* (M: marker; 1-6: recombinant strains; 7: wild-type strain; 8: plasmid pANxlnG2); (b) Xylanase activity (1: wild-type strain; 2 and 3: xylanase *xlnG2* of recombinant *A. niger* VTCC-F017).

promoter *GpdA*, and the reverse primer was used to amplify the xylanase gene. The PCR product had a size of about 1200 bp (Supplement 3C). Recombinant plasmids carrying the gene encoding *xlnG2* were denoted by pANxlnG2.

**3.4. Expression of Xylanase G2 Gene in *Aspergillus niger* VCCT-F017.** Recombinant plasmid pANxlnG2 was cut by restriction enzyme *EheI* and was inserted into the genome at the location of the gene encoding *GluA*. DNA from recombinant fungal strains was amplified by PCR reaction with primers in which forward primer was from promoter *GpdAF* and reverse primer was the primer in the gene coding xylanase G2 (Supplement 4).

For further examination, the DNA from the recombinant strain was cut with the restriction enzyme *XhoI* and examined with Southern blot with the probe of the xylanase G2 gene. According to calculations, when cutting the recombinant DNA with *XhoI*, the segment containing *xlnG2* would be 2700 bp (Figure 2(a)).

After checking the recombinant DNA strain carrying the gene coding xylanase G2, the recombinant and wild-type strains were cultured in the YPD medium. After 120 h of incubation, the recombinant *A. niger* VTCC-017 strains had higher xylanase activity than the wild-type strains (Figure 2(b)).

**3.5. Xylanase Purification.** Xylanase from crude extract obtained after 120 h of culture was purified through the  $\text{Ni}^{2+}$  affinity column. The purified xylanase G2 showed a single protein band of about 21 kDa on SDS-PAGE (Figure 3, lanes 3–6) and revealed a specific activity of  $1102.5 \pm 12.5$  U/mg protein. The yield of purification was 52.6%, and the

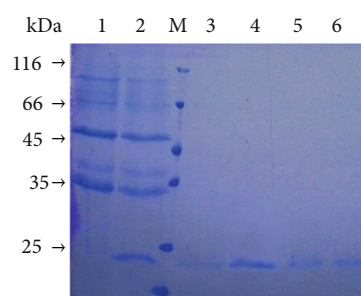


FIGURE 3: Electrophoresis of the xylanase G2: 1: extracellular extract of wild-type strain; 2: extracellular extract of recombinant strain; 3-6: purified xylanase G2; M: marker.

purification factor was 2.2 (Table 1). Family 11 xylanases from *A. oryzae* strain overexpressed in *A. oryzae* showed a molecular mass of 21 kDa [14]. However, this enzyme from *A. niger* strains expressed in *P. pastoris* showed a molecular mass of <21 kDa [24–27]. On the other hand, family 10 endo-1,4- $\beta$ -xylanases expressed in *P. pastoris* from *A. usarii* E001 [28] and *A. terreus* BCC129 [29] showed a molecular mass of >32 kDa. These results were obvious evidence of the diversity in *Aspergillus* xylanase production by different organisms.

### 3.6. Characterization of Recombinant Xylanase

**3.6.1. Optimization of Temperature and pH.** When the reaction temperature increased from 37 to 55°C, the xylanase activity also increased gradually from 56 to 100%. The highest activity of the enzyme was observed at 55°C

TABLE 1: Purification steps of recombinant xylanase G2.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification factor	Yield (%)
Culture supernatant	922.1 ± 8.6	1.84 ± 0.05	501.1 ± 7.8	1	100
Ni <sup>2+</sup> ProBond™ resin column	485.1 ± 13.2	0.44 ± 0.002	1102.5 ± 12.5	2.2	52.6

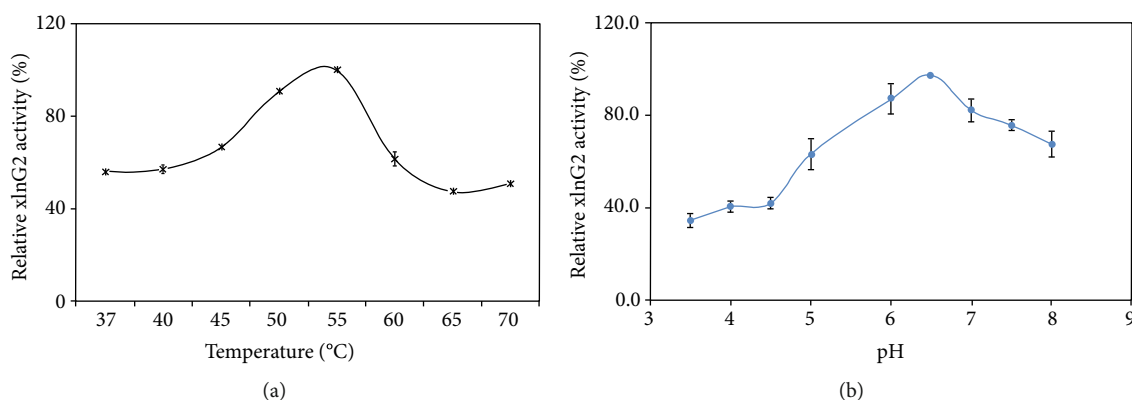


FIGURE 4: (a) Temperatures and (b) pH optimum of recombinant xylanase (the relative activity (%) was expressed as a percentage of optimal activity, and 100% xylanase activity was 1085 ± 36.8 U/mg at (a) 55°C and 852.3 ± 41.3 U/mg at (b) pH 7).

(1085 ± 36.8 IU/mg). When the reaction temperature continued to rise above 55°C, the hydrolysis activity tended to decrease. The optimal temperature for the recombinant xylanase was 55°C (Figure 4(a)).

At pH 4.0, the relative activity of xylanase is 38%. When the reaction pH increases to 6.5, the activity reached the highest point (1031 IU/ml). The residual activity decreased steadily to 50% at pH 9. So the recombinant xylanase activity was optimized in a neutral environment (Figure 4(b)).

The optimal temperature and pH for family 11 *Aspergillus* xylanases were observed at 50–60°C and pH 5–6 [13, 28, 29] while these of xylanase G2 from *A. oryzae* KBN616 were observed at 58°C and pH 6, respectively [14]. The optimal temperature and pH of a recombinant enzyme, purified from *A. niger* XZ-3S, which has a good stability in alkaline conditions, were 40°C and 5.0, respectively [30].

**3.6.2. Temperature and pH Stability.** Enzyme activity at temperatures of 25, 37, and 40°C was not much different (Figure 4(a)), and the overall trend was downward over time. The higher the temperature, the more rapid the enzyme activity loss. After 5 h of incubation at 25 and 40°C, enzyme activity decreased to 86 and 92%, respectively, compared to the initial activity. After 24 h of incubation at 50°C, activity plunged to 53% compared to the initial activity (Figure 5(a)).

Medium pH affects the stability of the enzyme, so it is important to select a suitable pH buffer for enzyme preservation. Xylanase from recombinant strains was relatively stable at pH 4.0–8.0. After 24 h of incubation, the activity remains 78.4% and 75.0%, respectively (Figure 5(b)). Therefore, this enzyme retained its function in both weak alkali and acidic environment. This characteristic of GH11 family xylanase was reported in previous study when the xylanase G2 from

*A. oryzae* KBN616 preserved its activity at pH 4.0–8.0 after incubated for 12 h at 25°C [14].

**3.6.3. Effect of Organic Solvents.** The organic solvent resistance of an enzyme is important to its catalytic capabilities. There are more and more advantages of employing enzymes as catalysts in organic solvents have been reported. Therefore, the search for organic solvent tolerant enzymes has been an extensive area of research. In our study, the effects of several widely used organic solvents such as methanol, ethanol, isopropanol, acetone, and n-butanol on the purified recombinant xylanase G2 were assessed. The purified recombinant xylanase G2 showed a high tolerance to nearly all the tested organic solvents with the residual activity of 62.4–84.6% (Table 2).

The highest tolerance was observed in presence of methanol. The xylanase activity was remained 84.6%, followed by acetone (reached 72.0%). The lowest was the solvent ethanol; the remaining xylanase activity was only 62.4%.

Previous studies also showed the high retained activity of xylanase from *A. awamori* VTCC-F312 63–86% [31] and xlnA from *Pichia pastoris* GS115 [16]. However, methanol and ethanol reduced significantly the activity of xylanase from *A. niger* C3486 [32] and completely inhibited the enzymatic activity from *Termitomyces* sp. [33].

**3.6.4. Effect of Detergents.** Detergents such as Tween 20 and SDS affect xylanase activity. The addition of 2% (v/v) of Tween 20 and SDS reduced the xylanase activity by 27% and 51%, respectively, after 1 h of treatment. Particularly, Tween 80 slightly increased the activity of xylanase 17% while Triton X-100 had no effect on the function of xylanase (Table 3). Our research was also coincident with Hmidasayari et al.'s study that the addition of Tween 80 increased the XAn11 *A. niger* xylanase activity by 33%, and an addition

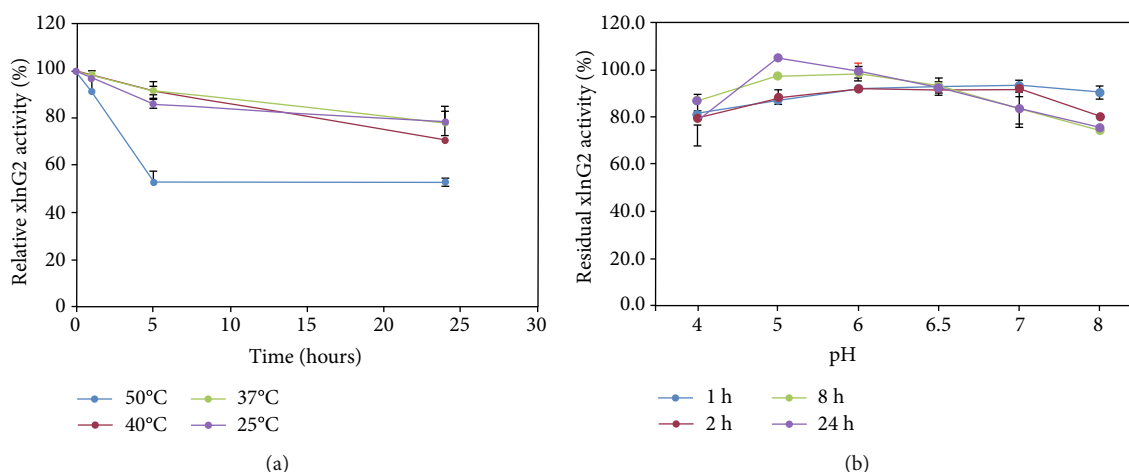


FIGURE 5: (a) Temperature and (b) pH stability of recombinant xylanase (the relative activity (%) was expressed as a percentage of the control reaction at time zero, and 100% xylanase activity was  $921.9 \pm 15.02$  U/mg and  $1097.2 \pm 76.3$  U/mg for temperature and pH stability, respectively).

TABLE 2: Effect of organic solvents on the recombinant xylanase G2 activity.

Organic solvents	Residual activity (%) <sup>a</sup>
MeOH	$84.6 \pm 0.4$
EtOH	$62.4 \pm 4.2$
IsOH	$66.5 \pm 1.7$
Act	$72.0 \pm 3.1$
n-BtOH	$40.8 \pm 2.7$

<sup>a</sup>Relative activity was expressed as a percentage of the control reaction without any additive (100% xlnG2 activity was  $927.21 \pm 38.3$  U/mg).

TABLE 3: Effect of detergents on the recombinant xylanase G2 activity.

Detergents	Residual activity (%) <sup>a</sup>
Triton X-100	$103 \pm 5.6$
Tween 80	$117 \pm 0.7$
Tween 20	$73 \pm 2.8$
SDS	$49 \pm 6.9$

<sup>a</sup>Relative activity was expressed as a percentage of the control reaction without any additive (100% xlnG2 activity was  $900.12 \pm 38.3$  U/mg).

of SDS strongly inhibited the enzyme with residual activity of 20% comparing to the initial activity [34].

**3.7. Effect of Metal Ions.** The effect of various metal ions and EDTA on the purified xylanase activity was investigated. After 2 h of incubation at the concentration of 10 mM, only  $Hg^{2+}$  completely inhibited enzyme activity while  $Mg^{2+}$ ,  $Ca^{2+}$ , and  $Ni^{2+}$  slightly reduced the enzyme activity. The presence of  $Fe^{2+}$ ,  $Zn^{2+}$ , and  $Ag^+$  partially reduced enzyme activity by 56, 57, and 57%, respectively (Table 4). On the other hand,  $K^+$  and EDTA slightly stimulated xylanase activity. The inhibition or stimulation of enzyme activity may be due to metal ions interacted with SH or carboxyl groups which led to an altered conformation of protein subsequent inactivation [35].  $Hg^{2+}$  is

TABLE 4: Effect of metal ions and EDTA on the recombinant xylanase G2 activity.

Additive (10 mM)	Residual activity (%) <sup>a</sup>
$Mg^{2+}$	$96 \pm 1.1$
$Fe^{2+}$	$44 \pm 5.8$
$Cu^{2+}$	$53 \pm 7.7$
$Ca^{2+}$	$99.6 \pm 13.5$
$Ag^+$	$43 \pm 9.1$
$Ni^{2+}$	$96 \pm 3.1$
$K^+$	$101 \pm 0.1$
EDTA	$106 \pm 10.4$
$Zn^{2+}$	$43 \pm 5.3$
$Hg^{2+}$	$0 \pm 0.0$

<sup>a</sup>Relative activity was expressed as a percentage of the control reaction without any additive (100% xlnG2 activity was  $881.0 \pm 65.4$  U/mg).

a strong inhibitor of most xylanases, including those produced by *Aspergillus* sp., such as *Aspergillus giganteus* [36], *Aspergillus awamori* [37], and *Aspergillus niger* [38]. The inhibition of xylanase by  $Hg^{2+}$  has been reported as related to the presence of histidine and tryptophan residues, which oxidize the indole ring, thereby inhibiting the enzyme activity. In addition, the inhibition by heavy metal ions (such as  $Zn^{2+}$  and  $Hg^{2+}$ ) may also occur due to the formation of a complex with the reactive groups of the enzyme, such as SH,  $CONH_2$ ,  $NH_2$ ,  $COOH$ , and  $PO_4$  or the catalysis of the cysteine thiol group auto-oxidation, which leads to the formation of intra- and intermolecular disulfide bonds or to the formation of sulfenic acid [39].

However, in our study, the addition of chelating agents, EDTA, did not affect xylanase activity. These data suggest that sulfhydryl groups are not related to the active site of the purified xylanase. Therefore, histidine and tryptophan might play an important role in the active site of the purified xylanase. Further structural studies are needed to confirm this hypotheses.



Some xylanases require  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  for their stability and activities, such as xylanase from *A. Niger* SCTCC 400264 [22], *A. niger* US368 [34], and *A. niger* BCC14405 [9]. However, in our study,  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  had no significant effects on xylanase activity. These results suggested that these metal ions do not play important role in the activation and protection of the active site of the purified enzyme.

#### 4. Conclusion

In this study, cloning of the gene encoding xylanase G2 from *A. oryzae* was performed successfully. The expression of recombinant xylanase G2 in *A. niger* was successful. The xylanase G2 activity reached 1025 IU/mg. Xylanase G2 displayed its optimal activity at 55°C, pH 6.5. This enzyme was stable at the temperatures ranging from 25 to 40°C, pH 5-7. Metal ions like  $\text{K}^+$  and EDTA slightly increased xylanase activity. On other hand, the purified recombinant xylanase was inhibited by Tween 20, SDS, heavy metal ions such as  $\text{Ag}^+$  and  $\text{Hg}^{2+}$ , and organic solvents. Tween 80 increased, and Triton X-100 had no effect on xylanase activity.

#### Data Availability

The nucleotide sequence is determined by the Sanger method, on the ABI PRISM 3100 Avant Genetic Analyzer. Nucleotide sequence analysis is performed by the DNASTar software.

#### Conflicts of Interest

The authors declare that they have no competing interests.

#### Authors' Contributions

DTT designed the experimental setup and assisted with data analysis and manuscript preparation. NTT and NSLT performed the experiments of cloning and purification of xylanase G10. NTT and NTHT performed and characterized the recombinant xylanase. LTH and NTC evaluated the data analysis and characterization of xylanase G2. DTT, DTMA, and NTC conducted the methodology verification, draft review, data validation, and manuscript editing. All authors read and approve the final manuscript.

#### Acknowledgments

This study was supported by the National Foundation for Science and Technology Development Vietnam (Nafosted), project 106.02-2018.347 "Engineering of recombinant *Aspergillus niger* to produce highly active xylanase for functional foods industry" 2019-2021.

#### Supplementary Materials

Supplement 1. The agarose gel electrophoresis of total RNA (A); PCR product *xlnG2* (B); PCR product from the with foreign gene (C). Supplement 2. PCR gene *xlnG2* product (A); Cutting plasmid with *Bam*HI: 1 and 2 are control, 3-8 are samples. (B). Supplement 3. (A), DNA electrophoresis prod-

ucts; pGEMT/*xlnG2/Bam*HI, (B) Purified *xlnG2* gene (C) PCR product from plasmid pAN*xlnG2*. Supplement 4. PCR products with DNA templates of recombinant strain and wild-type strain (A) M: Marker; 1-6, DNA template from recombinant strains; 7: wild-type strain; 8: from plasmid pAN*xlnG2*; (B) PCR products with primer pair *xlnG2F/xlnG2R* (*Supplementary Materials*)

#### References

- [1] M. Ayyachamy and T. M. Vasala, "Production and partial characterization of cellulase free xylanase by *Bacillus subtilis* C 01 using agriresidues and its application in biobleaching of nonwoody plant pulps," *Letters in Applied Microbiology*, vol. 45, no. 5, pp. 467-472, 2007.
- [2] M. L. Polizeli, A. C. Rizzatti, R. Monti, H. F. Terenzi, J. A. Jorge, and D. S. Amorim, "Xylanases from fungi: properties and industrial applications," *Applied Microbiology and Biotechnology*, vol. 67, no. 5, pp. 577-591, 2005.
- [3] R. J. Bernier, M. Desrochers, L. Jurasek, and M. G. Paice, "Isolation and characterization of a xylanase from *Bacillus subtilis*," *Applied and Environmental Microbiology*, vol. 46, no. 2, pp. 511-514, 1983.
- [4] R. Khandeparker and M. T. Numan, "Bifunctional xylanases and their potential use in biotechnology," *Journal of Industrial Microbiology and Biotechnology*, vol. 35, no. 7, pp. 635-644, 2008.
- [5] M. Mendis, E. Leclerc, and S. Simsek, "Arabinoxylans, gut microbiota and immunity," *Carbohydrate Polymers*, vol. 139, pp. 159-166, 2016.
- [6] S. I. Ooi, S. C. Pak, P. S. Micalos et al., "Rice bran arabinoxylan compound and quality of life of cancer patients (RBAC-QoL): study protocol for a randomized pilot feasibility trial," *Contemporary Clinical Trials Communications*, vol. 19, p. 100580, 2020.
- [7] M. A. Mendez-Encinas, E. Carvajal-Millan, A. Rascon-Chu, H. F. Astiazaran-Garcia, and D. E. Valencia-Rivera, "Ferulated arabinoxylans and their gels: functional properties and potential application as antioxidant and anticancer agent," *Oxidative Medicine and Cellular Longevity*, vol. 2018, Article ID 2314759, 22 pages, 2018.
- [8] D. Haltrich, B. Nidetzky, K. D. Kulbe, W. Steiner, and S. Župančič, "Production of fungal xylanases," *Bioresource Technology*, vol. 58, no. 2, pp. 137-161, 1996.
- [9] A. Krisana, S. Rutchadaporn, G. Jarupan, E. Lily, T. Sutipa, and K. Kanyawim, "Endo-1,4-beta-xylanase B from *Aspergillus cf. niger* BCC14405 isolated in Thailand: purification, characterization and gene isolation," *Journal of Biochemistry and Molecular Biology*, vol. 38, no. 1, pp. 17-23, 2005.
- [10] F. Lu, M. Lu, Z. Lu, X. Bie, H. Zhao, and Y. Wang, "Purification and characterization of xylanase from *Aspergillus ficuum* AF-98," *Bioresource Technology*, vol. 99, no. 13, pp. 5938-5941, 2008.
- [11] W. Panbangred, E. Fukusaki, E. C. Epifanio, A. Shinmyo, and H. Okada, "Expression of a xylanase gene of *Bacillus pumilus* in *Escherichia coli* and *Bacillus subtilis*," *Applied Microbiology and Biotechnology*, vol. 22, pp. 259-264, 1985.
- [12] X. L. Li, Z. Q. Zhang, J. F. Dean, K. E. Eriksson, and L. G. Ljungdahl, "Purification and characterization of a new xylanase (APX-II) from the fungus *Aureobasidium pullulans* Y-2311-1," *Applied and Environmental Microbiology*, vol. 59, no. 10, pp. 3212-3218, 1993, -8, 1993.



- [13] T. Kimura, H. Suzuki, H. Furuhashi et al., "Molecular cloning, characteriation, and expression analysis of the xynF3 gene from *Aspergillus oryzae*," *Bioscience Biotechnology and Biochemistry*, vol. 66, no. 2, pp. 285–292, 2002.
- [14] T. Kimura, H. Suzuki, H. Furuhashi et al., "Molecular cloning, overexpression, and purification of a major xylanase from *Aspergillus oryzae*," *Bioscience Biotechnology and Biochemistry*, vol. 64, no. 12, 2000.
- [15] N. N. Peij, J. Visser, and L. H. Graaff, "Isolation and analysis of XlnR, encoding a transcriptional activator co-ordinating xylanolytic expression in *Aspergillus niger*," *Molecular Microbiology*, vol. 27, no. 1, pp. 131–142, 1998.
- [16] T. T. Do, D. T. Quyen, T. N. Nguyen, and V. T. Nguyen, "Molecular characterization of a glycosyl hydrolase family 10 xylanase from *Aspergillus niger*," *Protein Expression and Purification*, vol. 92, no. 2, pp. 196–202, 2013.
- [17] D. T. Quyen, T. T. Nguyen, T. T. G. Le, H. K. Kim, T. K. Oh, and J. K. Lee, "A novel lipase/chaperone pair from *Ralstonia sp.* M1: analysis of the folding interaction and evidence for gene loss in *Ralstonia solanacearum*," *Molecular Genetics and Genomics*, vol. 272, pp. 538–554, 2004.
- [18] G. L. Miller, "Use of dinitrosalicylic acid reagent for determination of reducing sugars," *Analytical Chemistry*, vol. 31, no. 3, pp. 426–428, 1959.
- [19] U. K. Laemmli, "Cleavage of structure proteins during the assembly of the head of bacteriophage T4," *Nature*, vol. 227, no. 5259, pp. 680–685, 1970.
- [20] M. M. Bradford, "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding," *Analytical Biochemistry*, vol. 72, no. 1–2, pp. 248–254, 1976.
- [21] T. Kimura, N. Kitamoto, Y. Kito, S. Karita, K. Sakka, and K. Ohmiya, "Molecular cloning of xylanase gene xynGI from *Aspergillus oryzae* KBN 616, a shoyu koji mold, and analysis of its expression," *Journal of Fermentation and Bioengineering*, vol. 85, no. 1, pp. 10–16, 1998.
- [22] X. Yi, Y. Shi, H. Xu et al., "Hyperexpression of two *Aspergillus niger* xylanase genes in *Escherichia coli* and characterization of the gene products," *Brazilian Journal of Microbiology*, vol. 41, no. 3, pp. 778–786, 2010.
- [23] S. L. T. Nguyen and D. T. Quyen, "Cloning, sequence analysis of a gene encoding xylanase from *Aspergillus niger* DSM1957," in *The National Conference on Biotechnology 2009*, pp. 701–704, Thainguyen University Publisher, Thainguyen, 2009.
- [24] J. G. Berrin, G. Williamson, A. Puigserver, J. C. Chaix, W. R. McLauchlan, and N. Juge, "High-level production of recombinant fungal endo- $\beta$ -1,4-xylanase in the methylotrophic yeast *Pichia pastoris*," *Protein Expression and Purification*, vol. 19, no. 1, pp. 179–187, 2000.
- [25] P. Deng, D. Li, Y. Cao, W. Lu, and C. Wang, "Cloning of a gene encoding an acidophilic endo- $\beta$ -1,4-xylanase obtained from *Aspergillus niger* CGMCC1067 and constitutive expression in *Pichia pastoris*," *Enzyme and Microbial Technology*, vol. 39, no. 5, pp. 1096–1102, 2006.
- [26] M. Q. Liu, X. Y. Weng, and J. Y. Sun, "Expression of recombinant *Aspergillus niger* xylanase A in *Pichia pastoris* and its action on xylan," *Protein Expression and Purification*, vol. 48, no. 2, pp. 292–299, 2006.
- [27] V. Ruanglek, R. Sriprang, N. Ratanaphan et al., "Cloning, expression, characterization, and high cell-density production of recombinant endo-1,4- $\beta$ -xylanase from *Aspergillus niger* in *Pichia pastoris*," *Enzyme and Microbial Technology*, vol. 41, no. 1–2, pp. 19–25, 2007.
- [28] J. Q. Wang, X. Yin, M. C. Wu et al., "Expression of a family 10 xylanase gene from *Aspergillus usamii* E001 in *Pichia pastoris* and characterization of the recombinant enzyme," *Journal of Industrial Microbiology & Biotechnology*, vol. 40, no. 1, pp. 75–83, 2013.
- [29] D. Chantasingh, K. Pootanakit, V. Champreda, P. Kanokratana, and L. Eurwilaichitr, "Cloning, expression, and characterization of a xylanase 10 from *Aspergillus terreus* (BCC129) in *Pichia pastoris*," *Protein Expression and Purification*, vol. 46, no. 1, pp. 143–149, 2006.
- [30] G. Fu, Y. Wang, D. Wang, and C. Zhou, "Cloning, expression, and characterization of an GHF 11 xylanase from *Aspergillus niger* XZ-3S," *Indian Journal of Microbiology*, vol. 52, no. 4, pp. 682–688, 2012.
- [31] T. T. Do, D. T. Quyen, and T. H. Dam, "Purification and characterization of an acid-stable and organic solvent-tolerant xylanase from *Aspergillus awamori* VTCC-F312," *ScienceAsia*, vol. 38, pp. 157–165, 2012.
- [32] Y. Yang, Z. Wei, J. Huang et al., "Purification and characterization of an extracellular xylanase from *Aspergillus niger* C3486," *Africa Journal of Microbiology Research*, vol. 4, no. 21, pp. 2249–2256, 2010.
- [33] B. M. Faulet, S. Niamke, J. T. Gonnety, and L. P. Kouamé, "Purification and biochemical properties of a new thermostable xylanase from symbiotic fungus, *Termitomyces sp.*," *Africa Journal of Biotechnology*, vol. 5, no. 3, pp. 273–282, 2006.
- [34] A. Hmida-Sayari, S. Taktek, F. Elgharbi, and S. Bejar, "Biochemical characterization, cloning and molecular modeling of a detergent and organic solvent-stable family 11 xylanase from the newly isolated *Aspergillus niger* US368 strain," *Process Biochemistry*, vol. 47, no. 12, pp. 1839–1847, 2012.
- [35] P. R. Heinen, C. Henn, R. M. Peralta et al., "Xylanase from *Fusarium heterosporum*: properties and influence of thiol compounds on xylanase activity," *Africa Journal of Biotechnology*, vol. 13, no. 9, pp. 1047–1055, 2014.
- [36] M. B. Fialho and E. C. Carmona, "Purification and characterization of xylanases from *Aspergillus giganteus*," *Folia Microbiologica (Praha)*, vol. 49, no. 1, pp. 13–18, 2004.
- [37] M. A. Umsza-Guez, A. B. Diaz, I. de Ory, A. Blandino, E. Gomes, and I. Caro, "Xylanase production by *Aspergillus awamori* under solid state fermentation conditions on tomato pomace," *Brazilian Journal of Microbiology*, vol. 42, no. 4, pp. 1585–1597, 2011.
- [38] H. G. Chen, X. Yan, X. Y. Liu et al., "Purification and characterization of novel bifunctional xylanase, xynIII, isolated from *Aspergillus niger* A-25," *Journal of Microbiology and Biotechnology*, vol. 16, no. 7, pp. 1132–1138, 2016.
- [39] D. Verma, Y. Kawarabayasi, K. Miyazaki, and T. Satyanarayana, "Cloning, expression and characteristics of a novel alkalistable and thermostable xylanase encoding gene (Mxl) retrieved from compost-soil metagenome," *PLoS One*, vol. 8, no. 1, article e52459, 20132013.