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Cloning, purification, and characterization of recombinant endo-β-1,4-D-xylanase of *Bacillus* sp. From soil termite abdomen

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ABSTRACT

A novel endo-β-1,4-D-xylanase (*xynBT*) was identified from *Bacillus* sp. in soil termite abdomen and successfully cloned in *Escherichia coli* TOP10 and expressed in *Escherichia coli* BL21 (DE3) via pET-30a (+) as an expression vector. The full length gene consist of 801 bp ORF encoding a 267 amino acid polypeptide. The deduced amino acid sequence of *xynBT* displayed homology with glycoside hydrolase (GH) family 11 xylanase. Recombinant XynBT (r-XynBT), which was purified, showed an optimal pH and temperature of 5.5 and 40 °C, respectively. This enzyme was purified by the Immobilized Metal Affinity Chromatography (IMAC) method and has a molecular mass of 30 kDa, which was observed via sodium dodecyl polyacrylamide sodium electrophoresis (SDS-PAGE). Purified r-XynBT was the most stable at pH 5 for up to 120 min pre-incubation time and had a residual activity of 83%. Purified r-XynBT was also stable between 30 and 40 °C for 80 min of pre-incubation and had a residual activity of more than 50%. The presence of metal cations K^+ and Na^+ on r-XynBT increased its activity, while metal cations Mg^{2+}, Cu^{2+}, Zn^{2+}, and Fe^{3+} were inhibitors.

1. Introduction

Xylan is a polysaccharide in plant cells with complex highly branched heteropolysaccharides and has a linear backbone consisting of β-1,4-D-xylopyranoside residues (Collins et al., 2005). Xylan, which is the main element in hemicellulose, is the second most abundant resource in the world after cellulose (Fuzi et al., 2012; Wang et al., 2007). The complete hydrolysis of xylan requires the synergistic action of several hydrolytic enzymes due to its complex chemical structure. Among several hydrolytic enzyme, endo-β-1,4-D-Xylanase (β-1, 4-D-xylanase, EC 3.2.1.8) is the most important. This enzyme hydrolyzes the β-1,4-glycosidic bonds in the xylan backbone into short xylooligosaccharides (XOS) (Wang et al., 2010). The produced xylooligosaccharides have great potential in the health sector due to their prebiotic activity and the ability to reduce the risk of cancer (Va'zquez et al., 2000).

Classification systems based on the analysis of hydrophobic groups in terms of the catalytic domain and similarities in amino acid sequences have made the GH10 (F/10E) and GH 11 (G/11) families well-studied groups. GH10 and GH11 family members differ in their physicochemical properties, structure, mode of action, and substrate specificity (Liew et al., 2019; Motta et al., 2013). Compared to other GH families, family 11, as "true xylanases", is monospecific: it is only active on substrates containing D-xylose (Moukouli et al., 2011).

Endo-β-1,4-D-Xylanase is derived from many microorganisms, including bacteria, filamentous fungi, yeast, and actinomycetes. The microbial origin of xylanase has a broad application in food materials, bioethanol, waste treatment, and paper industries (Chen et al., 2019; Polizeli et al., 2005; Wang et al., 2010). At the same time, plant biomass is an abundant and universal renewable raw material for the formation of fresh bioproducts and biofuels (Michelin et al., 2011). This is in line with overall practices in which agricultural wastes that can be converted

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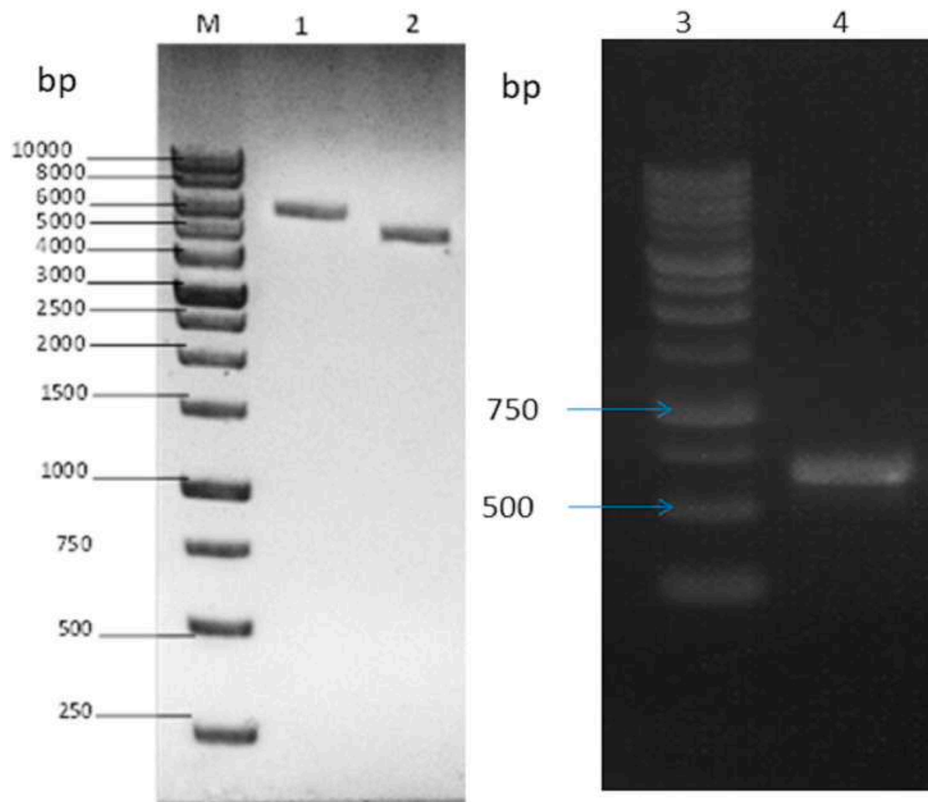

Fig. 1. Agarose gel electrophoresis of recombinant DNA. Lane M & 3: DNA marker 1 kb ladder; lane 1: *pET-xynBT*; lane 2: pET-30 (+); lane 4: PCR product.

via the fermentation process using microbes are typical modes of obtaining better by-products (Rajoka et al., 2012).

At the previous study, endo-β-1,4-D-Xylanase was isolated from *Bacillus* sp in soil termite abdomens (Ratnadewi et al., 2007). This enzyme belongs to the *Bacillus* sp group based on the phylogenetic tree of the 16s rRNA sequence previously determined by Basic Local Alignment Search Tool (BLAST) (Ratnadewi et al., 2013b). The enzyme produced activity with oat spelt xylan, arabinofuranoside, and glucuronide substrates. This enzyme can hydrolyze cassava pulp and coffee husks to produce XOS with 2, 3, 4, and 5 degrees of polymerization (Ratnadewi et al, 2016, 2019). Moreover, it is believed to produce XOS from cassava waste as a substrate. The prebiotic properties of produced XOS were tested in vitro and in vivo by observing the growth of the *Lactobacillus probiotic* bacteria. These bacterial growths were increasing and suppressing the growth of *Escherichia coli* (Ratnadewi et al., 2020).

Until now, many types of xylanases have been cloned and expressed (Chen et al., 2019; Liew et al., 2019; Moukouli et al., 2011; Polizeli et al., 2005). Typically, he superior activity and properties of heterologous hosts establish the successful expression of recombinant xylanases (Zhang et al., 2012). This research will conduct cloning, overexpression, and endoxylanase enzyme characteristics to determine the basic features of xylanase—specific activity, pH, temperature, pH and temperature stability, and resistance to cations—to help elucidate its many applications. While many researchers have reported the success of some xylanase genes cloned and expressed in a heterologous host (Bilgin et al., 2018; Jalal et al., 2009; Sriyapai et al., 2011; Zafar et al., 2016; Zhou et al., 2010), this is the first investigation into cloning the xylanase gene origin from *Bacillus* sp in soil termite abdomens using gene analysis and amino acid sequencing.

2. Materials and methods

2.1. Materials

An isolate from *Bacillus* sp bacteria from the soil termite abdominal system was obtained from 16s rRNA analysis (Ratnadewi et al., 2013b) and cultivated in a Luria-Bertani medium at 37 °C. *E. coli* TOP 10 (Invitrogen, Massachusetts-USA) and *E. coli* BL21 (DE3) (Promega, San Luis Obispo-US) were used for cloning and overexpression, respectively. The plasmid pET-30a (+) was obtained from Novagen (USA), and the oat xylan substrate was obtained from Sigma, USA. Taq DNA Polymerase was obtained from TOYOBO (Tokyo, Japan), and T4 DNA Ligase was obtained from Invitrogen (Massachusetts, USA).

2.2. Cloning and sequencing of the xynBT gene

The recombinant DNA technique was used as described by Sambrook (Sambrook and Russell, 2001). The gene sequence was amplified via a polymerase chain reaction (PCR). The nucleotide sequence of the gene with the accession number M36648 was used for the primer design. The primer design strategy based on a conserved sequence region for the amplification of the GH 11 xylanase gene from *Bacillus subtilis* (Gallardo et al., 2004; Helianti et al., 2008; Huang et al., 2006). The primers were designed for gene amplification without a signal peptide. The specific primers included a forward primer that contained a *SacI* restriction and a reverse primer that contained a *XhoI* restriction site. The primers forward were 5′- GGGGAGCTCATGTTTAAGTTTAAAAAGAATTTCTTAGTT -3′ and 5′- GCCTCGAGTTACCACACTGTTAC -3′ in reverse (restriction sites are underlined).

PCR was carried out using chromosomal DNA from *Bacillus* sp as a template and Taq DNA Polymerase from KOD FX Neo. The PCR conditions used were denaturation at 94 °C for 1 min, followed by 35 cycles at

```
gtt tgt aaa agt att tca att tcc cct ctt aga aat aat ttt gtt taa ctt taa gaa gga
gat ata cat atg cac cat cat cat cat cat tct tct ggt ctg gtg cca cgc ggt tct ggt
             M   H   H   H   H   H   H   S   S   G   L   V   P   R   G   S   G
atg aaa gaa acc gct gct gct aaa ttc gaa cgc cag cac atg gac agc cca gat ctg ggt
 M   K   E   T   A   A   A   K   F   E   R   Q   H   M   D   S   P   D   L   G
                                                             xynF
acc gac gac gac gac aag gcc atg gct gat atc gga tcc gaa ttc gag ctc atg ttt aag
 T   D   D   D   D   K   A   M   A   D   I   G   S   E   F   E   L   M   F   K
ttt aaa aag aat ttc tta gtt gga tta tcg gca gct tta atg agt att agc ttg ttt tcg
 F   K   K   N   F   L   V   G   L   S   A   A   L   M   S   I   S   L   F   S
gca acc gtc tct gca gct agc aca gac tac tgg caa aat tgg act gat ggg ggc ggt ata
 A   T   V   S   A   A   S   T   D   Y   W   Q   N   W   T   D   G   G   G   I
gta aac gct gtc aat ggg tct ggc ggg aat tac agt gtt aat tgg tct aat acc gga aat
 V   N   A   V   N   G   S   G   G   N   Y   S   V   N   W   S   N   T   G   N
ttt gct gtt ggt aaa ggt tgg act aca ggt tcg cca ttt agg acg ata aac tat aat gcc
 F   A   V   G   K   G   W   T   T   G   S   P   F   R   T   I   N   Y   N   A
gga gtt tgg gca ccg aat ggc aat gga tat tta act tta tat ggt tgg acg aga tca ccg
 G   V   W   A   P   N   G   N   G   Y   L   T   L   Y   G   W   T   R   S   P
ctc ata gaa tat tat gta gtg gat tca tgg ggt act tat aga cct act gga acg tat aag
 L   I   E   Y   Y   V   V   D   S   W   G   T   Y   R   P   T   G   T   Y   K
ggt act gta aaa agt gat ggg ggt aca tat gac ata tat aca act aca cgt tat aac gca
 G   T   V   K   S   D   G   G   T   Y   D   I   Y   T   T   T   R   Y   N   A
cct tcc att gat ggc gat cgc act act ttt acg cag tac tgg agt gtt cgc cag tcg aag
 P   S   I   D   G   D   R   T   T   F   T   Q   Y   W   S   V   R   Q   S   K
aga cca acc gga agc aac gct aca atc act ttc agc aat cat gtg aac gca tgg aag agc
 R   P   T   G   S   N   A   T   I   T   F   S   N   H   V   N   A   W   K   S
cat gga atg aat ctg ggc agt aat tgg gct tac caa gtc atg gcg aca gaa gga tat caa
 H   G   M   N   L   G   S   N   W   A   Y   Q   V   M   A   T   E   G   Y   Q
agt ggt gga agt tct aac gta aca gtg tgg taa ctc gag cac cac cac cac cac cac tga
                                    xynR
 S   G   G   S   S   N   V   T   V   W
gat ccg gct gct aca aag ccc gaa agg aag ctg agt tgg ctg ctg cca ccg ctg agc ata
act agc ata acc cct tgg gcc tct aaa cgg gtc ttg agg ggt ttt ttg ctg aag gag gaa
cta tat ccg gat tgg cga atg gga cgc gcc ctg tag cgg cgc att aag cgg cgg cgg gtg
tgg tgg tta cgc gca gcg tga cgc tta cac ttg cca agc gac cta gcg tcg ctc ttt ccg
ctt tct ttc cct cct ttc tac gtc acg tcg cgc ctt ccc ggt caa agc tct aat cgg ggc
tcg ata agg atc cga tat gct tta cga acc tcg atc cca aaa ctg aat tag ggt gag tgt
cta cac gga tgt gct
```

Fig. 2. Nucleotide sequence of the full-length *pET-xynBT* from *Bacillus* sp. soil termite abdomen and the deduced amino acid sequence of r-XynBT. The *grey italic letters ATG* and *TAA* represent the initiation codon and stop codon, respectively. The *bold arrows* below the letters represent the primers for the PCR amplification: forward primer (xynF) and reverse primer (xynR).

Fig. 3. Phylogenetic tree of endoxylanase GH family. GHs are represented by their family number followed by their Protein Data Bank (PDB) or GenBank accession number.

62, 60, and 58 °C for 30 s, and 72 °C for 1 min. The PCR product was purified (GenepHlowTM Gel/PCR Quick Kit) and ligated into the pET-30a (+) vector, forming a recombinant plasmid; *pET-xynBT* then was transformed into competent *E. coli* TOP 10. Colonies that appeared on the plate were randomly marked, and PCR colonies confirmed the positive clones for sequencing.

2.3. Analysis of sequence data

The recombinant clones were sequenced by the Bioneer Sequencing Team, Republic of Korea, and the sequence homology analysis was performed by the Basic Local Alignment Search Tool (BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple sequence alignments were conducted via the Clustal W program, and the signal peptide was analyzed by SignalP (http://www.cbs.dtu.dk/services/SignalP/). The isoelectric point (pI) and molecular weight were predicted by EXPASY (http://www.expasy.org). A Phylogenetic tree was constructed using MEGA-X software.

2.4. Expression and purification of recombinant XynBT (r-XynBT)

Recombinant plasmids with confirmed sequences were used for transforming the *E. coli* BL21 (DE3) cells using the heat shock method (Sambrook et al., 1989). Positive transformant selection was performed on a Luria Bertani (LB) medium contain kanamycin (50 μg/mL) at 37 °C. Induction protein expression was calculated using 0.2 mM/250 mL isopropyl-thio-β-D-thiogalactopyranoside (IPTG when the OD_{600} nm reached 0.6, and the culture was incubated for 16 h (Moayad et al., 2017). The cells were harvested via centrifugation and disrupted by sonication in a10 mM phosphate buffer with a pH of 7.5 containing 10 mM imidazole and 50 mM NaCl (Sittipol et al., 2019). All cell lysate was centrifuged, and the sample was purified using the Immobilized Metal Affinity Chromatography (IMAC) method (GE Healthcare Life Sciences). Proteins were eluted by imidazole at a concentration gradient 30, 60, 100, and 300 mM (Ratnadewi et al., 2013a). Pure protein was verified via SDS-PAGE analysis (Laemmli, 1970). Low-weight protein markers (GE Healthcare Life Sciences) were used as a standard.

2.5. Protein concentration and enzyme assay

Protein quantification was determined using the Bradford method (Bradford, 1976), and a calibration curve was made with bovine serum albumin. The r-XynBT activity was determined based on the concentration of reducing sugars released per minute as μmol min^{-1} (Miller, 1959). The reaction mixture consisted of enzyme and substrate (0.8% [w/v] xylan oat in a 50 mM phosphate buffer with a pH of 5.5) incubated with a control for 60 min at 40 °C. 3,5-Dinitrosalicylic acid (DNS) reagents were then added to the stopped reaction and re-incubated in boiling water for 15 min and cooled directly for 20 min. The resulting color demonstrated absorbance at a wavelength of 550 nm, and a calibration curve was made with D-Xylose.

2.6. Characterization of purified r-XynBT

Purified enzymes were characterized by the following parameters.

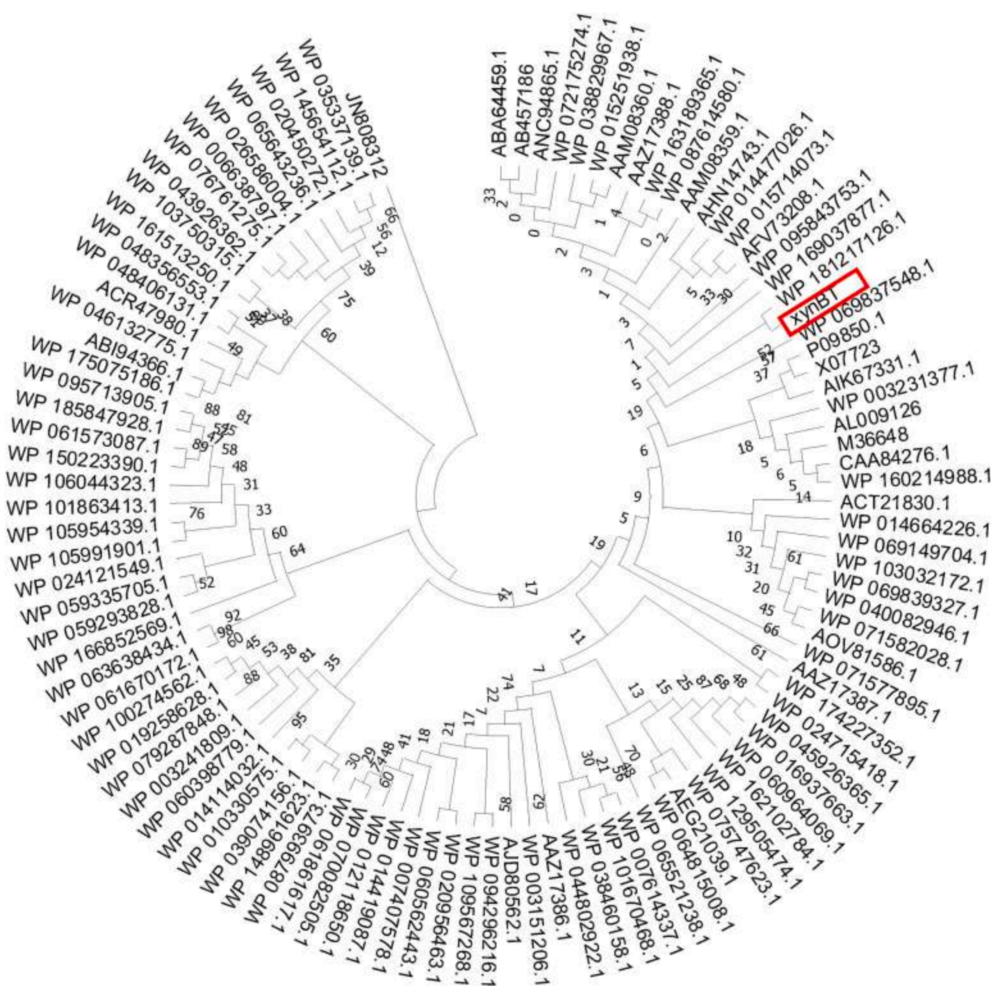

Fig. 4. Phylogenetic tree of endoxylanase GH 11 family. GH 11 are represented by their family number followed by GenBank accession number.

2.6.1. *Optimum temperature and thermal stability*

Enzyme activity was measured in a 50 mM phosphate-citrate buffer with a pH of 5.5 containing 0.8% (w/v) xylan oat substrate. The optimal temperature was tested in the range of 30–70 °C. For determining the thermal stability, r-XynBT was pre-incubated at different temperatures (30–60 °C) for 20, 40, 60, 80, 100, and 120 min. The residue activity and relative activity were both determined.

2.7. *Optimum pH and pH stability*

The optimal pH of r-XynBT was determined by the standard xylanase activity test method using 0.8% (w/v) xylan oat substrate within a pH range of 3.0–8.0 (range 1 pH unit); then, the buffer pH range is between pH 3.0 to 6.0 (range of 0.2 pH units). To determine the pH stability, r-XynBT was incubated at different pH levels (3.0–6.0) at 40 °C for 20, 40, 60, 80, 100, and 120 min.

2.8. *Effect of metal cations*

The effect of the metal cations (K^+, Na^+, Mg^{2+}, Cu^{2+}, Zn^{2+}, and Fe^{3+}) on the r-XynBT activity was observed by adding a metal solution to achieve the final concentration of 10 mM for 30 min at room temperature.

3. Result and discussion

3.1. *Cloning and sequencing of the xynBT gene*

The PCR product was about 600 bp (Fig. 1), and it was purified and ligated into the *SacI-XhoI* site of pET-30a (+) to generate *pET-xynBT*. A single band of *pET-xynBT* was observed in agarose gel and compared to pET-30a (+). The length of the *pET-xynBT* fragment appeared to be larger than that of the pET-30a (+) fragment. The results show that the *xynBT* gene fragment was inserted into pET-30a (+) as expected (Fig. 1).

The DNA sequencing result verified that the cloned fragment from the PCR using forward and reverse primers was exactly 639 bp in length encode 213 amino acid. The nucleotide sequence of the full-length *pET-xynBT* from *Bacillus* sp. in the soil termite abdomen was 1277 bp, containing a 69-bp 5́-untranslated region, an 801-bp ORF encoding a 267-aa xynBT, and a 407-bp 3́-untranslated region (Fig. 2). Recombinant XynBT (r-XynBT) consisted of 267 amino acid residues containing linker residues and a His-Tag in the N-terminus. The theoretical pI value and molecular mass (pI/Mw) predicted for the r-XynBT was estimated to be 7.14/29.26514, as determined using the SWISS-PROT tool (https://web.expasy.org/compute_pi/). The deduced amino acid of the gene also had a putative signal peptide, as predicted by signalP. The signal peptide was predicted to be located at the first 30 amino acids in the N-terminal region (Fig. 5).

The amino acid sequence of r-XynBT was aligned with other GH families retrieved from the CAZY database, and a phylogenetic tree was constructed. Phylogenetic analysis of the GH5, 8, 10, and 11 families showed that r-XynBT belongs to the GH11 family (Fig. 3). A study by

```
xynBT           MFKFKKNFLVGLSAALMSISLFSATVSAASTDYWQNWTDGGGIVNAVNGSGGNYSVNWSN 60
WP_069837548.1  MFKFKKNFLVGLSAALMSISLFSATASAASTDYWQNWTDGGGIVNAVNGSGGNYSVNWSN 60
WP_003231377.1  MFKFKKNFLVGLSAALMSISLFSATASAASTDYWQNWTDGGGIVNAVNGSGGNYSVNWSN 60
M36648          MFKFKKNFLVGLSAALMSISLFSATASAASTDYWQNWTDGGGIVNAVNGSGGNYSVNWSN 60
X07723          MFKFKKNFLVGLSAALMSISLFSATASAASTDYWQNWTDGGGIVNAVNGSGGNYSVNWSN 60
P09850.1        MFKFKKNFLVGLSAALMSISLFSATASAASTDYWQNWTDGGGIVNAVNGSGGNYSVNWSN 60
WP_160214988.1  MFKFKKNFLVGLSAALMSISLFSATASAASTDYWQNWTDGGGIVNAVNGSGGNYSVNWSN 60
CAA84276.1      MFKFKKNFLVGLSAALMSISLFPATASAASTDYWQNWTDGGGIVNAVNGSGGNYSVNWSN 60
WP_181217126.1  MFKFKKNFLVGLSAALMSISLFSATVSAASTDYWQNWTDGGGIVNAVNGSGGNYSVNWSN 60
AL009126        MFKFKKNFLVGLSAALMSISLFSATASAASTDYWQNWTDGGGIVNAVNGSGGNYSVNWSN 60
AB457186        MFKFKKNFLVGLSAALMSISLFSATASAASTDYWQNWTDGGGIVNAVNGSGGNYSVNWSN 60
WP_015251938.1  MFKFKKNFLVGLSAALMSISLFSATASAASTDYWQNWTDGGGIVNAVNGSGGNYSVNWSN 60
                ********************** **.**********************************

xynBT           TGNFAVGKGWTTGSPFRTINYNAGVWAPNGNGYLTLYGWTRSPLIEYYVVDSWGTYRPTG 120
WP_069837548.1  TGNFVVGKGWTTGSPFRTINYNAGVWAPNGNGYLTLYGWTRSPLIEYYVVDSWGTYRPTG 120
WP_003231377.1  TGNFVVGKGWTTGSPFRTINYNAGVWAPNGNGYLTLYGWTRSPLIEYYVVDSWGTYRPTG 120
M36648          TGNFVVGKGWTTGSPFRTINYNAGVWAPNGNGYLTLYGWTRSPLIEYYVVDSWGTYRPTG 120
X07723          TGNFVVGKGWTTGSPFRTINYNAGVWAPNGNGYLTLYGWTRSPLIEYYVVDSWGTYRPTG 120
P09850.1        TGNFVVGKGWTTGSPFRTINYNAGVWAPNGNGYLTLYGWTRSPLIEYYVVDSWGTYRPTG 120
WP_160214988.1  TGNFVVGKGWTTGSPFTTINYNAGVWAPNGNGYLTLYGWTRSPLIEYYVVDSWGTYRPTG 120
CAA84276.1      TGNFVVGKGWTTGSPFRTINYNAGVWAPNGNGYLTLYGWTRSPLIEYYVVDSWGTYRPTG 120
WP_181217126.1  TGNFVVGKGWTTGSPFRTINYNAGVWAPNGNGYLTLYGWTRSPLIEYYVVDSWGTYRPTG 120
AL009126        TGNFVVGKGWTTGSPFRTINYNAGVWAPNGNGYLTLYGWTRSPLIEYYVVDSWGTYRPTG 120
AB457186        TGNFVVGKGWTTGSPFRTINYNAGVWAPNGNGYLTLYGWTRSPLIEYYVVDSWGTYRPTG 120
WP_015251938.1  TGNFVVGKGWTTGSPFRTINYNAGVWAPNGNGYLTLYGWTRSPLIEYYVVDSWGTYRPTG 120
                ****.*********** *******************************************

xynBT           TYKGTVKSDGGTYDIYTTTRYNAPSIDGDRTTFTQYWSVRQSKRPTGSNATITFSNHVNA 180
WP_069837548.1  TYKGTVKSDGGTYDIYTTTRYNAPSIDGDRTTFTQYWSVRQMKRPTGSNATITFSNHVNA 180
WP_003231377.1  TYKGTVKSDGGTYDIYTTTRYNAPSIDGDRTTFTQYWSVRQSKRPTGSNATITFSNHVNA 180
M36648          TYKGTVKSDGGTYDIYTTTRYNAPSIDGDRTTFTQYWSVRQSKRPTGSNATITFSNHVNA 180
X07723          TYKGTVKSDGGTYDIYTTTRYNAPSIDGDRTTFTQYWSVRQSKRPTGSNATITFTNHVNA 180
P09850.1        TYKGTVKSDGGTYDIYTTTRYNAPSIDGDRTTFTQYWSVRQSKRPTGSNATITFTNHVNA 180
WP_160214988.1  TYKGTVKSDGGTYDIYTTTRYNAPSIDGDRTTFTQYWSVRQSKRPTGSNATITFSNHVNA 180
CAA84276.1      TYKGTVKSDGGTYDIYTTTRYNAPSIDGDRTTFTQYWSVRQSKRPTGSNATITFSNHVNA 180
WP_181217126.1  TYKGTVKSDGGTYDIYTTTRYNAPSIDGDRTTFTQYWSVRQTKRPTGSNATITFSNHVNA 180
AL009126        TYKGTVKSDGGTYDIYTTTRYNAPSIDGDRTTFTQYWSVRQSKRPTGSNATITFSNHVNA 180
AB457186        TYKGTVKSDGGTYDIYTTTRYNAPSIDGDRTTFTQYWSVRQTKRPTGSNATITFSNHVNA 180
WP_015251938.1  TYKGTVKSDGGTYDIYTTTRYNAPSIDGDRTTFTQYWSVRQTKRPTGSNATITFSNHVNA 180
                ***************************************** ************:*****

xynBT           WKSHGMNLGSNWAYQVMATEGYQSGGSSNVTVW     213     100%
WP_069837548.1  WKSHGMNLGSNWAYQVMATEGYQSSGSSNVTVW     213     98.12%
WP_003231377.1  WKSHGMNLGSNWAYQVMATEGYQSSGSSNVTVW     213     98.59%
M36648          WKSHGMNLGSNWAYQVMATEGYQSSGSSNVTVW     213     98.59%
X07723          WKSHGMNLGSNWAYQVMATEGYQSSGSSNVTVW     213     98.12%
P09850.1        WKSHGMNLGSNWAYQVMATEGYQSSGSSNVTVW     213     98.12%
WP_160214988.1  WKSHGMNLGSNWAYQVMATEGYQSSGSSNVTVW     213     98.12%
CAA84276.1      WKSHGMNLGSNWAYQVMATEGYQSSGSSNVTVW     213     98.12%
WP_181217126.1  WKSHGMNLGSNWAYQVMATEGYQSSGSSNVTVW     213     98.59%
AL009126        WKSHGMNLGSNWAYQVMATEGYQSSGSSNVTVW     213     98.59%
AB457186        WKSHGMNLGSNWAYQVMATEGYQSSGSSNVTVW     213     98.12%
WP_015251938.1  WKSHGMNLGSNWAYQVMATEGYQSSGSSNVTVW     213     98.12%
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(caption on next page)

Fig. 5. Alignment of amino acid sequences of xynBT compared to GH11 xylanase. *Bacillus* sp. (initial: XynBT, this work), *Bacillus subtilis* (WP_069837548.1), *Bacillus* (WP_003231377.1), *Bacillus subtilis* PAP115 (M36648), *Bacillus circulans* (X07723), *Bacillus circulans* (P09850.1), *Bacillus subtilis* (WP_160214988.1), *Bacillus subtilis* (CAA84276.1), *Bacillus subtilis* (WP_181217126.1), *Bacillus subtilis* 168 (AL009126), *Bacillus subtilis* R5 (AB457186), and *Bacillus* (WP_015251938.1). The bold and underlined amino acid sequence of *xynBT* is the predicted signal peptide (SignalP program). The red triangle shows the aspartate residue that plays a role in providing acid to the enzyme. The yellow highlighted regions indicate catalytic conservations. The four boxes define the segments with the highest similarities. The numbers on the right side of sequence represent the positions of the amino acid sequence, and the percentages indicate the percent of similarity between xynBT and GH11 xylanase. Sequence alignment was carried out using the CLUSTAL W program. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Helianti et al. (2008) showed that the genes from the 11 xylanase family were relatively conserved among various bacterial sources, and the BLAST results showed that the xylanase gene from family 11 is very well conserved among *Bacillus* species. Therefore, using the specific primer designed in this work would increase the likelihood of successful xyn11 isolation and facilitate the PCR cloning and xyn11 overexpression of different *Bacillus* species.

The BLAST analysis showed that r-XynBT was highly similar to the G/11 family xylanases and shares up to 98.59% similarity with the XynA from *Bacillus subtilis* (WP_181217126.1). The r-XynBT is well conserved, with more than 100 xylanases of the GH 11 family derived from the *Bacillus* family. The phylogenetic tree of r-XynBT compared with the GH11 family was constructed using MEGA software (Fig. 4). Multiple sequence alignment and molecular evolutionary relationships were constructed using the neighbor-joining method and bootstrap values. XynBT shared minimum evolutionary relationships with the xylanase GH11 family from *Bacillus subtilis* (WP_069837548.1), *Bacillus* (WP_003231377.1), *Bacillus subtilis* PAP115 (M36648), *Bacillus circulans* (X07723), *Bacillus circulans* (P09850.1), *Bacillus subtilis* (WP_160214988.1), *Bacillus subtilis* (CAA84276.1), *Bacillus subtilis* (WP_181217126.1), *Bacillus subtilis* 168 (AL009126), *Bacillus subtilis* R5 (AB457186), and *Bacillus* (WP_015251938.1).

The XynBT was 98.59% identical to that of the xylanase from *Bacillus* (WP_003231377.1), *Bacillus subtilis* PAP115 (M36648), *Bacillus subtilis* (WP_181217126.1), and *Bacillus subtilis* 168 (AL009126). However, the insert also revealed many substitutions (mutations with other sp. of *Bacillus*), which established that the *xynBT* from *Bacillus* sp. of soil termite abdomen is a novel gene. The nucleotides A, V, and G (accession number: M36648) at positions 26, 65, and 205 were replaced with V, A, and S, respectively, in the XynBT. This replacement did not have any effect on the amino acid properties. The replacement of A at position 26 by V resulted in the replacement of valine with alanine. Both valine and alanine are classified into the same class of amino acid on the basis of their structure and the general chemical characteristics of their R groups. They also belong to aliphatic group classes, since they have uncharged polar side chains, which make them hydrophilic in nature. Therefore, this replacement is not expected to have a significant effect on the secondary and tertiary structures of the protein (Jalal et al., 2009).

The amino acid sequences from the GH 11 family endoxylanase catalytic domain have been aligned. Eighty-two amino acid sequences with two glutamates acting as catalytic residues were conserved in all sequences, and four boxes defining the segments with the highest similarities were detected (Sapag et al., 2002; Wang et al., 2007). Four boxes, NGYLTLYGWT (+89 to +98), PLIEYYVVDSW (+101 to +111), SDGGTYDIYTTT (+128 to +139), and HVNAWKSHGMNLG (+177 to +189) were conserved motifs and recognized in all xylanases of GH family 11 studied thus far. Two glutamates in r-XynBT were also found

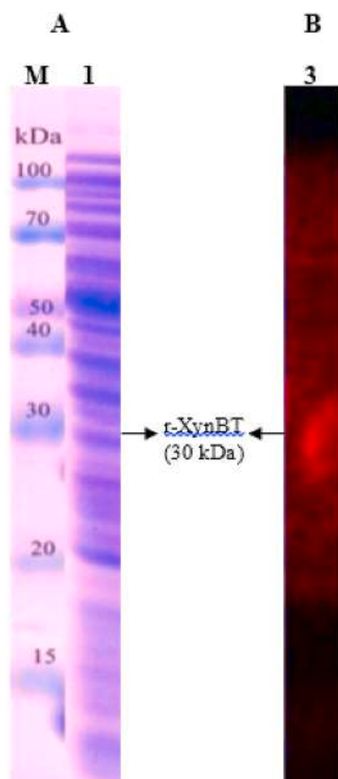

Fig. 6. SDS-PAGE of the r-XynBT. (a) Lane M: protein marker; Lane 1: crude r-XynBT, Zymogram of the r-XynBT (b) Lane 3.

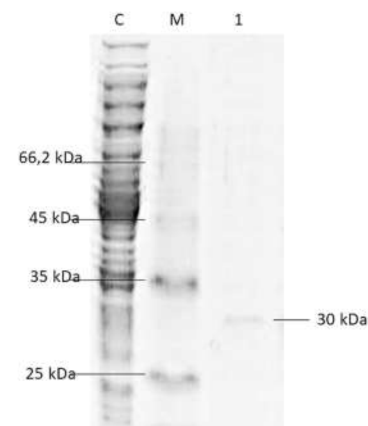

Fig. 7. SDS-PAGE of purified r-XynBT from *Escherichia coli* BL21 (DE3). C: crude extract; M: protein marker; 1: purified r-XynBT.

Table 1
Summary of r-XynBT purification.

Step	Activity (U)	Protein (mg)	Specific activity (U/mg)	Purification (-fold)	Yield (%)
Crude supernatant	4.90	8.73	0.56	1	100
Ni-NTA	0.59	0.11	5.45	10	12.48

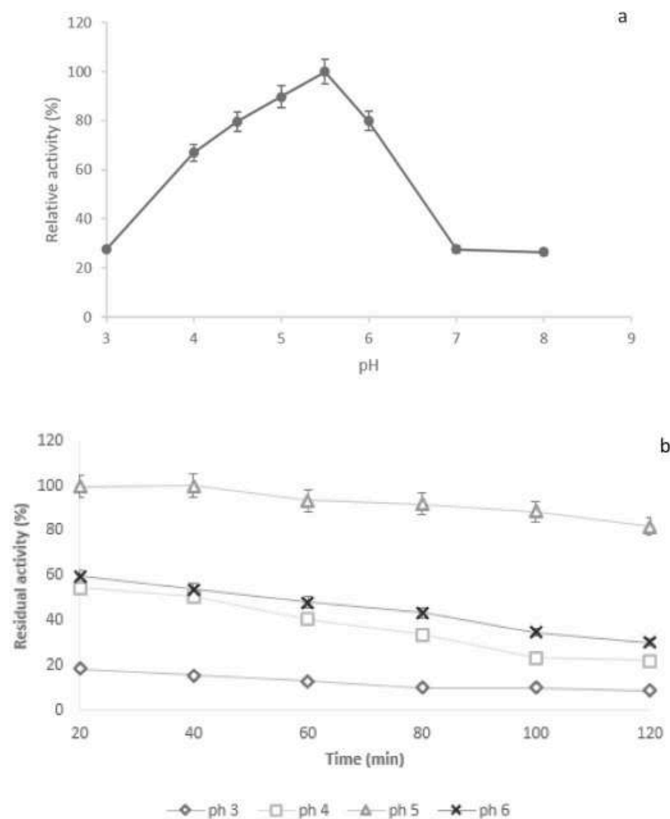

Fig. 8. Enzymatic characterization of r-XynBT. (a) Effect of pH on the activity of enzyme. (b) pH stability on enzyme activity. Data is represented in the means of triplicate experiments, and error bars expressed as standard deviation.

to be located at +104 and + 200, respectively (Fig. 5). These features verified that *xynBT* is a member of GH11.

3.2. Expression and purification of recombinant XynBT (r-XynBT)

The recombinant plasmid, *pET-xynBT*, is expressed in *E.coli* BL21 (DE3). *E. coli* BL21 (DE3) is a strain that has been widely used for many years for T7 RNA polymerases that depend on the high-level expression of a protein (Borgeaud and Blokesch, 2013). The success of the transformation can be determined using antibiotic resistance tests and verified with sequencing. It is important to note that r-XynBT has a His-tag. Transformants were successfully grown in a LB media containing kanamycin, whereas *E. coli* BL21 (DE3) did not show any colonies. The r-XynBT was successfully expressed, and the activity of xylanase was confirmed by SDS-PAGE zymogram through a clear zone in the band that was approximately 30 kDa, which was close to the expected 29.3 kDa (Fig. 6).

The specific activity of r-XynBT increased from 0.56 to 5.45 U/mg protein after purification with a 10-fold increase and a 12.48% recovery (Table 1). The specific activity of the purified r-XynBT was higher than that of the purified wild type, which was 2.865 U/mg. This result indicated that the expression of the *xynBT* gene in *E. coli* could increase the productivity of the xylanase.

The 6xHis-tagged recombinant xylanase (r-XynBT) was purified by Ni-NTA chromatography. The purified r-XynBT migrated on the gel as a single band with a molecular mass of around 30 kDa. The predicted molecular mass of the protein was 29.3 kDa, which is very close to the experimental molecular mass (Fig. 7). r-XynBT consisted of 267 amino acid residues containing linker residues and a His-Tag in the N-terminus, which caused the unusually large molecular weight compared to the other GH11 xylanases (Fig. 5). The other GH11 xylanases with unusual molecular weights were XylB8 (accession number: JN808312) from the

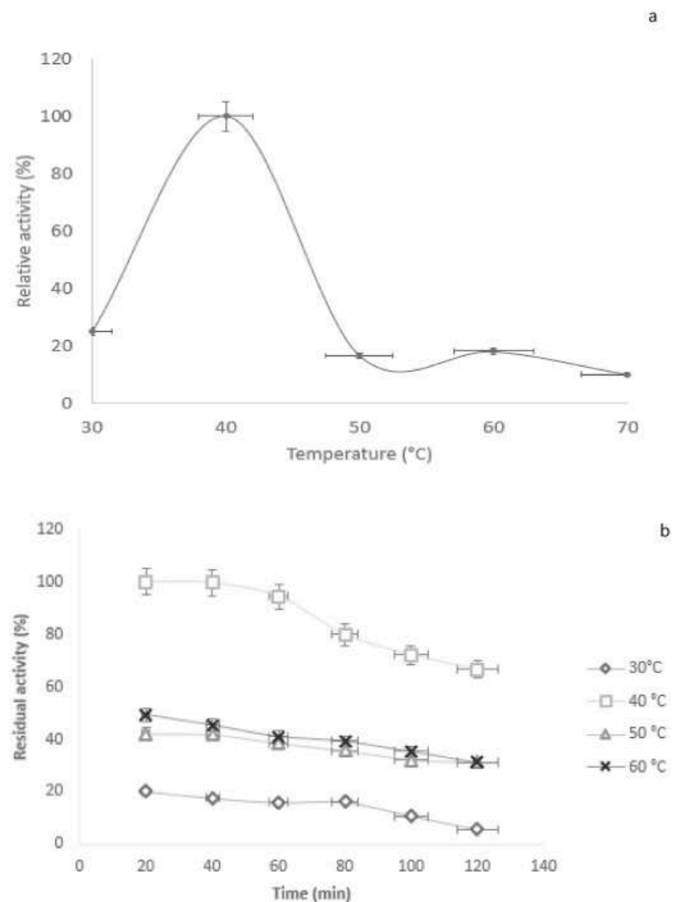

Fig. 9. Enzymatic characterization of r-XynBT. (a) Effect of temperature on the activity of r-XynBT. (b) Stability of temperature on the activity of r-XynBT. Data is represented as the mean of triplicate experiments, and error bars are expressed as standard deviation.

termite gut (*Reticulitermes santonensis*), which had a 29 kDa molecular mass, and Mxyl (accession number: AFP81696.1) from Compost-Soil Metagenome, which had a ~40 kDa molecular mass. Both xylanases had been purified by Ni-NTA chromatography (Mattéotti et al., 2012; Verma et al., 2013). The XylB8 molecular weight was quite close to that of XynBT, despite the low similarity of their amino acid sequences (57.97%).

The GH11 family, which is one of the best characterized GH families in terms of its bacterial and fungal members, is considered made up of true xylanases when compared to the other families due to its high substrate specificity. Indeed, compared to other xylanases, Xyl-11 displays several interesting properties: high substrate selectivity and high catalytic efficiency; small size (around 20 kDa); and variety of optimum pH and temperature. This makes them suitable under various conditions and in many applications (Paës et al., 2012). So, the unique nature of xylanase from soil termite abdominal bacteria is due to its activity and molecule size. Previous studies have reported that the nature the endoxylanase enzyme from soil termite abdominal bacteria showed activity not only with the oat-spelt xylan substrate but also with arabinofuranoside and glucuronide substrates. The molecular weight from r-XynBT purified is at about 30 kDa (with His-tag), which is close to the expected 29.3 kDa.

3.3. Characterization of purified r-XynBT

3.3.1. Optimum pH and pH stability

The activity of r-XynBT was determined within the pH range of 3–8. r-XynBT exhibited the highest activity around pH 5.5. Additionally, it

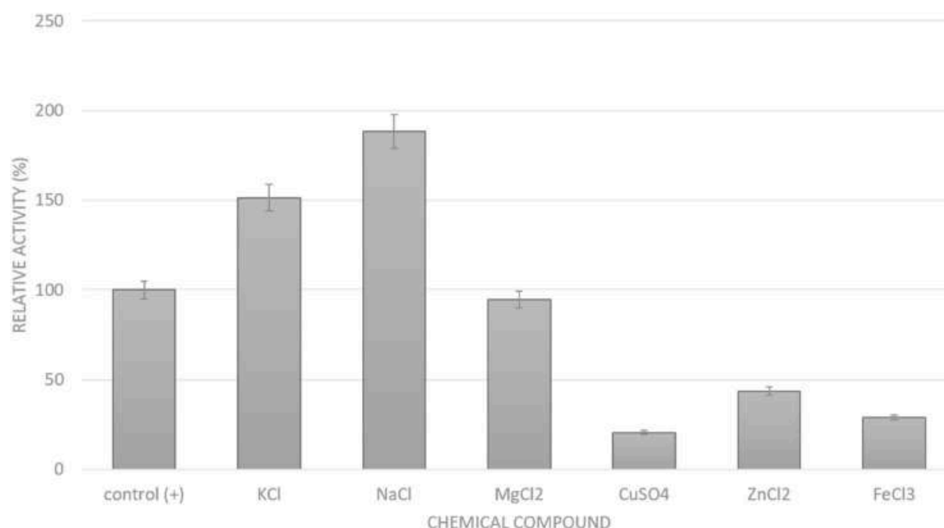

Fig. 10. Effect of the addition of metal cations on r-XynBT activity.

exhibited approximately 26–27% activity at pH 3.0 and 7.0–8.0 and 66–89% at pH 4.0–5.0. The results indicate that r-XynBT is stable under acidic conditions, but its activity declined significantly when the buffer pH was 7 (Fig. 8a). This enzyme retained 91.9% of its activity for 80 min at pH 5.0 before it decreased to 88% and 81.82% at 100 and 120 min, respectively (Fig. 8).

3.3.2. Optimum temperature and thermostability

The r-XynBT showed the highest activity at 40 °C (Fig. 9a). The temperature stability of r-XynBT was measured by incubating the enzyme for various amounts of time; then, residual activity was measured using standard assay methods. r-XynBT is stable up to 60 °C, and this enzyme has a relative activity of 66% after 2 h of incubation at 40 °C (Fig. 9b).

Several characteristic of xylanases from the *Bacillus* family of GH11 have been reported. Most of them have activity at pH of 5.0–9.0, and the optimum temperature can reach 40–60 °C, while some have reached 80 °C, with the highest stability at a temperature of 50–70 °C. Xylanase gene from *Bacillus subtilis* (WP_069837548.1), *Bacillus* (WP_003231377.1), *Bacillus subtilis* PAP115 (M36648), *Bacillus circulans* (X07723), *Bacillus circulans* (P09850.1), *Bacillus subtilis* (WP_160214988.1), *Bacillus subtilis* (CAA84276.1), *Bacillus subtilis* (WP_181217126.1), *Bacillus subtilis* 168 (AL009126), *Bacillus subtilis* R5 (AB457186), and *Bacillus* (WP_015251938.1) have ORF 639 bp encoding 231 amino acid. Almost all of them exhibited maximum activity at pH 5.0–7.0 and 50–60 °C, which this optimum condition are close to the r-XynBT optimum condition (Bernier et al., 1983; Jalal et al., 2009; Naufal et al., 2019; Niersbach et al., 1992; Ruller et al., 2006; Yang et al., 1988). The optimal pH and temperature of r-XynBT are also very close to those of GH 11 xylanase from the termite gut (*Reticulitermes santonensis*): 5.0 and 55 °C, respectively (Mattéotti et al., 2012).

From the optimum pH experiment, it can be observed that r-XynBT is an alkaline xylanase ($pH_{optimum} = 5.5$). The optimum pH values correlate with the residue adjacent to the acid/base catalysis, which can be determined via analysis of the alignment of various microbial xylanase sequences. Alkaline xylanases with optimum pH values above 5.0 have Asn residue, while acid xylanases have Asp residue with optimum pH values under 5.0 (Sapag et al., 2002). In Fig. 5 the preserved residue at position 63 of the amino acid sequence, namely Asn (N). This also confirms that xylanase from soil termite abdomen is alkaline xylanase.

3.4. Effect of metal cations

The effect of variations of metal cations on r-XynBT activity was also tested. The metal cations K^+ and Na^+ increased the relative enzyme activity by 51–88%, whereas Mg^{2+}, Cu^{2+}, Zn^{2+}, and Fe^{3+} on r-XynBT produced a slight decrease in the relative activity between 6 and 79% (Fig. 10). The presence of Mn^{2+} strongly inhibited the reAuXyn11D from *Aspergillus usamii* E001 (Zhang et al., 2012). According to Liu et al. (2012), the presence of metal cations K^+ and Na^+ stabilizes the catalytic side of r-XynBT from thermophilic bacteria *Geobacillus* sp, while metal cations such as Mg^{2+}, Cu^{2+}, Zn^{2+}, and Fe^{3+} inhibit the relative activity of enzymes (Liu et al., 2012). The inhibition of xylanase activity by Cu^{2+} is similar to the majority of xylanases (Mattéotti et al., 2012; Verma et al., 2013). Cation Na^+ increases the activity of XynRA1 from *Rhodothermaceae* bacterium RA, whereas Hg^{2+}, Mn^{2+}, Co^{2+}, Fe^{2+}, Pb^{2+}, Cu^{2+}, and Ag^{2+} inhibit the activity of this enzyme (Liew et al., 2019). However, in some other family 11 xylanases, the presence of Na^+ can inhibit enzyme activity (Zhang et al., 2012).

4. Conclusion

xynBT is the first xylanase cloned gene from *Bacillus* sp. in the soil termite abdomen. r-XynBT has a highly similar amino acid sequence to known GH11 xylanase. The increased in purified r-XynBT specific activity compared to purified wild type specific activity is expected to increase the effectiveness of the enzyme in the degradation of xylan from agroindustry waste and XOS production.

Declaration of competing interests

The authors whose names are listed in the manuscript certify that we all agree to submit the manuscript in Biocatalysis and Agricultural Biotechnology. All of us have NO competing of interests.

We also certify that the article is original work, has not received prior publication and is not under consideration for publication elsewhere.

Sincerely yours, Ni Nyoman Tri Puspaningsih and Anak Agung Istri Ratnadewi.

Corresponding authors.

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