Research Article

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

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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 BamHI. 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 Hg2+ and partially by Zn2+, Fe2+, and Ag+, while it was slightly stimulated by K+ 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-β-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 positive 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,*
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 (xhnG2).

Escherichia coli DH5α (F−, ϕ80d lacZDM15, DlacZYA-argF)U169, deoR, recA1, endA1, hsdR17 (rk=−, mk+), phoA, supE44, k, 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 μg 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 complex medium (CM) contains NaNO₃ 0.6, KCl 0.52, KH₂PO₄ 1.52, MgSO₄·7H₂O 0.52, sucrose 2, and 1 mL MNS. Complete media (CM) contain 10 mL solution A (Ca(NO₃)₂·4H₂O 10%), 10 mL solution B (KH₂PO₄ 2 g/L, MgSO₄·7H₂O 2.5 g/L, NaCl 1.5 g/L, and pH 5.3), yeast-casein 0.2/L, glucose 1 g/L, 1 mL MNS, and 900 mL H₂O. 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 xhnG2 available in GenBank, two oligonucleotides xhnG2F (5′-CG GGATCCATGTTGCTTCTCCTCCATCCC-3′) and xhnG2R (5′-CG GGATCCATGTTGCTTCTCCTCCATCCC-3′) were designed as primers to amplify the xhnG2 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-stand cDNA product was used to amplify xhnG2 using two primers xhnG2F and xhnG2R.

The PCR mixture contained 2.5 μL of 10 times PCR buffer, 2 μL of 2.5 mM dNTP, 1.5 μL of 25 mM MgCl₂, 2 μL of cDNA (50 ng) from control RT reaction, 0.5 μL of 5 unit Taq polymerase, and 1 μL of each primer (10 pmol), supplemented with 14.5 μL of distilled water to a final volume of 25 μL. 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 pJxhnG2 and transformed into the E. coli DH5α. The recombinant plasmid was confirmed by restriction enzyme analysis and DNA sequencing.

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 ProBond™ Nickel-chelating resin was from Invitrogen Corp. (Carlsbad, CA, USA). 3,5,5-Dinitrosaliislic acid (DNS), birchwood xylan, and SDS were from Sigma-Aldrich Co. (St. Luis, USA). Peptone, yeast extract, Tween 80, and Tween 20 were from BioBasic Inc. (Ontario, Canada). Triton X-100
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-funnel (100 μm), and washed 2 times with distilled water. The weight of the hyphae mixture was determined by measuring the concentration of reducing distill 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 been 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 fluorography.

2.7. DNA Isolation from A. niger. A square of 0.5 cm² 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 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 CaCl₂ 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).

2.8. 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 been 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 fluorography.

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 xlgG2 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™ Ni²⁺ affinity chromatography column containing 2 mL resin, which had been equilibrated with native binding buffer (250 mM NaH₂PO₄, 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 NaH₂PO₄, 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 NaH₂PO₄, 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 μL of the crude or purified xylanase containing 11 μg for each reaction was incubated with 400 μL 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 DNA was added. The reducing sugars were determined by measuring the absorbance at 540 nm [18]. D-xylene was used as a standard. One unit (U) of xylanase activity was defined as the amount of enzyme that released 1 mol of xylene 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+}, 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 DH5α. 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 DH5α. Recombinant plasmids carrying the gene encoding xlnG2 were denoted by pXlnG2.

3.2. Xylanase Gene Sequence Analysis. After nucleotide sequencing, the putative sequence of xylanase G2 gene in GenBank 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 hydroxidase family 11, which was completely similar to the xlnG2 (767 bp, 232 aa) (glycosyl hydroxidase family 11) [14], A. oryzae xlnG1 (725 bp, 189 aa) [21], xlnC (636 bp, 211 aa) [22], xlnA (glycosyl hydroxidase 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 pfXlnG2 by PCR reaction with specific primers containing BamHI 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 promoter GPDA from A. nidulans and a BamHI site was used. Plasmids containing the xylanase gene were cut by BamHI (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 DH5α.

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
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 
Ehe

I 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 
Xho

I and examined with Southern blot with the probe of the xylanase G2 gene. According to calculations, when cutting the recombinant DNA with 
Xho

I, 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 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 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-β-xylanases expressed in P. pastoris from A. usamii 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.
xylanases were observed at 50°C. 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 those 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 alkaline 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 Termittomyces 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 Hmida-Sayari et al.’s study that the addition of Tween 80 increased the XAn11 A. niger xylanase activity by 33%, and an addition

**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       |
| Ni²⁺ProBond™ resin column | 485.1 ± 13.2      | 0.44 ± 0.002       | 1102.5 ± 12.5            | 2.2                 | 52.6      |

![Figure 4](image-url)
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 2h 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 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 sulphydryl 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 Ca\(^{2+}\) and Mg\(^{2+}\) for their stability and activities, such as xylanase from \textit{A. Niger} SICTCC 400264 [22], \textit{A. niger} US368 [34], and \textit{A. niger} BCC14405 [9]. However, in our study, Mg\(^{2+}\) and Ca\(^{2+}\) had no significantly 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 \textit{A. oryzae} was performed successfully. The expression of recombinant xylanase G2 in \textit{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 able at the temperatures ranging from 25 to 40°C, pH 5-7. Metal ions like 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 Ag\(^{+}\) and 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. The 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 NTH 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.

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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 BamHI: 1 and 2 are control, 3-8 are samples. (B). Supplement 3. (A), DNA electrophoresis products; pGEMT/xlnG2/BamHI, (B) Purified xlnG2 gene (C) PCR product from plasmid pANxlnG2. 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 pANxlnG2; (B) PCR products with primer pair xlnG2F/xlnG2R (Supplementary Materials)

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