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Research article

Production optimization, purification, expression, and characterization of a novel α-L-arabinofuranosidase from *Paenibacillus polymyxa*

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ABSTRACT

Background: α-L-Arabinofuranosidase (EC 3.2.1.55) catalyzes the hydrolysis of terminal α-L-1,2-, -1,3-, and -1,5-arabinofuranosyl residues in arabinose-containing polymers, and hence, it plays an important role in hemicellulose degradation. Herein, the bacterium *Paenibacillus polymyxa,* which secretes arabinofuranosidase with high activity, was selected for enzyme production, purification, and characterization.

Results: Medium components and cultural conditions were optimized by the response surface method using shake flask cultures. Arabinofuranosidase production reached 25.2 U/mL under optimized conditions, which were pH 7.5, 28°C, and a basic medium supplemented with 1.5 g/L mannitol and 3.5 g/L soymeal. Furthermore, the arabinofuranosidase secreted by *P. polymyxa*, named as PpAFase-1, was partially purified from the supernatant using a DEAE Sepharose Fast Flow column and a hydroxyapatite column. The approximate molecular mass of the purified PpAFase-1 was determined as 56.8 kDa by SDS-PAGE. Protein identification by mass spectrometry analysis showed that the deduced amino acid sequence had significant similarity to the glycosyl hydrolase family 51. The deduced gene of 1515 bp was cloned and expressed in *Escherichia coli* BL21 (DE3) cells. Purified recombinant PpAFase-1 was active toward *p*-nitrophenyl-α-L-arabinofuranoside (pNPAra*f*). The K_m and k_{cat} values toward pNPAra*f* were 0.81 mM and 53.2 s^{-1}, respectively. When wheat arabinoxylan and oat spelt xylan were used as substrates, PpAFase-1 showed poor efficiency. However, a synergistic effect was observed when PpAFase-1 was combined with xylanase from *Thermomyces lanuginosus*.

Conclusion: A novel GH51 enzyme PpAFase-1 was cloned from the genome of *P. polymyxa* and expressed in *E. coli*. This enzyme may be suitable for hemicellulose degradation on an industrial scale.

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1. Introduction

Lignocellulose is the second most abundant organic polymer on the Earth. Lignocellulose mainly includes cellulose, hemicellulose, and lignin [1]. Hemicellulose, which accounts for approximately 25–30% of the total amount of lignocellulose [2], is composed primarily of pentoses (β-D-xylose and α-L-arabinose), hexoses (α-L-rhamnose, α-L-fucose, β-D-mannose, α-D-galactose, and β-D-glucose), and uronic acids (α-D-galacturonic acid, α-D-glucuronic acid, and α-D-4-*O*-methyl-glucuronic acid) [3]. The content and structure of hemicellulose vary in different organisms. In hardwood, hemicellulose mainly includes glucuronoxylan, glucomannan, and xyloglucan, whereas in softwood, the predominant constituents are galactoglucomannan and arabinogalactan. In gramineous plants, hemicellulose is composed of arabinoxylan, arabinoglucuronoxylan, and β-(1 → 3, 1 → 4)-glucan, in which arabinoxylan is the main constituent. The backbone of arabinoxylan is composed of β-l,4-linked D-xylose, with α-L-arabinose substituted at the *O*-2, *O*-3, or both positions [4].

The use of hemicellulose in food, pulping, pharmaceuticals, and fine chemical industries either directly or through structural modification has significant potential [5,6]. Hemicellulose is a complex structure that requires various enzymes for complete degradation, including endo-xylanase, β-xylosidase, and α-L-arabinofuranosidase [7,8]. The presence of L-arabinose in the side chain restricts the enzymatic hydrolysis of the xylan backbone by endo-xylanase. Therefore, α-L-arabinofuranosidase, which cleaves the α-L-arabinose from the side chain of arabinoxylan, is needed for complete degradation of arabinose-containing polymers [9,10]. α-L-Arabinofuranosidase (EC 3.2.1.55) catalyzes the hydrolysis of nonreducing α-1,2-, α-1,3-, and α-1,5-L-arabinofuranosyl residues leading to the release of arabinose

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from oligosaccharides or natural polysaccharides such as arabinoxylan, arabinan, and arabinogalactan [11]. α-L-Arabinofuranosidase plays an important role in hemicellulose degradation. Therefore, identification of new α-L-arabinofuranosidases that can efficiently convert hemicellulose is required. In addition, α-L-arabinofuranosidases have potential applications in improving animal feedstock digestibility and wine flavors, juice clarification, and delignification of pulp [12,13].

On the basis of amino acid sequence similarity, α-L-arabinofuranosidases are assigned to six different glycoside hydrolase (GH) families: GH3, 43, 51, 54, 62, and 93 [14,15]. The majority of the enzymes in the GH51 family hydrolyze the glycosidic linkage between the L-arabinofuranoside side chains of hemicelluloses. Currently, some GH51 α-L-arabinofuranosidases have been isolated from different organisms [16,17,18,19]. A bacterium, *Paenibacillus polymyxa*, was found to exhibit considerable α-L-arabinofuranosidase activity. Herein, the production of extracellular α-L-arabinofuranosidase by *P. polymyxa* was studied and optimized. The extracellular α-L-arabinofuranosidase was partially purified from the optimized medium; the enzyme was identified and heteroexpressed, and its potential use for the hydrolysis of hemicellulose was evaluated.

2. Materials and methods

2.1. Chemicals

p-Nitrophenyl-linked glycosides, oat spelt xylan, and *Thermomyces lanuginosus* xylanase were purchased from Sigma-Aldrich (St. Louis, MO, USA). Arabinobiose (O-ABI), xylobiose (O-XBI), sugar beet arabinan (P-ARAB), arabinogalactan (P-ARGAL), and wheat arabinoxylan (P-WAXYL) were purchased from Megazyme (Bray, Ireland). Restriction enzymes, T4 ligase, bacterial genome extraction kit, agarose gel extraction kit, and plasmid extraction kit were obtained from Takara (Tokyo, Japan). All other reagents were analytical or HPLC grade.

2.2. Culture conditions and enzyme production in shake flask cultures

The strain *P. polymyxa* KF-1 (CCTCC AB 2018146) was maintained on Luria-Bertani agar (LB) slants and stored at 4°C. α-L-Arabinofuranosidase production was carried out in 100 mL of basic medium using 250 mL of shake flasks. The composition of the basic medium was 2.5 g/L glucose, 1.5 g/L $(NH_4)_2SO_4$, 2.0 g/L KH_2PO_4, 2.0 g/L K_2HPO_4, 0.3 g/L $MgSO_4 \cdot 7H_2O$, 0.3 g/L $CaCl_2$, 0.01 g/L $FeSO_4 \cdot 7H_2O$, and 0.05 g/L KCl, pH 7.0. The overnight culture (2 mL) was used to inoculate the basic medium (100 mL), and the culture was shaken at 180 rpm and 30°C for 36 h. After incubation, bacterial cells were separated by centrifugation (5000 *g*, 4°C, 10 min). The cell-free supernatant was assayed for α-L-arabinofuranosidase activity using *p*-nitrophenyl-α-L-arabinofuranoside (pNPAra*f*, final concentration of 1 mM) as the substrate [20]. The reaction was performed in 20-mM Tris–HCl buffer (pH 7.0), and the reaction time was 30 min. One unit of enzyme activity was defined as the amount of enzyme required to release 1-μmol *p*-nitrophenol/min from pNPAra*f*. Enzymatic activities against other *p*-nitrophenyl-linked glycosides were determined by the same method.

2.3. Optimization for α-L-arabinofuranosidase production by P. polymyxa

2.3.1. Single-factor optimization

Various factors including inoculum volume (1, 2, 3, 4, and 5%), culture temperature (20, 25, 28, 33, and 38°C), initial pH (1.0–9.0), carbon source (dextrose, xylose, lactose, fructose, mannose, sucrose, arabinose, and mannitol), nitrogen source (peptone, yeast extract, beef extract, urea, soymeal, skim milk, KNO_3, $NaNO_3$, $(NH_4)_2SO_4$, and NH_4NO_3), and surfactant (Tween-20, Tween-40, Tween-60, Tween-80, PEG, SDS, and Triton X-100) were initially evaluated by single-factor optimization. The concentration of the carbon and nitrogen sources investigated in the test was 2.5 g/L, and the surfactants were evaluated at a concentration of 1.0 g/L. Cultures were fermented at 180 rpm for 36 h, and then, the α-L-arabinofuranosidase activities of the culture were determined as described in Section 2.2.

2.3.2. Box–Behnken design

The major variables that affect the α-L-arabinofuranosidase production by *P. polymyxa*, including pH, temperature, mannitol, and soymeal, were chosen for further optimization by the Box–Behnken design (BBD), which was designed and analyzed using Design-Expert software (version 8) [21]. α-L-arabinofuranosidase activity (U/mL) was taken as the response, and all experiments were conducted in triplicates. The model and second-order polynomial equation were validated by producing α-L-arabinofuranosidase under the conditions predicted by the model and comparing measured activities to predicted activities.

2.4. Purification of PpAFase-1 from P. polymyxa

The cell-free supernatant of *P. polymyxa* was purified using a DEAE Sepharose Fast Flow column (1 × 20 cm, column volume = 15 mL). The column was equilibrated with 20-mM Tris–HCl, pH 7.5 (buffer A) and eluted stepwise with 20-mL portions of 0.1, 0.2, 0.3, 0.4, and 0.5 M NaCl (in buffer A). Active fractions 10–14 were combined and further purified using a hydroxyapatite column (1 × 5 cm, column volume = 4 mL). Unbound materials were washed with 10 mL of 10-mM sodium phosphate buffer (pH 6.8), and bound proteins were eluted using a 30-mL linear gradient of 10- to 500-mM sodium phosphate buffer (pH 6.8). Fractions of 1 mL were collected. The active fractions were combined, named as PpAFase-1, and used for protein identification. The flow rates for column chromatography were 1 mL/min. The protein concentration of each fraction was determined by the Bradford method at 595 nm, with BSA as the standard [22].

2.5. Electrophoresis analysis and protein identification by mass spectrometry

The cell-free supernatant and partially purified PpAFase-1 were analyzed by SDS-PAGE with a 10% separating gel [23]. Partially purified PpAFase-1 was cut from the SDS-PAGE gel at the position of ~56.8 kDa. The protein was digested by trypsin, acidified with trifluoroacetic acid, and desalted using an in-house-prepared C18 tip. The desalted peptide samples were dried under vacuum and finally analyzed by nanoLC-MS/MS (Orbitrap Fusion Lumos Mass Spectrometer; Thermo Fisher Scientific, USA). The obtained MS file was searched against the Uniprot nonredundant protein database of *P. polymyxa* using Proteome Discoverer 1.4 (Thermo Fisher Scientific) [24].

2.6. Cloning, expression, and purification of recombinant PpAFase-1

The deduced gene of PpAFase-1 (accession number MH360731) was cloned from the genome of *P. polymyxa*. *Nde*I and *BamH*I restriction sites (underlined) were introduced by the primers PpAFase-1F: 5′-GGAA TTCCATATGGTGGCAGCAGCTGTAAAAGC-3′ and PpAFase-1R: 5′-CGGGATCCTTAATGAGATTTAGCAAGACG-3′. Gene amplification was carried out by PrimeSTAR HS DNA polymerase (Takara) according to the manufacturer's instruction. The PCR product was double digested, ligated to pET-28a, and transformed into *E. coli* BL21 (DE3) cells. The recombinant plasmid was extracted and identified by DNA sequencing, with T7 and T7 terminator as sequencing primers. Molecular cloning was manipulated as described in literature [25].

Recombinant *E. coli* BL21 (DE3) cells were incubated at 37°C and 180 rpm. When the OD_{600nm} reached 0.7, enzyme expression was induced by addition of IPTG (final concentration of 0.4 mM). The induction lasted for 12 h at 28°C. Then, the cells were collected by centrifugation at 5000 rpm, 4°C for 10 min. The cells were then sonicated and centrifuged, and the obtained supernatant was purified using an Ni Sepharose Fast Flow column (GE, bed volume of 10 mL) as

described in literature [25]. The column was eluted using a 100-mL linear gradient of 10- to 200-mM imidazole. The active fractions were combined, dialyzed in 20-mM phosphate buffer (pH 7.0), and used for enzyme characterization. The purification procedure was analyzed by SDS-PAGE.

2.7. Characterization of PpAFase-1

The pH optimum of PpAFase-1 was determined at different pH values using pNPAra*f* as the substrate. The following buffers were used: 25-mM NaAc buffer, pH 2.0–6.0; 25-mM Na_2HPO_4-citrate buffer, pH 6.0–8.0; and 25-mM Glycine-NaOH buffer, pH 8.0–11.0 [25]. For pH stability assay, the purified enzyme was incubated at 4°C for 24 h in buffers without the substrate. Then, the residual activity was measured under standard assay conditions. The optimum temperature was determined by measuring enzyme activity for a temperature range of 20–80°C. Maximal activity observed was defined as 100%. Thermostability was determined by analyzing residual activity after incubation of the enzyme at different temperatures (20–80°C) for 1 h. The initial activity determined without preincubation was defined as 100%.

The effects of metals and chemicals on PpAFase-1 activity were determined. After incubation in the presence of metals or chemicals (final concentration of 10 mM) at 25°C for 1 h, the remaining α-L-arabinofuranosidase activity was determined using pNPAra*f* as the substrate in 20-mM Tris–HCl buffer (pH 7.0). The initial activity determined without preincubation was defined as 100%.

The influence of D-xylose and L-arabinose on α-L-arabinofuranosidase activity was measured by varying the concentration of the sugar (5, 10, 20, 50, and 100 mM) in the assay mixture. The relative α-L-arabinofuranosidase activity was measured with pNPAra*f* as the substrate and compared with that of the control.

The K_m and V_{max} values of purified PpAFase-1 were determined by measuring the hydrolytic rate of pNPAra*f*. The reaction was performed by incubating pNPAra*f* at concentrations ranging from 0.25 to 4.0 mM under standard assay conditions for 15 min. The kinetic values were determined from Lineweaver–Burk plots generated in GraphPad Prism V5.

2.8. Hydrolysis of disaccharides and natural polysaccharides and synergistic action of PpAFase-1 with xylanase

Hydrolysis of arabinobiose, xylobiose, xylan, arabinan, arabinogalactan, and arabinoxylan was performed by incubating substrate (10 mg/mL) with PpAFase-1 (0.5 U/mL) in 50-mM phosphate buffer (pH 7.0) at 40°C and 150 rpm for 8 h. The amount of reducing sugars released from the reaction at different reaction times was measured using the DNS reagent, with L-arabinose as the standard [26].

To investigate the synergistic effect of PpAFase-1 in combination with xylanase, the reaction mixture contained 10 mg/mL of oat spelt xylan, or wheat arabinoxylan, and *T. lanuginosus* xylanase (Xyn, 0.5 U/mL) and PpAFase-1 (0.5 U/mL) either individually or in combination. The reaction was incubated at 40°C for 8 h. Subsequently, the released reducing sugars were measured by the DNS method. The hydrolysis products were further analyzed by thin-layer chromatography (TLC). The TLC silica gel G60 F_{254} plates (Merck, Darmstadt, Germany) were developed using ethyl acetate/methanol/water (16/6/1, v/v/v) as the developing solvent. After developing, the plates were sprayed with sulfuric acid/ethanol (5/95, v/v) followed by heating at 100°C until the products were visible. The hydrolytic products were identified by high-performance anion exchange chromatography (HPAEC), detected using a pulsed amperometric detector. The CarboPac PA-200 analytical column (3 × 250 mm, P/N062896) was eluted at 0.5 mL/min by 0.05-M NaOH for 10 min. The hydrolytic products were compared with standards.

3. Results and discussion

3.1. Time course of α-L-arabinofuranosidase production

P. polymyxa, previously known as *Bacillus polymyxa*, is a soil bacterium that secretes multiple extracellular xylanases, thus showing hemicellulase degradation activity [27]. Therefore, *P. polymyxa* may be a suitable organism to produce enzymes required for the hydrolysis of hemicellulose. The α-L-arabinofuranosidase production and cell growth of *P. polymyxa* were assayed in basic medium. The α-L-arabinofuranosidase activity reached the maximum (18.1 U/mL) at 36 h. Longer incubation periods did not promote further increases in enzyme production (Fig. S1). Therefore, fermentation was terminated at 36 h in further studies.

3.2. Optimization for α-L-arabinofuranosidase production by the single-factor method

Different initial inoculum sizes (1, 2, 3, 4, and 5%) were examined for their effects on α-L-arabinofuranosidase production. The results showed that inoculum size did not affect α-L-arabinofuranosidase production significantly (Fig. 1a). Therefore, an initial inoculum size of 1% was selected for further optimization.

The pH of the medium is a critical environmental parameter that affects bacterium growth and enzyme production. The bacterium produced a maximum concentration of α-L-arabinofuranosidase (19.41 U/mL) at an initial medium pH of 7.0. An increase in pH caused lower enzyme production. At an initial medium pH of 10.0, more than 50% of its activity was lost. A decrease in the initial pH of the medium from 5.0 to 1.0 also caused a decrease in α-L-arabinofuranosidase production (Fig. 1b).

Temperature is another critical environmental parameter that affects enzyme production. The optimization of incubation temperature (20, 25, 28, 33, and 38°C) for the production of α-L-arabinofuranosidase indicated that the production gradually increased from 20 to 33°C, and maximum α-L-arabinofuranosidase production was observed at 33°C. Enzyme production was observed to decrease at 38°C (Fig. 1c).

Extracellular enzyme production depends significantly on medium composition, especially the carbon and nitrogen sources. Among the tested carbon sources, maximum enzyme production (14.33 U/mL) was measured when mannitol was used as the carbon source. Other sugars tested were not efficient carbon sources for α-L-arabinofuranosidase production (Fig. 1d). Increasing the mannitol concentration resulted in an increase in enzyme production, with a concentration of 3 g/L yielding the maximum enzyme production (data not shown). Further increase in the mannitol concentration caused a decrease in enzyme production. Mannitol was previously reported to promote the production of some secreted enzymes such as β-glucosidase from the *Bacillus stratosphericus* strain SG9. It was also reported that mannitol was an optimal carbon source for bacterial cellulose synthesis by *Acetobacter xylinum* [28]. Organic nitrogen sources tested were found to enhance enzyme production. Soymeal as a nitrogen source yielded the highest enzyme production level (14.72 U/mL). Inorganic nitrogen sources including $NaNO_3$, $(NH_4)_2SO_4$, and NH_4NO_3 resulted in poor levels of enzyme production (Fig. 1e).

Surfactants may enhance the production of hydrolytic enzymes [29]. Among the tested surfactants, Triton X-100 was found to increase α-L-arabinofuranosidase production slightly, with a maximum enzyme activity of 11.22 U/mL observed. Other surfactants tested had no significant effect or decreased α-L-arabinofuranosidase production (Fig. 1f). These results are consistent with those observed in earlier studies, such as protease production from *Saccharopolyspora* sp. [29] and an amylase from *Bacillus tequilensis* RG-01 [30].

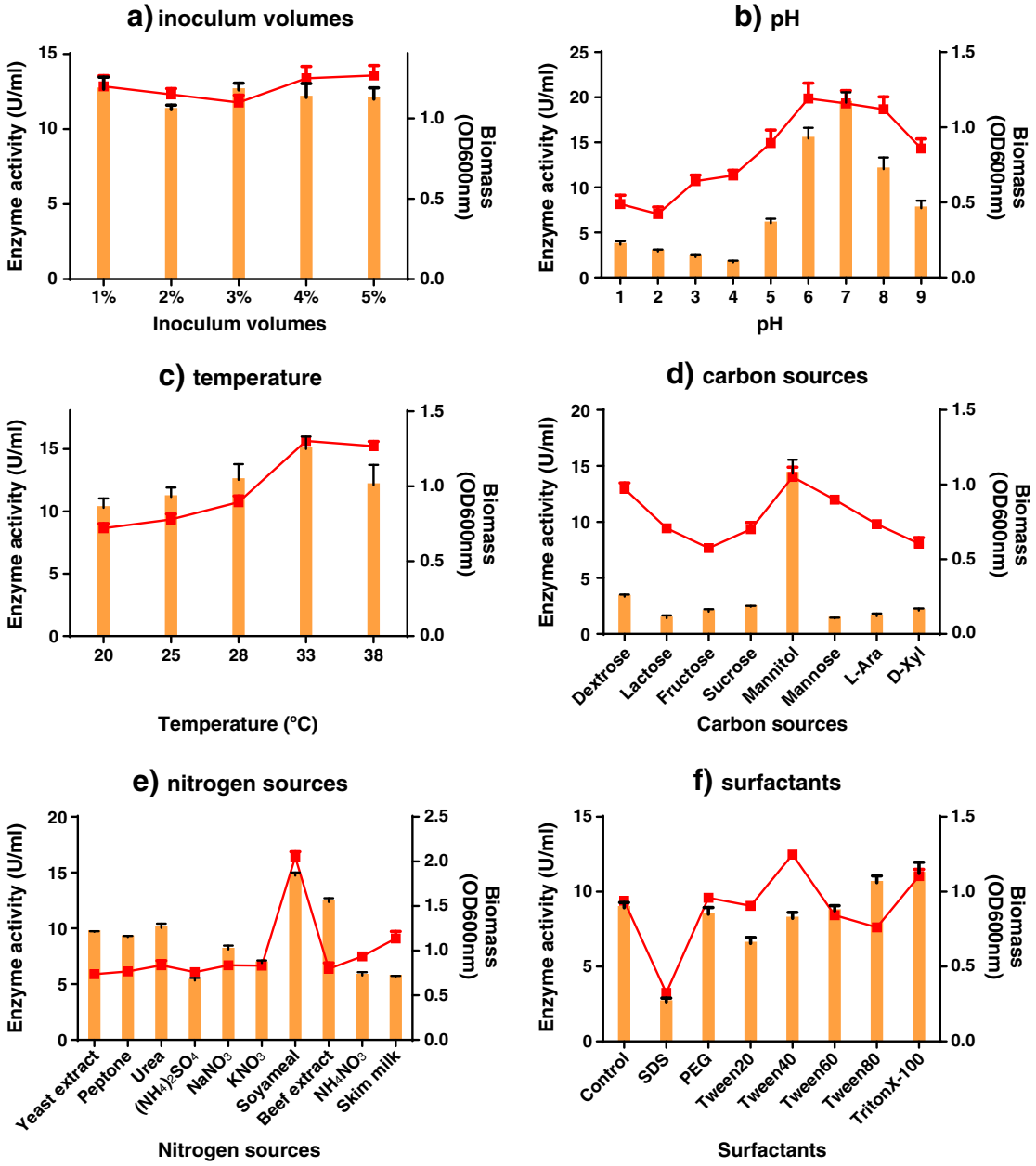

Fig. 1. Single-factor optimization of arabinofuranosidase production by *P. polymyxa*. (a) inoculum volumes, (b) pH, (c) temperature, (d) carbon sources, (e) nitrogen sources, and (f) surfactants. The bar chart represents enzymatic activity, and the line chart represents biomass ($OD600_{nm}$). Values presented are the mean value of three independent experiments.

3.3. Optimization of parameters for α-L-arabinofuranosidase production by response surface methodology

On the basis of the results of single-factor experiments, four variables, namely, pH, temperature, mannitol, and soymeal, were found to affect α-L-arabinofuranosidase production significantly. To study the effect of their interactions on α-L-arabinofuranosidase production, a BBD experiment was performed. The design matrix and the corresponding α-L-arabinofuranosidase yield are shown in Table S1. Multiple regression analysis was used to analyze the experimental data (Table S2). The independent variables and the dependent variables are related by the following second-order polynomial equation:

$$Y = +0.53 + 0.065^{*}X_1 - 0.12^{*}X_2 - 0.082^{*}X_3 + 0.13^{*}X_4 - 0.034^{*}X_1{}^{*}X_2 + 7.500E{-}003^{*}X_1{}^{*}X_3 - 3.250E{-}003^{*}X_1{}^{*}X_4 - 0.024^{*}X_2{}^{*}X_3 - 0.11^{*}X_2{}^{*}X_4 - 0.19^{*}X_3{}^{*}X_4 - 0.20^{*}X_1{}^{2} + 0.077^{*}X_2{}^{2} - 4.467E{-}003^{*}X_3{}^{2} + 0.081^{*}X_4{}^{2}$$

where Y is the activity of α-L-arabinofuranosidase (U/mL) and X_1, X_2, X_3, and X_4 represent the variables for pH, temperature (°C), mannitol (g/L), and soymeal (g/L), respectively. Multiple nonlinear regressions using Design-Expert software version 8.0 gave an F value of 15.96, and the corresponding probability of failure value ($P > F$) of 0.0985 for the lack-of-fit test further supports the significance of the model. Values of "Prob > F" less than 0.05 indicate that the model terms are significant. In this experiment, X_1, X_2, X_3, X_4, X_2X_4, X_3X_4, X_1^2, X_2^2, and X_4^2 were significant model terms (Fig. S2).

The optimum values were calculated from the model equation. The corresponding uncoded optimum values are pH 7.49, temperature 28.1°C, mannitol 1.48 g/L, and soymeal 3.48 g/L. For convenient operation, the parameters were amended to pH 7.5, temperature 28°C, mannitol 1.5 g/L, and soymeal 3.5 g/L. Verification of the model equation was performed by conducting experiments in triplicates for α-L-arabinofuranosidase production under the above conditions. Maximal α-L-arabinofuranosidase production of 25.2 ± 0.5 U/mL was

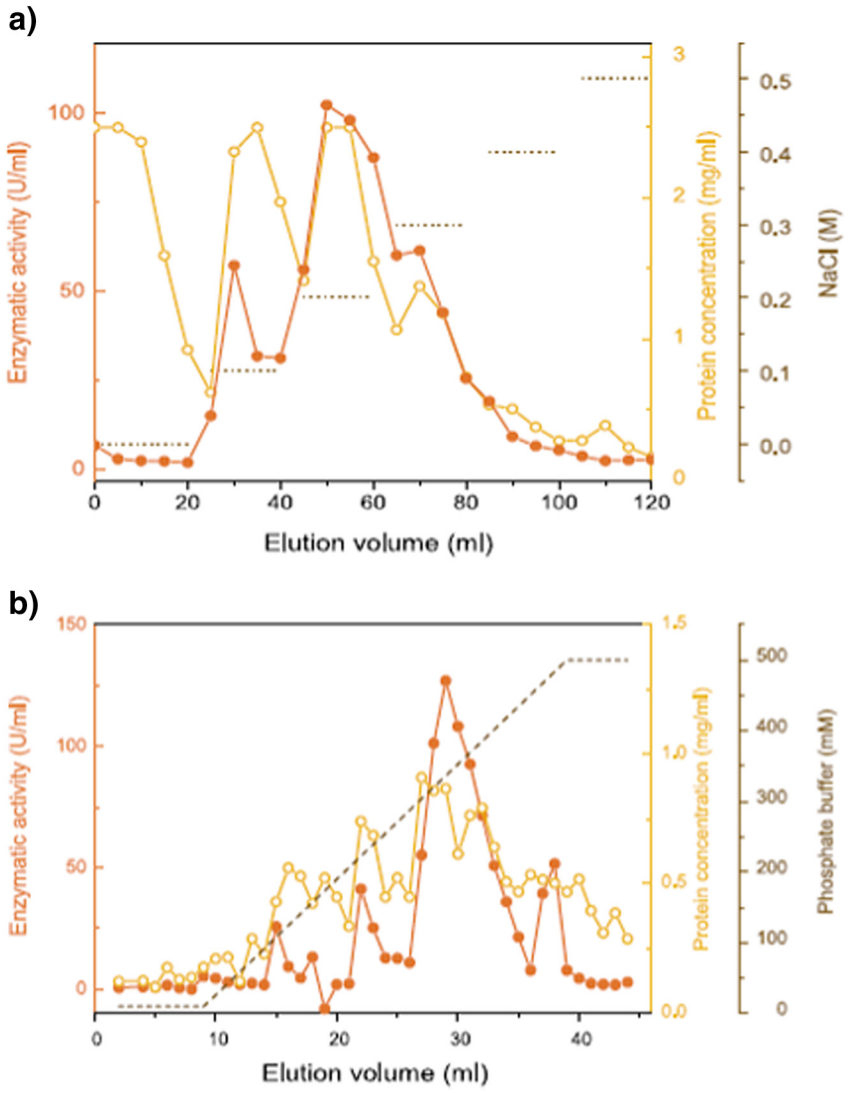

Fig. 2. Purification of PpAFase-1 from the fermentation supernatant of *P. polymyxa* using the (a) DEAE Sepharose Fast Flow column and (b) hydroxyapatite column. Solid circle represents arabinofuranosidase activity assayed with pNPAra*f* as a substrate; hollow circle represents the protein concentration assayed by the Bradford method ($OD595_{nm}$); dotted line represents the concentration of NaCl (a) and phosphate buffer (b).

achieved following incubation for 36 h, whereas production in basic medium was 18.1 U/mL. The predicted response for α-L-arabinofuranosidase production (25.6 U/mL) was detected to be in close agreement to the actual response. The excellent correlation between theoretical and experimental values validates the model.

3.4. Purification of PpAFase-1

A two-step procedure involving ion exchange chromatography and hydroxyapatite column was performed to purify the α-L-arabinofuranosidase. The fraction numbers 10–14 from DEAE Sepharose Fast Flow chromatography showed the highest α-L-arabinofuranosidase activity and was pooled to obtain a final aliquot of 20 mL (Fig. 2a). The active fractions were further purified by hydroxyapatite column chromatography. The fraction numbers 24–30 showed the highest enzyme activity (Fig. 2b). After purification, a final specific activity of 50.2 U/mg and a yield of 5.2% were achieved (Table 1).

3.5. Electrophoresis and mass spectrometry analysis

The crude extract and partially purified PpAFase-1 were analyzed by SDS-PAGE (Fig. 3a). The arabinofuranosidase produced by *P. polymyxa*

Table 1
Purification of PpAFase-1 from *P. polymyxa*.

Procedure	Volume (ml)	Total activity (kU)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification
Purification						
Fermentation broth	800	17.25	1026.2	16.8	100.0	1.0
DEAE Sepharose Fast Flow column	20	1.95	52.5	37.1	11.3	2.2
Hydroxyapatite chromatography	7	0.89	17.8	50.2	5.2	3.0
Heteroexpression						
Crude enzyme extract	50	12.5	304	41.1	100	1.0
Ni Sepharose Fast Flow column	10	4.48	57.1	78.4	35.8	1.91

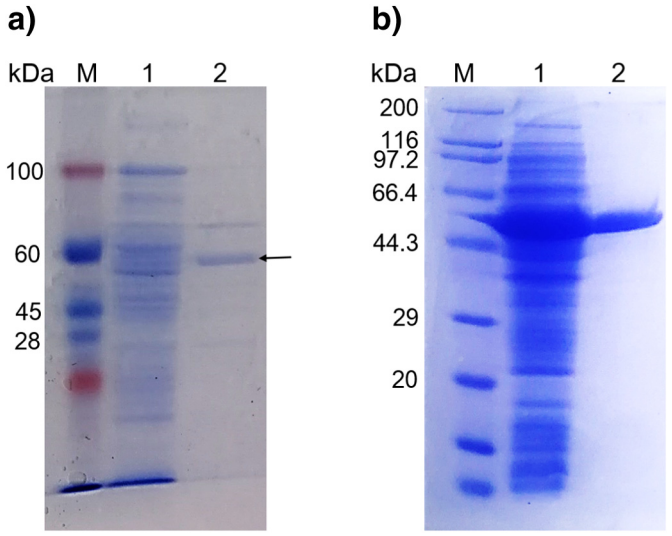

Fig. 3. SDS-PAGE analysis: (a) fermentation supernatant of *P. polymyxa* and partially purified PpAFase-1 after passing through the hydroxyapatite column: M, Protein Marker (Tiagen); 1, fermentation supernatant of *P. polymyxa*; 2, partially purified PpAFase-1 after passing through the hydroxyapatite column; the arrow indicates the band (~56.8 kDa) cut for mass spectrometry analysis; (b) recombinant PpAFase-1 analysis: M, Premixed Protein Marker (Broad) (Takara); 1, culture lysate of recombinant *E. coli* BL21 (DE3) cells after IPTG induction; 2, PpAFase-1 purified from an Ni Sepharose Fast Flow column. The gel was stained with Coomassie Brilliant Blue R-250.

was purified as a predominant protein band with approximate molecular mass of 56.8 kDa. Previously, the molecular masses of the two polypeptides exhibiting α-L-arabinofuranosidase activity encoded by the *xynD* gene from *P. polymyxa* were determined to be 64 and 53 kDa [27]. Other GH51 α-L-arabinofuranosidases possess similar molecular masses, such as the ~57-kDa rAbfA enzyme from *Paenibacillus* sp. DG-22 [31], the 59-kDa AbfATK4 protein from *Geobacillus caldoxylolyticus* TK4 [32], the 56-kDa α-L-arabinofuranosidase from *B. pumilus* [33], and the 56-kDa AbfD3 from *Thermobacillus xylanilyticus* [34].

Inspection of the genome sequence of *P. polymyxa* revealed that the predicted Mr of the gene products annotated as putative α-L-arabinofuranosidase is between 52 and 57 kDa. Therefore, the band at 56.8 kDa on the SDS-PAGE gel was excised, and nanoLC-MS/MS analysis was performed. Twenty-three peptides were identified from the band and to belong to *P. polymyxa* α-L-arabinofuranosidase (Fig. S3). The peptides were spliced and compared with the sequences in GenBank using the BLASTp program. The deduced amino acid sequence of PpAFase-1 was consistent with the GH family 51 α-L-arabinofuranosidase with the protein ID WP_019685877.1. Previously, several GH51 α-L-arabinofuranosidases from different microorganisms have been characterized. Among the genus of the *P. polymyxa*, the two polypeptides AF64 and AF53 show α-L-arabinofuranosidase activity and are encoded by the *P. polymyxa xynD* gene. The AF64 and AF53 polypeptides share the same sequence at their N-terminus but differ from that of PpAFase-1 [27].

The majority of the GHs from family 51 hydrolyze the glycosidic bond between the L-arabinofuranoside side chains of hemicelluloses. Currently,

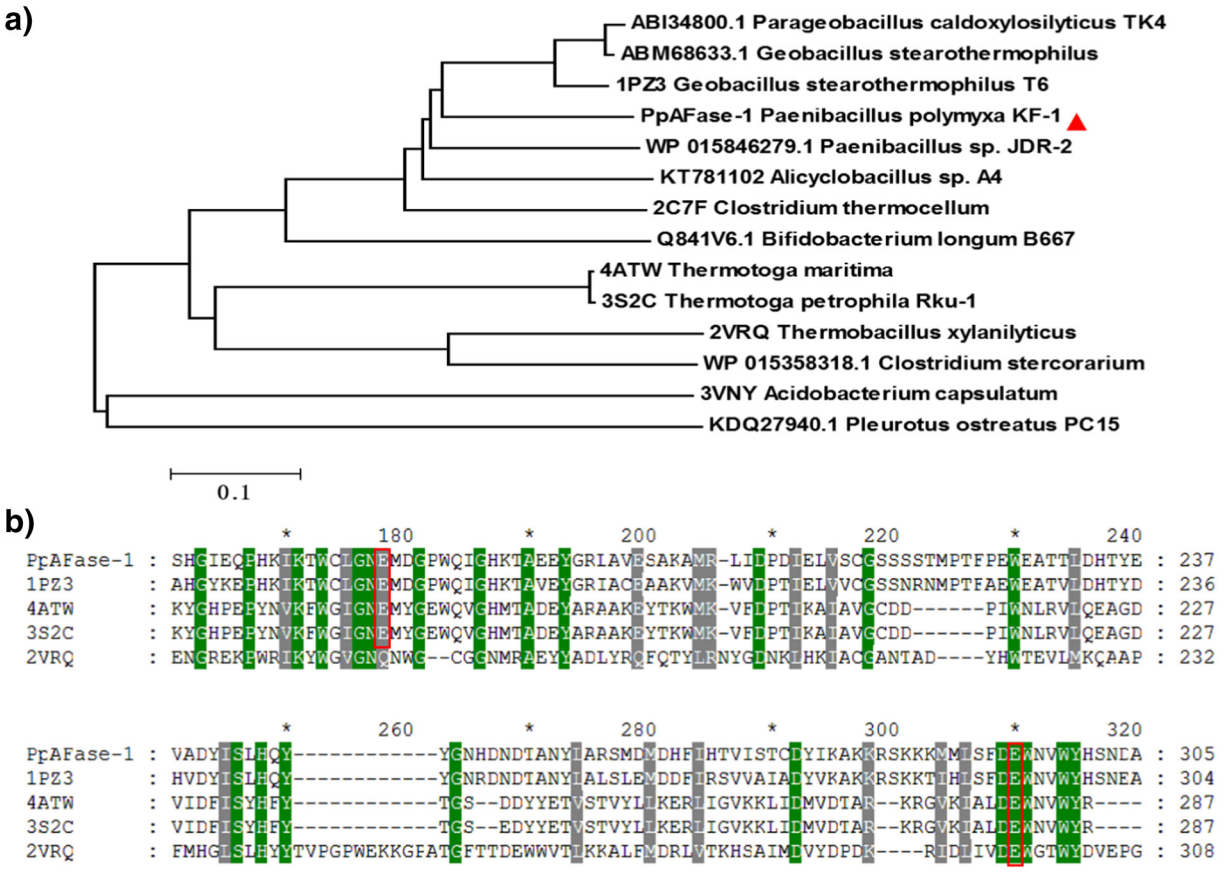

Fig. 4. Phylogenetic analysis (a) and multiple amino acid sequence alignment (b) of PpAFase-1 with GH51 enzymes. The two catalytic Glu residues are enclosed in red boxes. The phylogenetic tree was drawn by MEGA6. The alignment was carried out using MEGA6, and the figure was edited using GeneDoc.

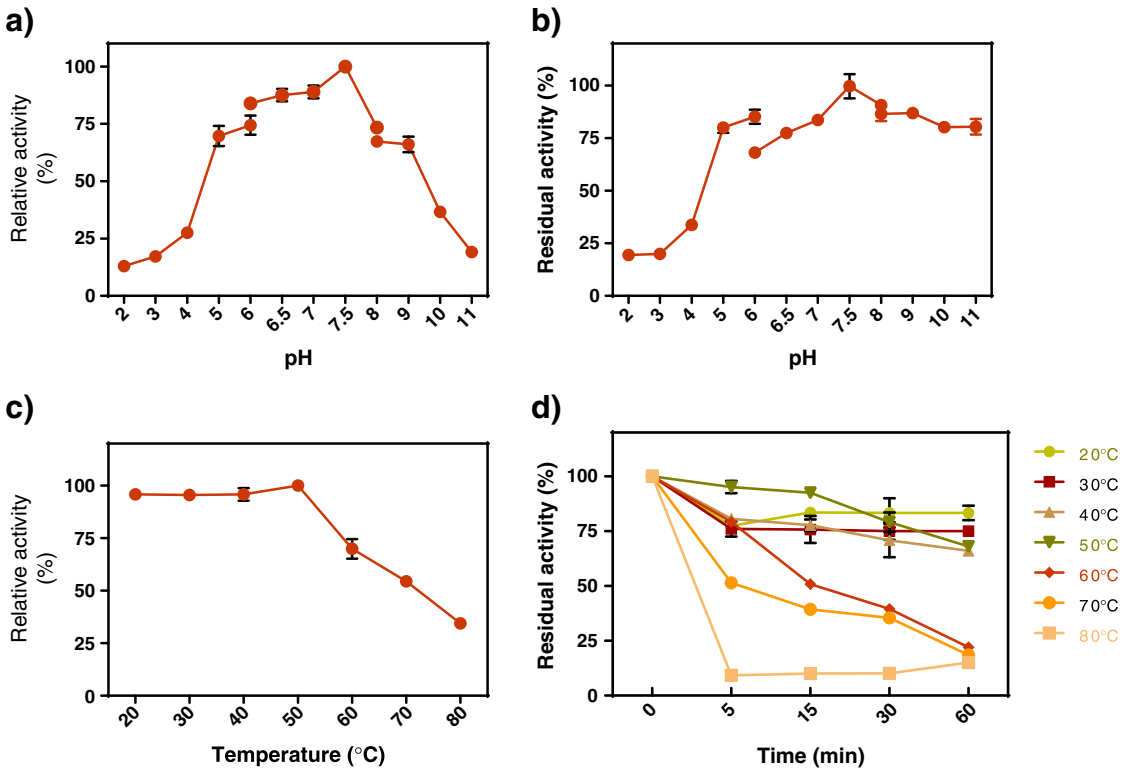

Fig. 5. Effects of pH and temperature on the activity and stability of PpAFase-1. (a) Optimal pH; (b) pH stability; (c) optimal temperature; and (d) thermostability. Values presented are the mean value of three independent experiments.

several crystal structures of GH51 α-L-arabinofuranosidase isolated from different microorganisms including *Geobacillus stearothermophilus*, *Clostridium thermocellum*, *Thermotoga xylanilyticus*, *Thermotoga petrophila*, and *Thermotoga maritime* have been reported [17,19,34,35,36]. Multiple sequence alignment of PpAFase-1 with the characterized GH51 proteins is shown in Fig. 4. PpAFase-1 showed identities with the GH51 arabinofuranosidases from *G. stearothermophilus* T-6 (74% identity, ABM68633), *Paenibacillus* sp. JDR-2 (69%, YP_003013427), *C. thermocellum* ATCC 27405 (65% identity, YP_001038942), and *T. petrophila* RKU-1 (36% identity, YP_001244227). Analysis of these reported structures revealed that two essential Glu residues, which are highly conserved in the aligned sequences, are required for catalysis in GH51 α-L-arabinofuranosidase enzymes. For the *G. stearothermophilus* T-6 α-L-arabinofuranosidase, the Glu175 residue in the consensus motif NEM was identified as the acid/base and the Glu294 residue in the consensus motif DEW was identified as the nucleophile [17]. The corresponding Glu176 and Glu295 residues in PpAFase-1 may have similar catalytic roles. SignalP 4.1 software (http://www.cbs.dtu.dk/services/SignalP/) analysis showed that no signal sequence was present.

Table 2
Effects of metal ions and chemicals on PpAFase-1 activity.

Compound (10 mM)[a]	Relative activity (%)[b]	Statistical significance[c]
Control	100.00 ± 2.13	
Na^+	70.66 ± 1.51	**
K^+	71.36 ± 1.52	**
Ca^{2+}	85.92 ± 1.83	*
Mg^{2+}	62.21 ± 1.33	**
Fe^{2+}	30.28 ± 0.65	**
Mn^{2+}	88.73 ± 1.89	*
Cu^{2+}	47.18 ± 1.01	**
Zn^{2+}	87.09 ± 1.86	*
Al^{3+}	99.06 ± 2.11	–
Co^{2+}	104.23 ± 2.22	–
Fe^{3+}	127.00 ± 2.71	**
EDTA	65.96 ± 1.41	**
DTT	81.92 ± 1.75	*
SDS	61.97 ± 1.32	**
Tween-40	67.14 ± 1.43	**
Tween-60	64.08 ± 1.37	**
Tween-80	61.50 ± 1.31	**
TritonX-100	65.96 ± 1.41	**

[a] All reagents were used in their chloride forms and dissolved in deionized water.
[b] The activity assayed in the absence of compounds was taken as 100%. Values presented are the mean value of three independent experiments.
[c] Statistical significance: –, not significant; * $P < 0.05$, significant; ** $P < 0.01$, very significant.

3.6. Cloning, expression, and purification of PpAFase-1

The deduced gene of PpAFase-1 (accession number MH360731) was cloned from the genome of *P. polymyxa* and expressed in *E. coli* BL21

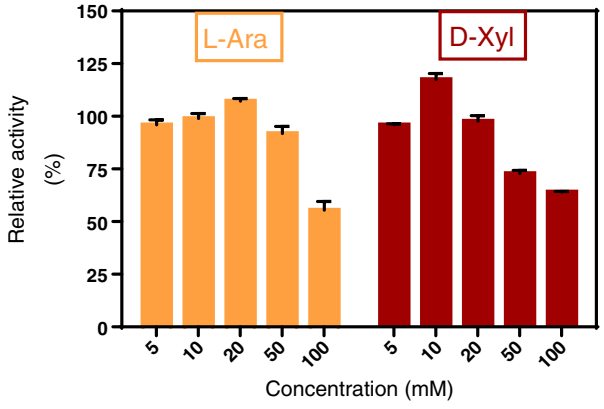

Fig. 6. Effects of L-arabinose and D-xylose on PpAFase-1 activity. Values presented are the mean value of three independent experiments.

Table 3
Specificity of PpAFase-1 for different substrates.

Substrate	Specific activity (U/mg)[a]
p-Nitrophenyl-α-L-arabinofuranoside	76.7 ± 1.2
p-Nitrophenyl-α-L-arabinopyranoside	ND
p-Nitrophenyl-α-D-glucopyranoside	ND
p-Nitrophenyl-β-D-glucopyranoside	2.75 ± 0.09
p-Nitrophenyl-β-D-mannopyranoside	ND
p-Nitrophenyl-β-D-xylopyranoside	2.97 ± 0.05
Arabinobiose	15.5 ± 0.74
Xylobiose	ND
Arabinan	0.55 ± 0.02
Arabinogalactan	ND
Oat spelt xylan	3.05 ± 0.12
Wheat arabinoxylan	3.52 ± 0.06

ND: not detected.
[a] Specific activity values are represented as mean ± SD (n = 3).

(DE3) cells. The recombinant enzyme was purified using an Ni Sepharose Fast Flow column. Data on enzyme purification from a 200-mL culture are summarized in Table 1. After purification, the recombinant enzyme was purified to 1.91-fold and a yield of 35.8% was achieved; 57.1 mg of purified PpAFase-1 was obtained from a 200-mL culture. The specific activity of the purified recombinant enzyme was 78.4 U/mg. SDS-PAGE analysis showed a single band with a molecular mass of approximately 56.8 kDa (Fig. 3b).

3.7. Characterization of the purified PpAFase-1

3.7.1. Effect of temperature and pH

The purified PpAFase-1 was found to be most active at pH 7.5. The enzyme was remarkably active across the pH range of 5.0–9.0 (Fig. 5a). This optimal pH range is typical for α-L-arabinofuranosidases isolated from bacteria, with usually slightly acidic pH values ranging from 5.5 to 7.0, whereas most fungal α-L-arabinofuranosidases have pH optimum of less than 5.0 [13]. The enzyme exhibited stability across a wide pH range. After incubating the enzyme in buffers with a pH range between 5.0 and 11.0 for 24 h and 4°C, more than 80% of the activity of PpAFase-1 was retained (Fig. 5b), thus indicating that this enzyme may be suitable for potential use in industrial applications because of its considerable stability across a wide pH range.

The optimum temperature of PpAFase-1 was found to be 50°C with pNPAra*f* as the substrate for the reaction of 30 min. Almost 70% of α-L-arabinofuranosidase activity remained at 60°C (Fig. 5c). Previously reported GH51 α-L-arabinofuranosidases have higher optimal temperatures such as 60°C for rAbfA from *Paenibacillus* sp. DG-22 [31], 75–80°C for AbfATK4 from *G. caldoxylolyticus* TK4 [32], and 75°C for

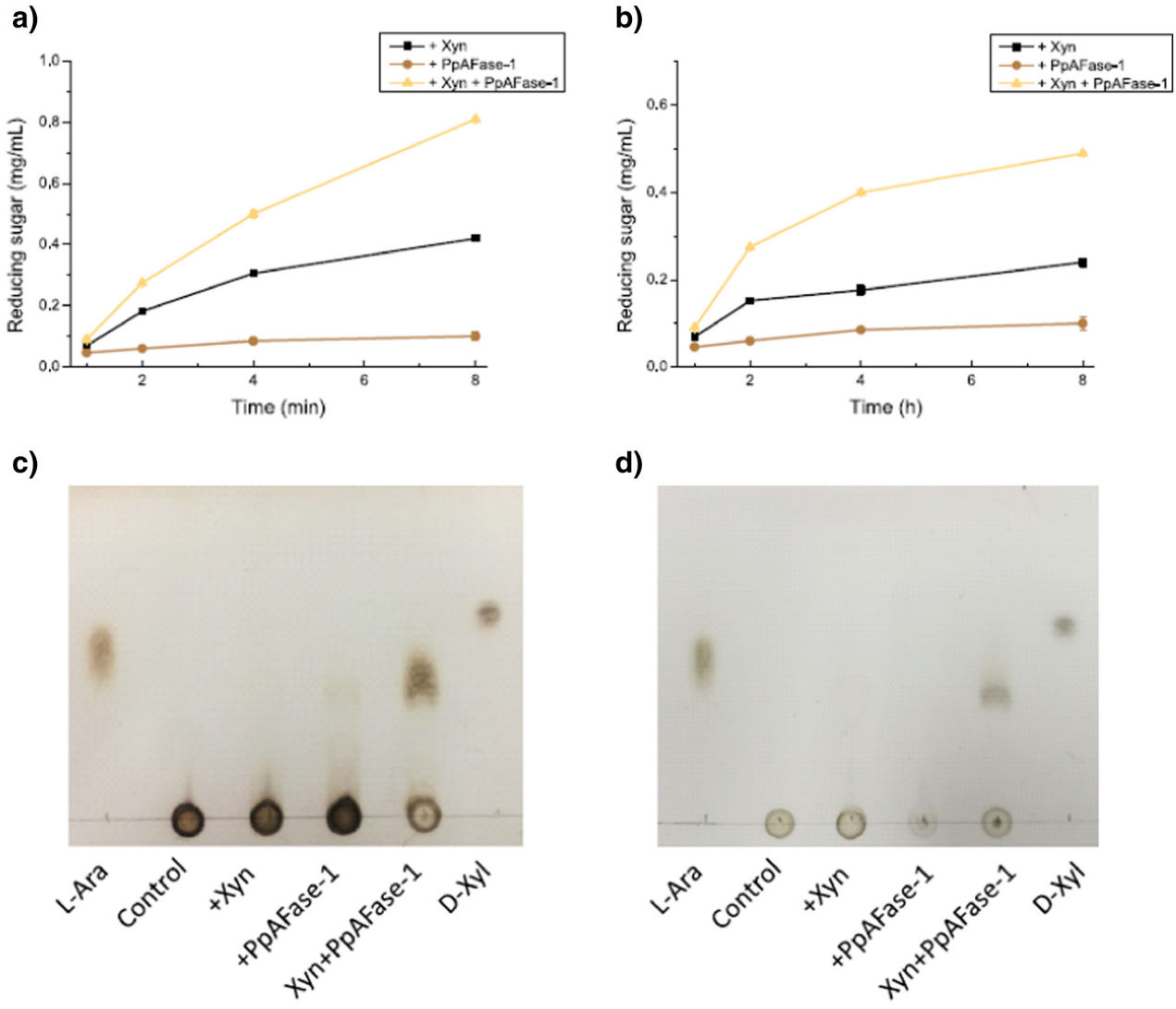

Fig. 7. Hydrolysis of oat spelt xylan and wheat arabinoxylan by the combination of purified PpAFase-1 and *T. lanuginosus* xylanase (Xyn). The amounts of reducing sugars released during hydrolysis of xylan (a) and arabinoxylan (b) were determined using the DNS reagent; the hydrolysis of xylan (c) and arabinoxylan (d) after 8 h was analyzed by the TLC method.

AbfD3 from *T. xylanilyticus* [34]. The thermal denaturation study revealed that *P. polymyxa* α-L-arabinofuranosidase exhibited reasonable thermostability. After 60 min of incubation, approximately 68% of the enzyme retained activity at 50°C (Fig. 5d). The half-life of PpAFase-1 was ~105 min at 50°C.

3.7.2. Effects of metal ions and chemicals

The effect of various metal ions and chemicals on α-L-arabinofuranosidase activity was determined (Table 2). Addition of Fe^{3+} enhanced the α-L-arabinofuranosidase activity by 27%. Addition of Co^{2+} and Al^{3+} did not affect α-L-arabinofuranosidase activity significantly. Cu^{2+}, Hg^{2+}, and Zn^{2+} are known inhibitors for some α-L-arabinofuranosidases from this family [37]. Herein, Zn^{2+} was found to reduce the enzyme activity but only modestly (less than 15%), whereas Cu^{2+} strongly inhibited the activity of PpAFase-1. In addition, Mn^{2+} and Ca^{2+} reduced enzyme activity slightly, whereas Fe^{2+} strongly inhibited the activity of PpAFase-1. The chemicals DTT, EDTA, SDS, Tween-40, Tween-60, Tween-80, and Triton X-100 partially inhibited the activity of PpAFase-1 with more than 50% activity retained.

3.7.3. Effects of L-arabinose and D-xylose

In hemicellulosic biomass degradation, L-arabinose and D-xylose are the main monosaccharides present in the reaction system; therefore, tolerance to L-arabinose and D-xylose has become a vital property for α-L-arabinofuranosidase application. Herein, the effects of L-arabinose and D-xylose on PpAFase-1 were examined, and the results indicate that PpAFase-1 exhibited remarkable tolerance to L-arabinose and D-xylose. In the presence of 50-mM L-arabinose and D-xylose, 91.8% and 72.8% relative activity of the enzyme was retained, respectively. In the presence of 100-mM L-arabinose and D-xylose, 55.4% and 64% relative activity of the enzyme was retained, respectively (Fig. 6).

3.8. Substrate specificity and kinetics analysis

When using *p*-nitrophenyl-linked glycosides as substrates, PpAFase-1 was highly active toward pNPAra*f*, and it showed minor activity toward *p*-nitrophenyl-β-D-xylopyranoside (pNPXyl). No activity was observed toward other *p*-nitrophenyl-linked glycosides tested (Table 3). Several GH51 enzymes have both β-xylosidase and α-L-arabinosidase activities, such as AbfATK4 from *G. caldoxylolyticus* TK4 [32]. Similar to AbfATK4, PpAFase-1 showed a higher arabinofuranosidase activity than xylosidase activity. In addition, PpAFase-1 showed activity toward arabinobiose, with specific activity of 15.5 U/mg. The kinetic parameters of PpAFase-1 were investigated using pNPAra*f* as the substrate. The enzyme exhibited typical Michaelis–Menten kinetics with K_m, V_{max}, and k_{cat} values of 0.81 ± 0.06 mM, 56.2 ± 2.06 U/mg protein, and 53.2 s^{-1}, respectively.

Natural arabinose-containing polymers including arabinan, arabinogalactan, xylan, and arabinoxylan were incubated with PpAFase-1. The results showed that no detectable reducing sugars could be measured when arabinogalactan was used as the substrate. However, PpAFase-1 exhibited minor hydrolytic activity toward sugar beet arabinan, oat spelt xylan, and wheat arabinoxylan, with specific activities of 0.55, 3.05, and 3.52 U/mg, respectively (Table 3).

3.9. Hydrolysis of xylan and arabinoxylan by PpAFase-1 and synergy with xylanase

Previously, synergistic effects between α-L-arabinofuranosidase and xylanase in xylan degradation have been reported [31]. We mixed PpAFase-1 with *T. lanuginosus* xylanase (Xyn) to determine the synergistic effects between xylanase and α-L-arabinofuranosidase. The TLC results showed that PpAFase-1 exhibited very poor hydrolytic activity against oat spelt xylan (Fig. 7c) and wheat arabinoxylan (Fig. 7d). After incubation with PpAFase-1 for 8 h, only a small amount of arabinose was released (Fig. 7a and b). Combining PpAFase-1 and Xyn significantly improved the release of reducing sugars from oat spelt xylan and wheat arabinoxylan, and synergistic effects were observed between PpAFase-1 and Xyn on the degradation of oat spelt xylan and arabinoxylan, with a 1.56- and 1.44-fold increase in the amount of reducing sugar released, respectively (Table 4). The hydrolytic products were arabinose, xylose, and xylobiose, identified by HPAEC (Fig. 8). This is probably because PpAFase-1 removes the arabinose residues from the side chain, and this eliminates steric hindrance and exposure of additional sites that can be converted by xylanase.

Table 4
Synergistic action of Xyn and PpAFase-1 on the degradation of oat spelt xylan and wheat arabinoxylan.

Enzyme	Liberated reducing sugar (mg/mL)[a]	Degree of synergy[b]
Oat spelt xylan		
Xyn	0.42 ± 0.01	
PpAFase-1	0.10 ± 0.01	
Xyn + PpAFase-1	0.81 ± 0.02	1.56
Wheat arabinoxylan		
Xyn	0.24 ± 0.01	
PpAFase-1	0.10 ± 0.01	
Xyn + PpAFase-1	0.49 ± 0.01	1.44

[a] Reducing sugar was measured by the DNS method.
[b] Degree of synergy = synergistic activity (Xyn + PpAFase-1)/the sum of the activities of each enzyme separately.

Previously, several α-L-arabinofuranosidases isolated from the fungi *Aspergillus* sp. and *Penicillium* sp. were reported. AbfA from *Aspergillus niger* was specifically active toward pNPAra*f* and 1,5-α-L-linked arabino-oligosaccharides. AbfB from *A. niger* had activity toward beet and apple arabinan as well as arabinoxylan [38]. The *Penicillium chrysogenum* α-L-arabinofuranosidase belonging to the GH family 51 was characterized in a study. The enzyme exhibited broad substrate specificity and released arabinose from arabinan, arabinoxylan, arabinogalactan, and arabino-oligosaccharides [39]. The GH43 α-L-arabinofuranosidase isolated from *P. chrysogenum* was active on sugar beet L-arabinan; the enzyme preferentially degraded single-

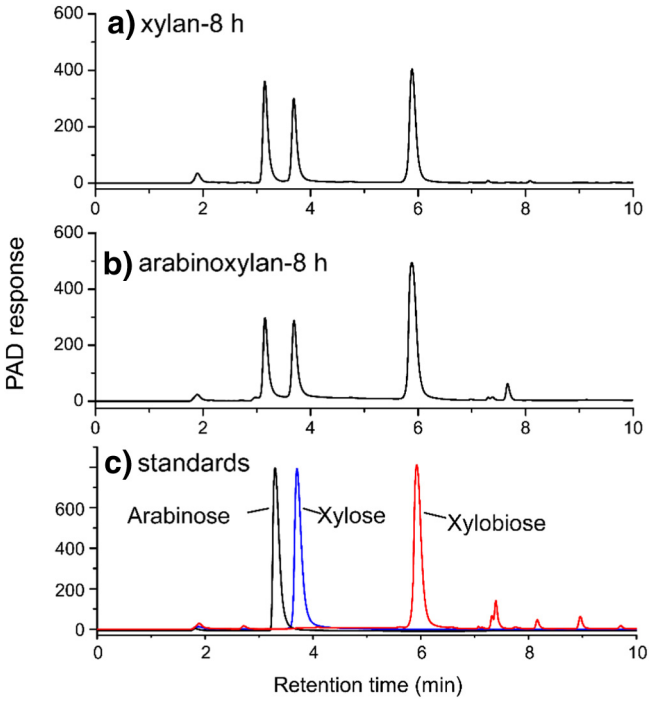

Fig. 8. Identification of hydrolytic products from xylan and arabinoxylan by the combination of PpAFase-1 and *T. lanuginosus* xylanase (Xyn) by HPAEC. (a) Products from xylan, (b) products from arabinoxylan, (c) standards L-arabinose, D-xylose, and xylobiose.

substituted arabinosyl side chains from arabinan [40]. The GH51 Ac-Abf51A from *Alicyclobacillus* sp. A4 exhibited a synergistic effect with endo-xylanase on the degradation of wheat arabinoxylan [10], which was similar to that exerted by PpAFase-1. However, the catalytic efficiency of PpAFase-1 was still lower than that of some reported arabinofuranosidases, which promote us to optimize the catalytic process.

4. Conclusion

A novel extracellular α-L-arabinofuranosidase was cloned from *P. polymyxa* and identified to belong to GH family 51. The enzyme with striking thermostability, wide pH stability, and tolerance to arabinose and xylose might be suitable for use in industrial applications such as hemicellulose degradation.

Conflict of interest

The authors declare no competing interests.

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Supplementary material

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References

[1] Li Q, Song J, Peng S, et al. Plant biotechnology for lignocellulosic biofuel production. Plant Biotechnol J 2014;12(9):1174–92. https://doi.org/10.1111/pbi.12273 PMID: 25330253.
[2] Peng P, She D. Isolation, structural characterization, and potential applications of hemicelluloses from bamboo: a review. Carbohydr Polym 2014;112(4):701–20. https://doi.org/10.1016/j.carbpol.2014.06.068 PMID: 25129800.
[3] Pauly M, Gille S, Liu L, et al. Hemicellulose biosynthesis. Planta 2013;238(4):627–42. https://doi.org/10.1007/s00425-013-1921-1.
[4] Scheller HV, Ulvskov P. Hemicelluloses. Annu Rev Plant Biol 2010;61:263–89. https://doi.org/10.1146/annurev-arplant-042809-112315.
[5] Zhang L, Peng X, Zhong L, et al. Lignocellulosic biomass derived functional materials: synthesis and applications in biomedical engineering. Curr Med Chem 2017;24:1–18. https://doi.org/10.2174/0929867324666170918122125.
[6] Liu ZH, Fatehi P, Sadeghi S, et al. Application of hemicelluloses precipitated via ethanol treatment of pre-hydrolysis liquor in high-yield pulp. Bioresour Technol 2011;102(20):9613–8. https://doi.org/10.1016/j.biortech.2011.07.049 PMID: 21865032.
[7] Girio FM, Fonseca C, Carvalheiro F, et al. Hemicelluloses for fuel ethanol: a review. Bioresour Technol 2010;101(13):4775–800. https://doi.org/10.1016/j.biortech.2010.01.088 PMID: 20171088.
[8] Jia L, Budinova GALG, Takasugi Y, et al. Synergistic degradation of arabinoxylan by free and immobilized xylanases and arabinofuranosidase. Biochem Eng J 2016;114:268–75. https://doi.org/10.1016/j.bej.2016.07.013.
[9] Zhang JH, Siika-Aho M, Tenkanen M, et al. The role of acetyl xylan esterase in the solubilization of xylan and enzymatic hydrolysis of wheat straw and giant reed. Biotechnol Biofuels 2011;4(1):60. https://doi.org/10.1186/1754-6834-4-60 PMID: 22185437.
[10] Yang WX, Bai YG, Yang PL, et al. A novel bifunctional GH51 exo-α-L-arabinofuranosidase/endo-xylanase from *Alicyclobacillus* sp. A4 with significant biomass-degrading capacity. Biotechnol Biofuels 2015;8(197). https://doi.org/10.1186/s13068-015-0366-0 PMID: 26628911.
[11] Wilkens C, Andersen S, Dumon C, et al. GH62 arabinofuranosidases: structure, function and applications. Biotechnol Adv 2017;35(6):792–804. https://doi.org/10.1016/j.biotechadv.2017.06.005 PMID: 28669588.
[12] Numan MT, Bhosle NB. α-L-Arabinofuranosidase: the potential applications in biotechnology. J Ind Microbiol Biotechnol 2006;33(4):247–60. https://doi.org/10.1007/s10295-005-0072-1. PMID: 16385399.
[13] Saha BC. α-L-Arabinofuranosidase: biochemistry, molecular biology and application in biotechnology. Biotechnol Adv 2000;18(5):403–23. https://doi.org/10.1016/S0734-9750(00)00044-6 PMID: 14538102.
[14] Cantarel BL, Coutinho PM, Rancurel C, et al. The Carbohydrate-Active EnZymes database (CAZy). An expert resource for glycogenomics. Nucleic Acids Res 2009;37(sup 1):D233–8. https://doi.org/10.1093/nar/gkn663 PMID: 18838391.
[15] Carapito R, Imberty A, Jeltsch JM, et al. Molecular basis of arabinobio-hydrolase activity in phytopathogenic fungi: crystal structure and catalytic mechanism of *Fusarium graminearum* GH93 exo-alpha-L-arabinanase. J Biol Chem 2009;284(18):12285–96. https://doi.org/10.1074/jbc.M900439200 PMID: 19269961.
[16] Amore A, Iadonisi A, Vincent F, et al. Identification of the nucleophile catalytic residue of GH51 α-L-arabinofuranosidase from *Pleurotus ostreatus*. AMB Express 2015;5(1):79. https://doi.org/10.1186/s13568-015-0164-x PMID: 26690659.
[17] Hövel K, Shallom D, Niefind K, et al. Crystal structure and snapshots along the reaction pathway of a family 51 α-L-arabinofuranosidase. EMBO J 2003;22(19):4922–32. https://doi.org/10.1093/emboj/cdg494 PMID: 14517232.
[18] Souza TACB, Santos CR, Souza AR, et al. Structure of a novel thermostable GH51 α-L-arabinofuranosidase from *Thermotoga petrophila* RKU-1. Protein Sci 2011;20(9):1632–7. https://doi.org/10.1002/pro.693 PMID: 21796714.
[19] Taylor EJ, Smith NL, Turkenburg JP, et al. Structural insight into the ligand specificity of a thermostable family 51 arabinofuranosidase, Araf51, from *Clostridium thermocellum*. Biochem J 2006;395(1):31–7. https://doi.org/10.1042/BJ20051780 PMID: 16336192.
[20] Hu Y, Yan X, Zhang H, et al. Cloning and expression of a novel α-1,3-arabinofuranosidase from *Penicillium oxalicum* sp. 68. AMB Express 2018;8(1):51. https://doi.org/10.1186/s13568-018-0577-4 PMID: 29611040.
[21] Yoon JH, Kim JH, Ham SS, et al. Optimal processing conditions of *Boswellia carteri* Birdw. using response surface methodology. Pharmacogn Mag 2018;14(54):235–41. https://doi.org/10.4103/pm.pm_140_17 PMID: 29720838.
[22] Bradford MM. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72(1-2):248–56. https://doi.org/10.1016/0003-2697(76)90527-3 PMID: 942051.
[23] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227(5259):680–5. https://doi.org/10.1038/227680a0 PMID: 5432063.
[24] Patel H, Kumar AK, Shah A. Purification and characterization of novel bi-functional GH3 family β-xylosidase/β-glucosidase from *Aspergillus niger* ADH-11. Int J Biol Macromol 2018;109:1260–9. https://doi.org/10.1016/j.ijbiomac.2017.11.132 PMID:29174354.
[25] Yuan Y, Ji L, Hu YB, et al. High yield preparation of ganglioside GM1 using recombinant sialidase from *Cellulosimicrobium cellulans*. Process Biochem 2017;58:92–7. https://doi.org/10.1016/j.procbio.2017.04.010.
[26] Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 1959;31(3):426–8. https://doi.org/10.1021/ac60147a030.
[27] Morales P, Sendra JM, Pérez-González JA. Purification and characterization of an arabinofuranosidase from *Bacillus polymyxa* expressed in *Bacillus subtilis*. Appl Microbiol Biotechnol 1995;44(1-2):112–7. https://doi.org/10.1007/BF00164489 PMID: 8579824.
[28] Ramana KV, Tomar A, Singh L. Effect of various carbon and nitrogen sources on cellulose synthesis by *Acetobacter xylinum*. World J Microbiol Biotechnol 2000;16(3):245–8. https://doi.org/10.1023/A:1008958014270.
[29] Raut GR, Chakraborty S, Chopade BA, et al. Isolation and characterization of organic solvent stable protease from alkaliphilic marine *Saccharopolyspora* species. Indian J Geo Mar Sci 2013;42:131–8.
[30] Tiwari S, Shukla N, Mishra P, et al. Enhanced production and characterization of a solvent stable amylase from solvent tolerant *Bacillus tequilensis* RG-01: thermostable and surfactant resistant. Sci World J 2014;972763. https://doi.org/10.1155/2014/972763 PMID: 25401163.
[31] Lee SH, Lee YE. Cloning, expression, and characterization of a thermostable GH51 α-L-arabinofuranosidase from *Paenibacillus* sp. DG-22. J Microbiol Biotechnol 2014;24(2):236–44. https://doi.org/10.4014/jmb.1308.08078 PMID: 24248090.
[32] Canakci S, Belduz AO, Saha BC, et al. Purification and characterization of a highly thermostable alpha-L-arabinofuranosidase from *Geobacillus caldoxylolyticus* TK4. Appl Microbiol Biotechnol 2007;75(4):813–20. https://doi.org/10.1007/s00253-007-0884-1 PMID: 17361432.
[33] Degrassi G, Vindigni A, Venturi V. A thermostable α-arabinofuranosidase from xylanolytic *Bacillus pumilus*: purification and characterisation. J Biotechnol 2003;101(1):69–79. https://doi.org/10.1016/S0168-1656(02)00304-8 PMID: 12523971.
[34] Debeche T, Cummings N, Connerton I, et al. Genetic and biochemical characterization of a highly thermostable α-L-arabinofuranosidase from *Thermobacillus xylanilyticus*. Appl Environ Microbiol 2000;66(4):1734–6. https://doi.org/10.1128/AEM.66.4.1734-1736.2000 PMID: 10742272.
[35] Paës G, Skov LK, O'Donohue MJ, et al. The structure of the complex between a branched pentasaccharide and *Thermobacillus xylanilyticus* GH-51 arabinofuranosidase reveals xylan-binding determinants and induced fit. Biochemistry 2008;47(28):7441–51. https://doi.org/10.1021/bi800424e PMID: 18563919.
[36] Dumbrepatil A, Park JM, Jung TY, et al. Structural analysis of α-L-arabinofuranosidase from *Thermotoga maritima* reveals characteristics for thermostability and substrate specificity. J Microbiol Biotechnol 2012;22(12):1724–30. https://doi.org/10.4014/jmb.1208.08043 PMID: 23221536.
[37] Pei J, Shao W. Purification and characterization of an extracellular α-L-arabinosidase from a novel isolate *Bacillus pumilus* ARA and its over-expression in *Escherichia coli*. Appl Microbiol Biotechnol 2008;78:115–21. https://doi.org/10.1007/s00253-007-1295-z PMID: 23221536.
[38] Rombouts FM, Voragen AGJ, Searle-van Leeuwen MF, et al. The arabinanases of *Aspergillus niger*—purification and characterisation of two α-L-arabinofuranosidases and an endo-1,5-α-L-arabinanase. Carbohydr Polym 1988;9:25–47. https://doi.org/10.1016/0144-8617(88)90075-6.
[39] Sakamoto T, Kawasaki H. Purification and properties of two type-B α-L-arabinofuranosidases produced by *Penicillium chrysogenum*. Biochim Biophys Acta Gen Subj 2003;1621(2):204–10. https://doi.org/10.1016/S0304-4165(03)00058-8 PMID: 12726996.
[40] Shinozaki A, Kawakami T, Hosokawa S, et al. A novel GH43 α-L-arabinofuranosidase of *Penicillium chrysogenum* that preferentially degrades single-substituted arabinosyl side chains in arabinan. Enzyme Microb Technol 2014;58-59:80–6. https://doi.org/10.1016/j.enzmictec.2014.03.005 PMID: 24731829.