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Expression and Characterization of the *Dictyoglomus thermophilum* Rt46B.1 Xylanase Gene (*xynB*) in *Bacillus subtilis*

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Abstract To obtain extracellular and high-level expression of the *Dictyoglomus thermophilum* Rt46B.1 xylanase B gene, this gene was integrated into the α-amylase gene site of a host strain of *Bacillus subtilis* WB800. The extreme thermophile xylanase gene was successfully integrated and expressed in the host, measured at 24±0.4 XUs/mL in the Luria broth medium supernatant. The recombinant enzyme was purified by ammonium sulfate precipitation, anion exchange chromatography, and gel filtration. The molecular mass and p*I* value of xylanase were estimated to be 24 kDa and 4.3, respectively. The optimal pH level and temperature of the purified enzyme were 6.5 and 85 °C, respectively. Xylanase showed reasonable activity at temperatures up to 95 °C and remained stable at 4 °C for 1 week. The purified enzyme retained most of its activity in 1 mM ethylenediaminetetraacetic acid or dithiothreitol and 0.1% Tween-20 or Triton X-100. However, strong inhibition was observed in the presence of 5 mM Mn^{2+}, 0.5% sodium dodecyl sulfate, Tween-20, or Triton X-100; a strong stimulating effect was also observed in the presence of Fe^{2+}. The K_m and V_{max} values of the recombinant xylanase for birchwood xylan were calculated to be 2.417±0.36 mg/mL and 325±41 μmol/min mg, respectively. Xylanase was found to be useful in the prebleaching process of paper pulps.

Keywords Thermostable xylanase · Purification · Characterization · *Bacillus subtilis*

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Introduction

Xylan is a main component of hemicellulose compounds present in plant cell walls and middle lamella. It consists of a backbone of β-1,4-linked xylopyranose residues with branches containing arabinofuranosyl, acetyl, and glucuronosyl residues [1–3]. The hydrolysis of the xylan backbone requires the combined actions of several enzymes, all of which act cooperatively to convert xylan into its constituent sugars. Of these enzymes, endoxylanase acts to convert xylan to xylooligosaccharides and is crucial to this process [3, 4].

Xylanases have received growing attention due to their biotechnological uses and potential applications in industrial processes, such as the modification of cereal-based food products, the improvement of the digestibility of animal feed stocks [5], and the bioconversion of lignocellulosic materials and agrowastes to fermentable products. In recent years, scientists have paid more attention to the xylanase enzymes that are active at high temperatures and pH levels with the hopes that these enzymes might reduce the need for toxic chemicals in the pulp and paper industry, such as chlorine and chlorine dioxide [6–8], which are used in the bleaching process of pulps. Thus, these enzymes have the potential to promote the development of more environmentally friendly industrial processes [9–11].

Xylanases are widely found in fungi and bacteria [12–14]. Most are optimally active in neutral or acidic pH ranges with optimal temperatures at or below 45 °C. For application in the prebleaching of kraft pulp, enzymes that are optimally active and stable both at higher temperatures and pH levels are of great interest [15–17]. As such, the thermostable xylanases from thermophiles and mesophiles have attracted considerable interest. Although thermostable xylanases have many advantages, they are normally produced at low levels in extremely thermophilic bacteria. Since the advent of protein engineering techniques, a molecular approach to promoting the expression of foreign proteins in prokaryotic systems has become recognized as a good method for achieving rapid extracellular overexpression of proteins.

The gene of *xynB* (GenBank accession number DTU76545) was isolated from *Dictyoglomus thermophilum* Rt46B.1 [18], which was isolated from a deep-sea hydrothermal field. Although we have successfully expressed the proteins in *Escherichia coli* DB1 strain, the expression was low level and intracellular and thus not suitable for large-scale industrial applications. In order to actualize high-level expression and study the relationship between the structure and function of the gene, we synthesized the gene, revised seven rare codons, and changed codons 26 and 203. We report that we have successfully expressed the enzyme in the host *Bacillus subtilis*, purified the extracellular protein, and performed further characterization of the protein.

Materials and Methods

Strains, Plasmid, Enzymes, Chemicals, and Growth Conditions

E. coli DH5α was used as the host for plasmid propagation. *B. subtilis* WB800 was used as a host strain for the integrative expression of xylanase. *E. coli* and *B. subtilis* were grown at 37 °C in Luria broth (LB) medium or on agar (1.5% *w*/*v*) plates supplemented with ampicillin (50 μg mL^{-1}) or chloramphenicol (5 μg mL^{-1}), when these antibiotics were required for plasmid selection and protein expression, respectively. The integration vector

pDG364 and the plasmid of pET-xynB containing the *D. thermophilum* Rt46B.1 *xynB* gene were stored at −20 °C in our laboratory. The restriction/ligation enzymes, *Taq* DNA polymerase, and a gel extraction kit were obtained from Takara Company. Primers were synthesized by Sangon. Birchwood xylan was purchased from Sigma Chemical Company. A dig probe detection kit was purchased from Innogent Company. All other chemicals used were analytical grade reagents, unless otherwise stated.

Construction of Integration Plasmid and Integration of the *xynB* Gene

The plasmid of pET-xynB containing the *xynB* gene was digested with *Eco*RI and *Bgl*II; the fragment was then recovered and ligated into the integration vector of pDG364, which had been linearized by *Eco*RI and *Bam*HI (Fig. 1a), *E. coli* competent cells were transformed with the ligation mixture, and the cells were plated on LB–ampicillin medium. The positive colonies were screened by digestion with *Eco*RI and *Bam*HI [19]. The recombinant plasmid was named pDG364-xynB and was propagated with *E. coli*.

The recombinant-linearized (*Pst*I) pDG364-xynB plasmid was transformed into competent cells of *B. subtilis* [20]; the cells were then plated on LB–chloramphenicol medium (http://www.bgsc.org/Catalogs/Catpart4.pdf). The positive recombinants in which the foreign genes were integrated into the amyE region of the *B. subtilis* chromosome by a double-crossover mechanism [21] were named *B. subtilis-xynB* (Fig. 1b). Taking *B. subtilis-xynB* chromosomal DNA as the template, polymerase chain reaction (PCR) amplification with primers (forward [atgcaaacgtctataaca] and reverse [ttcactaactacttccact]) was used to confirm that a single double-crossover event had occurred, and the fragments were verified by DNA sequencing.

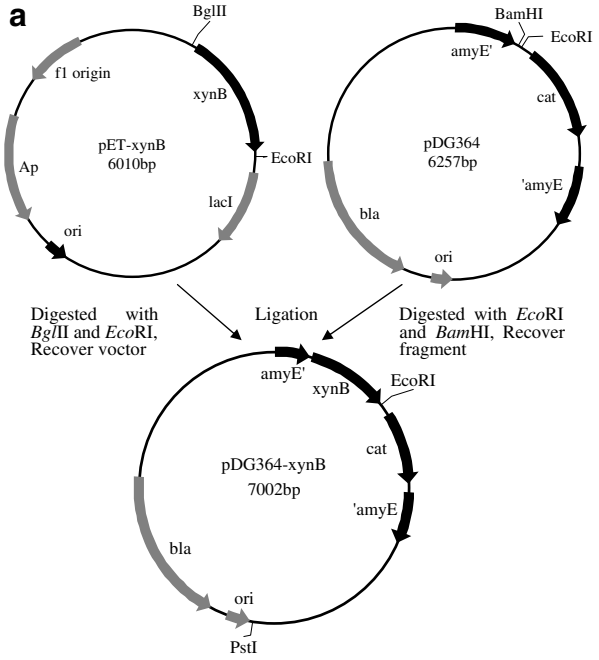
Amylase and Xylanase Activity Assay

The positive recombinants were screened using the *B. subtilis* strain as a control. The α-amylase activity was assayed as described by selected methods (http://www.bgsc.org/Catalogs/Catpart5.pdf).

The thermostable xylanase activity was quantitatively determined in LB culture supernatants using birchwood xylan (Sigma Chemical, St. Louis, MO, USA) as the substrate. The enzymatic release of the reducing sugar was determined by a modification of the method of Lever [22]. A 60-μL sample of culture supernatant, appropriately diluted in 50 mM sodium phosphate buffer, pH 6.5 at 85 °C, was mixed with 1,140 μL of xylan solution (0.25% birchwood xylan in the same buffer) and incubated at 85 °C for 10 min. The reaction was terminated by the addition of 1,800 μL of coloring reagent (50 mM *para*-hydroxybenzoic acid hydrazide, 300 mM NaOH, 50 mM Na_2SO_3, 15 mM $CaCl_2$, and 200 mM trisodium citrate). The mixture was boiled for 5 min, and A_{405} was measured. The concentration of the reducing sugar present was determined using D-xylose as a standard. The enzyme activity was expressed in xylanase units (XUs) where 1 XU is the amount of enzyme required to release 1 mmol of reducing sugar from xylan per minute [23]. For each assay in this study, the experiments were performed in triplicate and the reported results represent averages.

Culture and Crude Xylanase Purification

The transformants were cultured in 50 mL of LB medium containing 5 μg/mL chloramphenicol in 500-mL Erlenmeyer flasks. The flasks were incubated in a shaker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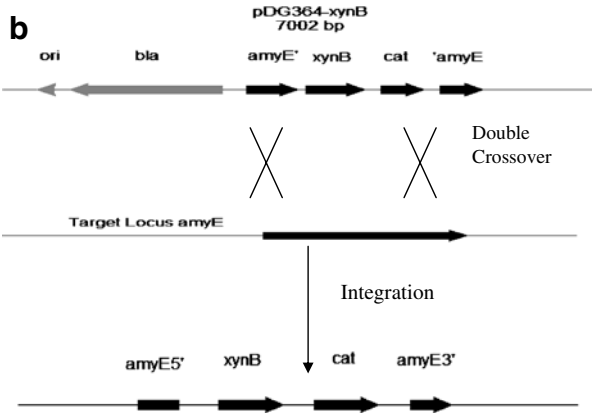
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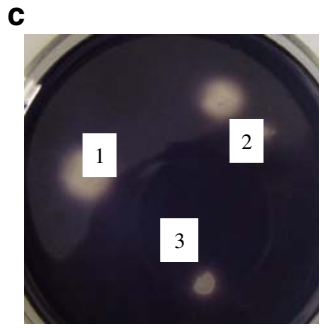
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incubator at 37 °C with constant shaking at 180 rpm for 22 h. The cells were removed by centrifugation (4,000×*g*, 10 min, 4 °C), and the liquid supernatant was used as the enzyme source. All of the following operations were performed at 4 °C, unless otherwise mentioned. The crude xylanase purification was carried out by ammonium sulfate precipitation [24]. Solid ammonium sulfate was slowly added to the supernatant with constant stirring to achieve 30% saturation. After centrifugation at 12,000×*g* for 10 min, the precipitate was discarded and the supernatant was subsequently adjusted to 70% saturation by the addition of calculated amounts of ammonium sulfate. The final precipitate was recovered by centrifugation (12,000×*g*, 10 min) and was then dissolved in a minimal amount of water and dialyzed against deionized ice-cold water for at least 16 h; the water was replaced three times to remove the residual ammonium sulfate. Finally, the enzyme solution was equilibrated with 50 mM Tris–HCl buffer, pH 6.5. The dialysate was centrifuged, and the undissolved material was removed. The clear supernatant was put on a DEAE Sepharose Fast Flow (Amersham Biosciences, Uppsala, Sweden) column equilibrated with the same buffer. Elution was performed with a linear gradient of 0–0.5 M NaCl. The fractions exhibiting xylanolytic activity were pooled and concentrated. The concentrate was fractionated using a Sephadex G-75 (Amersham Biosciences, Uppsala, Sweden) column at a flow rate of 8 mL/h. The xylanolytic fractions were pooled, concentrated, and refractionated on the same G-75 column at a flow rate of 4 mL/h. The enzyme obtained after the second gel filtration was used for characterization.

Protein Analysis

The homogeneity of the purified enzyme was verified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using a 12% polyacrylamide gel [25]. Protein bands were stained with Coomassie brilliant blue R-250 (Bio-Rad). The isoelectric point of the purified protein was determined by Rotofor using broad-range ampholytes (Bio-Rad Laboratories, Hercules, CA, USA). The dialyzed, $(NH_4)_2SO_4$-precipitated protein was subjected to isoelectric focusing after the addition of ampholytes (IEF 3-10). The pH level and xylanase activity of each fraction were determined. The protein concentration was determined following a bicinchonic acid method using bovine serum albumin as a standard [26].

Optimum Temperature and Thermostability of the Xylanase

The optimum temperature was determined by performing a xylanase activity assay at various temperatures ranging from 50 to 95 °C at pH 6.5 (optimum). The thermostability of the xylanase was investigated at temperatures ranging from 50 to 100 °C after the enzyme solutions had been incubated without substrate at the optimum pH level for 60 min at different temperatures. Residual activities were determined under xylanase activity assay conditions. All enzyme assays were performed in triplicate.

Optimum pH Level and pH-Dependent Stability of the Xylanase

The effect of the pH level on the xylanase activity was evaluated at the optimal temperature at various pH levels. The pH levels used varied from 4.0 to 10.0. Four different buffers

◀ **Fig. 1** **a** Construction of recombinant plasmid pDG364-xynB. **b** Double crossover between linearized pDG364-xynB and chromosome of *B. subtilis* in amyE site. **c** Test for the production of α-amylase. *1* and *2* show *B. subtilis* with a clearing halo and *3* shows *B. subtilis-xynB* without a clearing halo on starch-containing medium

(50 mM) were used: sodium acetate (pH 4.0 to 6.0), sodium phosphate (pH 6.0 to 8.0), Tris–HCl (pH 7.5 to 9.0), and glycine–NaOH (pH 9.0 to 10.0). Under the xylanase activity assay conditions, further study of the pH-dependent stability of the xylanase was carried out at 85 °C by preincubation of the enzyme solutions in the aforementioned buffer systems (pH 4.5 to 8.5) in the absence of substrate for 15, 30, 45, 60, 75, and 90 min, respectively. After the pH values of the various reaction solutions were adjusted to 6.5, the residual enzyme activities were measured following the xylanase activity assay.

Effects of Metal Ions on the Xylanase Activity

The effect of metal ions on the xylanase activity was determined by measuring the residual activity after preincubation of the enzyme solutions with various metal ions (1.0 and 5.0 mM) at 85 °C for 10 min. To avoid the formation of insoluble phosphates, 50 mM succinic acid buffer (pH 6.5) was used instead of the phosphate buffer.

Effect of Inhibitors and Detergents on the Xylanase Activity

To determine the effect of inhibitors such as SDS, ethylenediaminetetraacetic acid (EDTA), and dithiothreitol (DTT), the xylanase was preincubated with these inhibitors at concentrations of 0.1%/1 mM or 0.5%/5 mM at 85 °C for 10 min, followed by a xylanase activity assay. The effect of the detergents, for example, TritonX-100 and Tween-20 at a final concentration of 0.1% (*w*/*v*) and 0.5% (*w*/*v*), on the xylanase activity was also monitored by the same procedure.

Kinetic Parameters of the Xylanase

Kinetic studies of the xylanase were performed at a constant enzymatic concentration using ten different concentrations of birchwood xylan ranging from 1 to 10 mg/mL. Each concentration was analyzed in triplicate. All xylanase activity experiments were carried out at 85 °C in a phosphate buffer (50 mM, pH 6.5). A typical Lineweaver–Burk plot [27] was obtained by plotting $1/[v]$ against $1/[S]$. The kinetic parameters (K_m and V_{max}) were estimated by linear regression from the Lineweaver–Burk plot.

Results

Construction and Expression of the *B. subtilis-xynB* Strain Containing an Additional Thermostable Xylanase Gene in the amyE Locus

The construction of the xynB integration vector is shown in Fig. 1a. The *B. subtilis-xynB* strain is derived from the *B. subtilis* strain and carries an additional copy of the *xynB* gene encoding thermostable xylanase, which comes from the *D. thermophilum* Rt46B.1. The *xynB* gene and the chloromycetin gene were integrated into the amyE locus of *B. subtilis* by a double crossover via the integration vector pDG364-xynB. The *xynB* gene and the chloromycetin gene were controlled by the *amyE* gene promoter which is inducible by starch (Fig. 1b). Further PCR analysis confirmed the integration of the *xynB* gene in the amyE locus; the right fragment was verified by DNA sequencing. To examine the change in enzyme activity, a batch culture was performed in a 500-mL Erlenmeyer flask with 50 mL of LB medium. As

expected, the thermostable xylanase activity of the *B. subtilis-xynB* strain was significantly increased in the cultured supernatant and failed to digest the starch (Fig. 1c). The specific activity of the thermostable xylanase was observed in the crude extract of the *B. subtilis-xynB* strains and *B.subtilis* by measuring the activity of the thermostable xylanase.

Enzyme Purification and Characterization

The xylanase was purified from the culture supernatant of *B. subtilis-xynB*, following the steps indicated in Table 1; the total activity (in units), total protein (in milligrams), and specific activity (in xylanase units per milligram) of the xylanase in the crude enzyme filtrate are also listed. A homogenous enzyme preparation was obtained, as analyzed by SDS-PAGE (Fig. 2). This gene has been expressed in the *E. coli* DB1 strain. The molecular weight and p*I* value of the enzyme were estimated to be 24 kDa and 4.3, respectively.

Effect of Temperature on Activity and Thermostability

The recombinant xylanases exhibited optimal activity at 85 °C with similar activity levels measured at temperature ranging from 75 to 100 °C (Fig. 3a). Thermostability assays indicated that the recombinant xylanases remained stable at 50 to 100 °C (pH 6.5) for at least 1 h. However, the half-life of the recombinant xylanase was about 60 min at 85 °C (pH 6.5), and the recombinant xylanase lost more than 55% of its activity after a 60-min incubation at 95 °C (Fig. 3b).

Effect of pH Level on Activity and Stability

The investigation of xylanase activity at various pH values revealed that the optimum pH value for the recombinant xylanases was 6.5 (Fig. 4a). The recombinant xylanases were stable in acidic pH values ranging from 5.5 to 7.5, retaining more than 80% of their initial activity. However, the activity of the recombinant xylanases decreased sharply at pH levels below 5.0 or above 8.0. The recombinant xylanase seemed to exhibit good tolerance at partially acidic pH levels. It presented higher stability after incubation at 85 °C for 30 min, showing 88.1% and 84.4% residual activity at pH 6.5 and 5.5, respectively (Fig. 4b).

Effect of Additives on Activity

The effects of different metal ions on the recombinant xylanase are shown in Table 2. The metal ions Zn^{2+}, Hg^{2+}, DTT, SDS, EDTA, Tween-20, and Triton X-100 showed partial

Table 1 Summary of *B. subtilis-xynB* xylanase purification.

Purification steps	Volume (mL)	Total activity (U)	Total protein (mg)	Specific activity (XU/mg)	Recovery (%)
Culture supernatant	1,000	2,520	452	5.58	100
Ammonium sulfate ppt	50	1751	218	8.03	69.5
Ion exchange chromatography	98	1212	119	10.18	48.1
First gel filtration	89	631	11.2	56.3	25.0
Second gel filtration	72	313	1.4	223.6	12.4

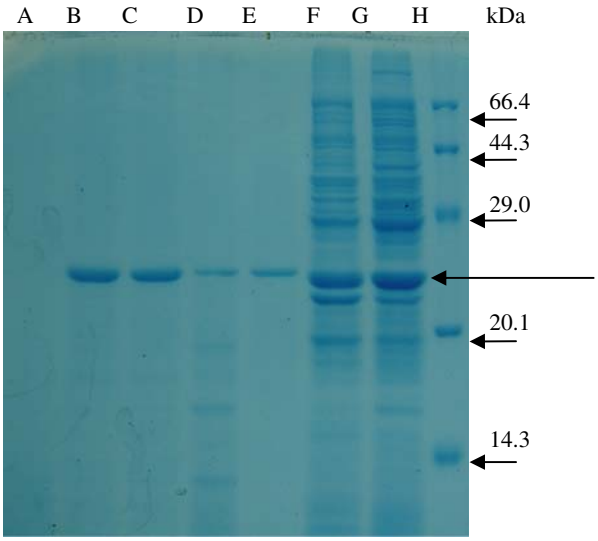

Fig. 2 12% SDS-PAGE analysis of the purified xylanase. *Lane A* purified protein of *B. subtilis* (control); *lanes B*, *C*, and *E B. subtilis-xynB* (second gel filtration with different loading quantity); *lane D B. subtilis-xynB* (first gel filtration); *lanes F* and *G* expressed xylanase from recombinant *E. coli* DB1 strain; *lane H* protein molecular weight markers

inhibition of the activity at 0.1%/1 mM concentrations, while the divalent ions of heavy metals, such as Mn^{2+}, as well as EDTA, DTT, SDS, Tween-20, and Triton X-100, gave strong inhibition at 5 mM concentrations. Monovalent cations, such as Na^{+} and K^{+}, had a small stimulating effect on the enzymatic activity, but Fe^{2+} had strong stimulating effect on the enzymatic activity.

Enzyme Kinetics

The kinetic parameters of the recombinant xylanase were obtained from Lineweaver–Burk plots of specific activities at 85 °C and pH 6.5 with different substrate concentrations. The results show that its K_m and V_{max} values were 2.417±0.36 mg/mL and 325±41 μmol/mg min, respectively.

Discussion

As thermophiles have adapted to survive at high temperatures, they produce thermostable biocatalysts capable of functioning under extreme conditions, comparable to those present in various industrial processes. The enzymes from thermophiles, therefore, are of great interest for industrial applications. The *xynB* gene was cloned years ago, but it still encodes for one of the most heat-resistant xylanases and is thus a good study material for examining the relationship between gene structure and function.

We first expressed the gene in *E. coli*; the activity results have been previously reported as 0.005–25 XU/mL [28]. As the nature of the target proteins plays a critical role in determining the production yield in the expression system, when the gene was synthesized, seven rare codons were revised and codons 26 (serine to threonine) and 203

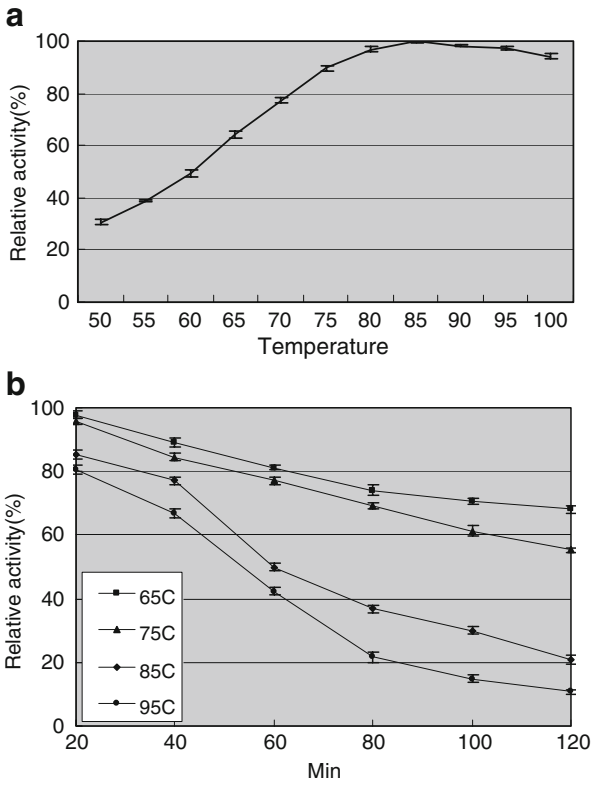

Fig. 3 **a** Effect of temperature on *B. subtilis-xynB* xylanase activity. The enzyme activity was determined by incubating the enzyme with 0.25% (*w*/*v*) birchwood xylan dissolved in water, pH 6.5. The maximum activity is set to 100%. The experiment was performed in triplicate. **b** Effect of temperature on *B. subtilis-xynB* xylanase activity. The residual enzyme activity was monitored after incubation at 65, 75, 85, and 95 °C for different time periods at pH 6.5. The maximum activity is set to 100%. The experiment was performed in triplicate

(serine to glycine) were changed. As a result, in the recombinant strain, the expression of the enzyme activity was increased, but the optimum temperature, compared with the original reported value, was reduced [18]. Nevertheless, the optimum enzyme temperature was still higher than that of xylanase isolated from heat-resistant fungi by over 10 °C [13] and was also higher than those of other xylanases isolated from the heat-resistant bacteria [29]. In the following enzyme characteristics study, we found that the recombinant xylanase from *B. subtilis-xynB* was easily purified because the recombinant xylanase was secreted in the liquid supernatant of the fermentation media. This characteristic would facilitate the application of xylanase in industrial processes.

In this study, we encountered two very difficult issues. One issue resulted from the fact that the host of the *B. subtilis* strain itself also exhibits xylanase activity; however, through a number of xylanase activity tests, we found that, at temperatures greater than 55 °C, no activity was detected. Thus, we concluded that the of *B. subtilis-xynB* strain showed extreme thermophile xylanase activity as a result of the xynB expression. The other issue was that, although the host bacteria have a defective eight-protease gene strain, when the extreme thermophile xylanase was expressed, the *B. subtilis-xynB* strain secreted a variety

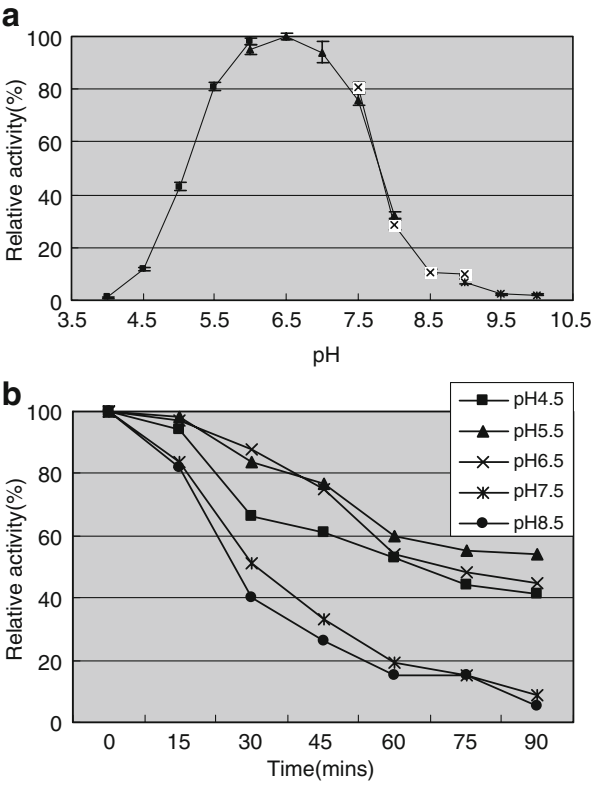

Fig. 4 **a** The effect of pH level on xylanase activity was evaluated at the optimal temperature over a pH range of 4.0 to 10.0 using four different buffers (50 mM): sodium acetate (pH 4.0 to 6.0), sodium phosphate (pH 6.0 to 8.0), Tris–HCl (pH 7.5 to 9.0), and glycine–NaOH (pH 9.0 to 10.0). The maximum activity is set to 100%. The experiment was performed in triplicate. **b** Effects of pH level on the recombinant xylanase stability. The enzyme activity was determined by incubating the enzyme with 0.25% (*w*/*v*) birchwood xylan dissolved in water (pH 6.5). The maximum activity is set to 100%. The experiment was performed in triplicate

of hydrolases; as such, the expression levels of the thermophile xylanase gene negatively affected fermentation. As the host strain can express its own amylase at high levels, the *B. subtilis-xynB* strain should be able to express high levels of the thermophile xylanase in order to meet the requirements of industrial production, if we optimize the fermentation conditions of the *B. subtilis-xynB* strain.

The recombinant xylanase obtained in this study was highly active at temperatures ranging from 75 to 95 °C with an optimal temperature of 85 °C at pH 6.5. This trend is similar to the characteristics of the strain *D. thermophilum* Rt46B.1. In this paper, the xylanase expressed by *B. subtilis-xynB* was characterized; the molecular weight and p*I* value of the enzyme were estimated to be 24 kDa and 4.3, respectively. In accordance with previous reports [28], the xylanase exhibited high activity and good stability under mildly acidity conditions, making it a good candidate for fermentative products. In particular, xylanases that are operationally stable at higher temperatures are more beneficial in the prebleaching process of paper pulps due to the potential for

Table 2 Effect of metal salts, DTT, and EDTA on *B. subtilis-xynB* xylanase activity.

Metal ion/chemical agent	Concentration	Relative activity (%)	Concentration	Relative activity (%)
None (mM)	0	100±0	0	100±0
$FeCl_2$ (mM)	1	213±6.51	5	233±4.93
$ZnCl_2$ (mM)	1	89±3.51	5	76±1.53
$MgCl_2$ (mM)	1	110±2.51	5	108±3.06
$MnCl_2$ (mM)	1	55±3.51	5	24±1.00
$CaCl_2$ (mM)	1	105±2.52	5	100±2.52
$CuCl_2$ (mM)	1	101±1.53	5	68±2.00
$CoCl_2$ (mM)	1	108±2.53	5	79±2.00
KCl (mM)	1	100±2.54	5	104±1.53
NaCl (mM)	1	100±3.00	5	101±1.53
$HgCl_2$ (mM)	1	87±1.52	5	85±2.52
EDTA (mM)	1	94±2.53	5	41±1.53
DTT (mM)	1	95±3.51	5	40±1.53
SDS (%)	0.1	72±3.06	0.5	34±1.00
Tween-20 (%)	0.1	95±1.52	0.5	23±1.53
Triton X-100 (%)	0.1	91±2.50	0.5	32±2.52
β-Mercaptoethanol (%)	0.1	101±1.55	0.5	105±3.00

The maximum activity is set to 100%. The experiment was performed in triplicate

savings in cooling cost and time. Thus far, only a few xylanases with optimum activity temperatures exceeding 70 °C have been reported [30]. There are very few similar enzyme characteristics that can be used for comparison. In this regard, the present xylanase is expected to operate under conditions close to those of most mills (high temperature). Due to high temperature fermentation and acidity conditions, the acidic active xylanases may also find other potential applications: for example, in waste management programs, xylanases can be used to hydrolyze xylan in industrial and municipal waste.

It is generally known that xylanases may be inhibited or activated by metal ions or other reagents. Therefore, the influence of different metal ions and other reagents was investigated, as shown above. The xylanase activity was strongly inhibited by Mn^{2+}. In contrast, it was significantly activated by Fe^{2+} (213%) in concentrations as small as 1 mM. The activity of this xylanase was not significantly inhibited by the presence of different metal ions, while the majority of xylanases from different sources are sensitive to those metal ions [31]. In fact, many impurities, such as metal ions, which can potentially inhibit the activity of xylanase, exist in industrial waste. In view of the processing of impure pulp and other environmental applications, the resistance of this xylanase to different metal ions and chelating agents could be an attractive feature.

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