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EXPERIMENTAL ARTICLES

Characterization of the Novel Xylanase from the Thermophilic *Geobacillus thermodenitrificans* JK1[1]

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Abstract—Thermophilic strain JK1 was isolated from compost using xylan as a single carbon source. On the basis of 16S rRNA gene phylogenetic analysis and *spo0A* gene sequence similarity analysis, strain JK1 was identified as *Geobacillus thermodenitrificans* strain. During the exponential culture growth, the strain JK1 was found to produce the single xylan degrading enzyme ~45 kDa in size. Xylose was not an inducer of this xylanase. Cloning, expression and characterization of the recombinant xylanase were performed. Xylanase of *G. thermodenitrificans* JK1 was cellulase-free; pH and temperature optimums were found to be 6.0 and 70°C, respectively. The metal ions Na^+, K^+, Ca^{2+}, and Co^{2+} showed partial inhibition of the activity, while Mn^{2+} had slight stimulating effect on the enzymatic activity. Recombinant xylanase was thermostable over the temperature range of 55–70°C. It presented the highest stability after incubation at 55°C for 60 min showing 84% residual activity. 50% residual activity was revealed after incubation at 60°C for 60 min as well as at 65 and 70°C for 30 min. Results of the thermostability experiments showed xylanase of JK1 having quite low thermostability when compared with the respective enzymes of the other geobacilli.

Keywords: Geobacillus, G. thermodenitrificans, endospore-forming, low thermostability, thermostable xylanase, xylan

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INTRODUCTION

Xylanases (endo-1,4-β-xylanase, EC 3.2.1.8) are glycosidases that catalyze the hydrolysis of xylan, a major component of hemicellulose complex. Xylanases have been successfully used for pretreatment of animal feed and pulp bleaching, and are considered to be a key player in the biodegradation of renewable resources to useful end products. In most of these processes, a thermostable xylanase would be beneficial since the enzyme load could be decreased and the possibility of microbial contamination could be reduced [1].

Thermophilic endospore-forming bacteria of the genus *Geobacillus* have gained a great deal of attention as the potential producers of the biotechnologically relevant enzymes. Xylanases are not the exception. Xylanases of *Geobacillus thermoleovorans* [2], *Geobacillus stearothermophilus* [3–5] and those of unidentified *Geobacillus* species [6] were described. *xynA* gene was identified in the complete sequenced genome of *Geobacillus thermodenitrificans* NG80-2; some aspects of xylanolytic activity were reported for wild type strains of *G. thermodenitrificans* [7]. It should be noted, however, that Canakci et al. [7] carried out xylanolytic assay only in the supernatants of the cultures, no cloning and purification of the enzymes were performed. Recombinant DNA technologies were reported to be very important for improving various aspects of hemicellulolytic enzymes [8]. But only two xylanases of geobacilli have been cloned and expressed yet [6, 9].

On the other hand, xylanases of the glycoside hydrolase family 10 (GH10) were supposed to be a good example of enzymes with a TIM-barrel fold, the most common enzyme fold [5]. They can be used in the studies on relationships between structure and their properties, especially high thermal stability.

In this paper we report the cloning, expression and characterization of *G. thermodenitrificans* JK1 xylanase from GH10 family—the first extensively characterized xylanase of this thermophilic bacterial species. A few unique characteristics of this xylanase, including low thermostability, were established.

MATERIALS AND METHODS

Isolation and taxonomic characterization of the xylanase producing thermophilic strain JK1. Strain JK1 was isolated from compost using birch wood xylan as a sole carbon source. The genomic DNA was extracted from fresh cell culture using the Genomic DNA Purification Kit (Thermo Fisher Scientific). Amplification and cloning of 16S rRNA gene as well as GEOSPO-PCR were done according to Kuisiene et al. [10, 11]. 16S rRNA gene of the strain JK1 as well as the GEOSPO-PCR product were sequenced, the obtained sequences were edited and sequence similarity

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was determined using the SEQBUILDER and MEGALIGN components of LASERGENE 6 (DNASTAR). 16S rRNA gene sequences of the type strains as well as those of *Geobacillus* strains with the completely sequenced genomes were obtained from the GenBank. The 16S rRNA gene sequences were aligned using the MEGA 4.0 program [12]. The size of the 16S rRNA gene used for alignment was 1391 nucleotides. The phylogenetic tree was constructed using the MEGA 4.0 program by the neighbour-joining method [13]. Pairwise-deletion option was used. Bootstrap analysis of the neighbour-joining data, using 1000 resamplings, was carried out in order to evaluate the validity and reliability of the tree topology.

Preparation of crude xylanase from the wild type strain JK1. In order to analyse the pattern of xylanase secretion, *G. thermodenitrificans* wild type strain JK1 was grown in the medium (100 mL) containing (g/L): xylan, 5.0; yeast extract, 3.0; $NaNO_3$, 3.0; K_2HPO_4, 2.5; NaCl, 1.0; pH 7.0. In some cases xylose was reported to be an inducer of the xylanase secretion [14]. The impact of xylose on JK1 xylanase was analysed in the same medium with xylose (1.0, 5.0 and 10.0 g/L) instead of xylan. Bacteria were cultivated in 250 mL Erlenmeyer flasks at a temperature of 60°C at 180 rpm. Growth was determined by measuring optical density at 590 nm. The pattern of xylanase secretion was tested using well diffusion method. The wells were cut in the medium containing (g/L): xylan, 5.0; agar, 15.0. Aliquots of the culture (200 μL) were taken every two hours, cells were removed by centrifugation (10000 *g*, 5 min) and supernatant of the culture (100 μL) was poured into the well. After 24 h at 60°C the medium with the wells was stained using Congo red solution according to Ng et al. [15].

For crude xylanase preparation *G. thermodenitrificans* JK1 was grown for 10 hours (the exponential growth phase). Cells were removed by centrifugation (7000 *g*, 20 min, 4°C), and solid ammonium sulphate was slowly added to the supernatant with constant stirring to achieve 40% saturation. After centrifugation at 12000 *g* at 4°C for 20 min, the precipitate was discarded, and the supernatant was subsequently adjusted to 60% saturation by addition of calculated amounts of ammonium sulphate. The final precipitate was recovered by centrifugation (12000 *g*, 20 min, 4°C), then dissolved and dialyzed against 50 mM Tris-HCl buffer (pH 7.0/60°C).

Cloning and analysis of the extracellular xylanase gene. In order to characterize the extracellular xylanase gene, the primers GeXyl-F27 (5'-ATG CCC CAC AAT TGG ATC AAC GCT ACA-3') and GeXyl-R27 (5'-GGC GCA TCT TTT CCT TTC CCT TTT TCC-3') were designed. Sequences EU599644 (*G. thermodenitrificans* T-2), GQ857066 and EU931582 (*Geobacillus* sp. strains TC-W7 and WB1) as well as *xynA* sequences extracted from the genomic sequences of *G. thermodenitrificans* NG80-2 (CP000557, 1862421...1863644) and *G. stearothermophilus* T-6 (DQ868502, 68530...69768) were obtained from the GenBank and used for the construction of these primers. The primers were designed using the PRIMERSELECT component of LASERGENE 6 (DNASTAR). Xylanase gene was amplified (GEXYL-PCR) in 50 μL of reaction mixture containing PCR buffer with $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 0.2 mM each dNTP, 0.25 μM each GEXYL primer, 1.25 U recombinant *Taq* DNA Polymerase and 10 ng of bacterial genomic DNA. Reaction mixture was supplemented with 10% (v/v) DMSO. GEXYL-PCR was conducted under the following conditions: initial denaturation at 95°C for 2 min followed by 29 cycles each consisting of 95°C for 1 min, 60°C for 2 min and 72°C for 3 min with a final extension step at 72°C for 7 min in an Eppendorf thermal cycler. Products of amplification were analysed by electrophoresis through 1% agarose gel.

PCR amplicons containing the complete xylanase coding sequence were prepared using the primers JULXYL-F (5'-GGT CGG ATC CCC ATG TTG AAA AGA TCG-3') and JULXYL-R (5'-GGG GAA GCT TTC ACT TAT GAT CGA TAA T-3'). A *Bam*HI site (underlined) was incorporated into the forward primer and a *Hind*III site (underlined) was incorporated into the reverse primer for cloning into pET-28c(+). JULXYL-PCR conditions were the same as stated for GEXYL-PCR, except that native *Pfu* DNA Polymerase ("Thermo Fisher Scientific") was used instead of aforementioned recombinant *Taq* DNA Polymerase. JULXYL-PCR products were cloned into *Escherichia coli* DH5α using the CloneJET™ PCR Cloning Kit ("Thermo Fisher Scientific"). Plasmid DNA was isolated from clones with inserts using the GeneJET™ Plasmid Miniprep Kit (Thermo Fisher Scientific) and digested with *Bam*HI and *Hind*III ("Thermo Fisher Scientific") according to the manufacturer's instructions. The xylanase gene of *G. thermodenitrifcans* JK1 was ligated into the pET-28c(+) vector, and the products were transformed into *E. coli* BL21 (DE3). The sequences were aligned and analysed using MEGA 4.0 program [12]. Signal sequence prediction was performed using both SignalP 3.0 and SIG-Pred servers. Motif and domain scan were performed using PROSITE server.

Expression and purification of the recombinant xylanase. Transformants were grown in Luria-Bertani medium containing 30 μg kanamycin/mL. Protein expression was induced with 0.5 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) when OD590 reached 0.8, and the incubation was continued for another 5 h at 30°C. Cultures were harvested by centrifugation (7000 *g*, 20 min, 4°C) and resuspended in phosphate buffered saline. Cells were disrupted by sonication, and then the cell debris was removed by centrifugation at 10000 *g* for 20 min. His-Spin Protein Miniprep™ kit ("Zymo Research") was used for the purification of His-tagged recombinant xylanase.

Determination of protein concentration. Protein concentration was determined by Bradford's assay using bovine serum albumin as a standard [16].

SDS-PAGE and zymographic analysis. SDS-PAGE was performed by the method of Laemmli [17] on a 12% running gel, and protein bands were visualized by staining with the PageBlue™ Protein Staining Solution ("Thermo Fisher Scientific").

Zymograms of xylanase activity were performed in SDS/polyacrylamide gels containing 0.15% birch wood xylan. After electrophoresis, the gel was washed twice for 20 min in 0.1 M acetate buffer (pH 6.0) containing 1.0% (w/v) Triton X-100. After further incubation for 80 min at 60°C, the gel was soaked in 0.3% Congo red solution for 20 min and washed with 1 M NaCl until excess dye was removed from the active band.

Xylanase activity and substrate specificity assay. The assay mixture consisted of equal volumes of appropriately diluted enzyme solution and 0.1 M Tris-HCl buffer (pH 7.0) containing 0.5% (w/v) of birch wood xylan. The amount of released reducing sugars was determined by the dinitrosalicylic acid (DNS) method [18] at 60°C for 30 min. One unit of enzyme activity was defined as the amount of the enzyme releasing 1 μmol of xylose equivalent per min under the conditions described above.

The hydrolytic activity against 0.5% (w/v) of birch wood xylan, avicel and CM-cellulose in 0.1 M Tris-HCl buffer (pH 7.0) were determined to evaluate the substrate specificity of xylanase. The assay as described above was performed in triplicate.

Effect of pH, temperature and metal ions on the enzyme activity. In order to establish the effect of temperature and pH, recombinant enzyme assay was carried out at different temperatures (50–80°C) and pH (4.0–10.0). The effect of pH was tested using 0.1 M acetate (pH 4.0–6.0), 0.1 M Tris-HCl (pH 7.0–8.0) and 0.1 M glycine-NaOH (pH 9.0–10.0) buffers. The effect of temperature and metal ions was determined using 0.1 M acetate buffer (pH 6.0). The effect of different metal ions on the recombinant xylanase activity was determined by the addition of the corresponding ion at a concentration of 1.0 mM to the reaction mixture. The tested ions included Na^+, K^+, Ca^{2+}, Mg^{2+}, Mn^{2+} and Co^{2+}. Reaction mixtures were incubated for 30 min. Released reducing sugars were determined by DNS method described above.

Effect of temperature on the enzyme stability. The thermostability of xylanase was investigated at temperatures 55, 60, 65 and 70°C after incubation of the enzyme solutions in absence of a substrate for 5, 15, 30, 45 and 60 min. Residual activities were determined by DNS method as described above, except that the assay mixture was incubated for 10 min at pH 6.0.

RESULTS

Determination of the taxonomic position of strain JK1. Strain JK1 was isolated from the compost using birch wood xylan as a single carbon source. In order to reveal the taxonomic position of the strain JK1, 16S rRNA gene was cloned, sequenced and subjected for the phylogenetic analysis. Strain JK1 appeared to form a separate phylogenetic branch, with the type strain DSM 465^T and strain NG80-2 of *G. thermodenitrificans* to be the closest phylogenetic neighbours (data not shown). 16S rRNA analysis does not allow the exact identification of the prokaryotic species—the use of DNA-DNA hybridization or other phylogenetic markers is needed for this purpose. *spo0A* gene was shown to be a good marker for the identification of *G. thermodenitrificans* [11]. In order to determine whether the strain JK1 definitely belongs to this species, *spo0A* gene was amplified, sequenced and compared with that of the other strains of *G. thermodenitrificans*. The similarity of *spo0A* gene of the strain JK1 with that of the strain DSM 465^T was 99.5%, and it was completely identical with that of the strain NG80-2. So, on the basis of our results, we can conclude that thermophilic strain JK1 belongs to the species *G. thermodenitrificans*.

Characterization of crude xylanase of *G. thermodenitrificans* JK1. The pattern of xylanase production was tested using well diffusion method. Assay of xylanase production was carried out using the medium with xylan for culture growth. Xylanolytic activity in the culture supernatant was detected in the early exponential phase, at the 4th hour of growth. Maximum activity was recorded at the 10th hour of growth, at the late exponential phase (data not shown). Xylanolytic activity was not detected when the medium with xylose was used for the culture growth, suggesting xylose was not an inducer or was even a suppressor of xylanase production.

Crude xylanase was salted out from the supernatant of the 10th hour culture. Only the fraction obtained using 40–60% saturated ammonium sulphate possessed xylanolytic activity. In order to determine the exact number of xylanolytic enzymes secreted by strain JK1, zymographic analysis of crude xylanase was performed. Results of this analysis showed that only one xylanase with a molecular weight of ~45 kDa (Fig. 1a) is secreted by JK1.

Sequence analysis of the extracellular xylanase of *G. thermodenitrificans* JK1. In order to characterize the only extracellular xylanase of JK1, GEXYL-PCR was performed. The PCR product in size of ~1030 bp was obtained using the genomic DNA of *G. thermodenitrificans* JK1. GEXYL-PCR product was sequenced, and the obtained sequence was compared with the xylanase gene sequences of the other geobacilli. GEXYL-PCR product of the strain JK1 was completely identical with the respective fragment of the xylanase gene of *G. thermodenitrificans* strains

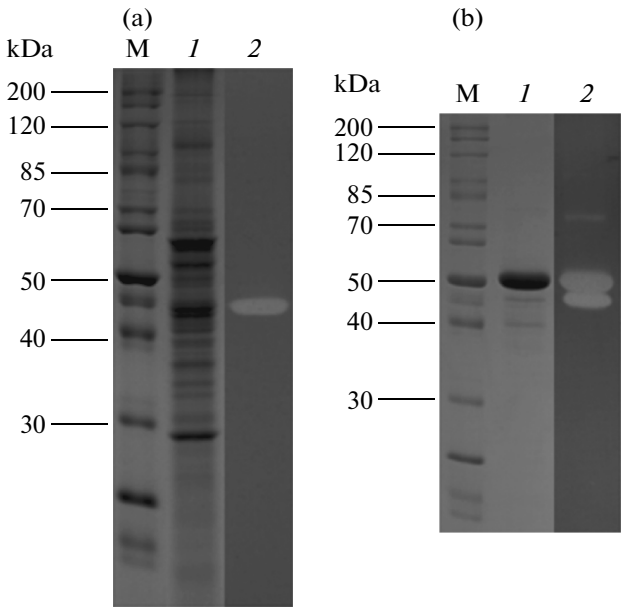

Fig. 1. SDS-PAGE and xylan zymogram of (a) extracellular proteins of *G. thermodenitrificans* JK1 grown on xylan and (b) recombinant xylanase. (a) lanes: *1*—SDS-PAGE of extracellular proteins; *2*—xylan zymogram of extracellular proteins, (b) lanes: *1*—SDS-PAGE of purified recombinant xylanase; *2*—xylan zymogram of recombinant xylanase. M—PageRuler^{TM} Unstained Protein Ladder (Thermo Fisher Scientific).

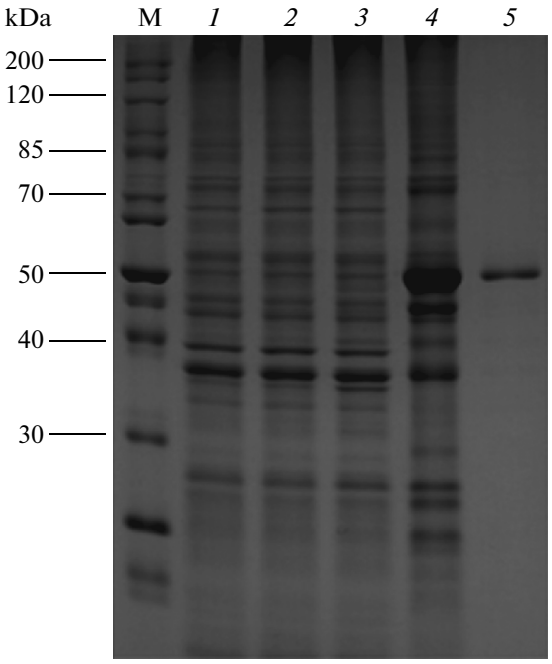

Fig. 2. SDS-PAGE of expressed and purified recombinant JK1 xylanase. Lanes: *1*—control (lysate of *E. coli* BL21(DE3)); *2*—control (*E. coli* BL21(DE3) with empty vector); *3*—control (uninduced recombinant *E. coli* BL21(DE3)); *4*—induced recombinant *E. coli* BL21(DE3); *5*—purified His-tagged xylanase. M—PageRuler^{TM} Unstained Protein Ladder (Thermo Fisher Scientific).

NG80-2 (CP000557, 1862421...1863644) and T-2 (EU599644). Consequently, the latter two sequences were used for the construction of the primers JULXYL-F and JULXYL-R in order to obtain the full-length xylanase gene. JULXYL-PCR was performed, and the PCR product was sequenced.

The xylanase gene of *G. thermodenitrificans* JK1 was 1224 bp in length (GenBank acc. no. JN209933) coding the single-domain protein of 407 amino acids with a signal sequence of 28 amino acids in length. Molecular weight was calculated to be 47.4 kDa for the protein with the signal sequence and 44.3 kDa for the protein without it, and it was in accordance with zymographic analysis of the crude xylanase.

Xylanase of *G. thermodenitrificans* JK1 was determined to be endo-1,4-β-xylanase (EC 3.2.1.8) belonging to the GH10. It differed from the other two aforementioned sequences of *G. thermodenitrificans* in one nucleotide, but this difference was reflected in the content of amino acids—363rd amino acid in the xylanase of JK1 was alanine, and that in the other two sequences—threonine. The similarity with the xylanase gene of *Geobacillus* sp. strain TC-W7 (GQ857066) was 99%, and with that of the other geobacilli—86% and below. The xylanase of JK1 and of *Geobacillus* sp. TC-W7 differed in 4 amino acids, but it should be noted that the same differences were noticed between the xylanase of *Geobacillus* sp. TC-W7 and the other two xylanase genes of *G. thermodenitrifcans*. *G. stearothermophilus* T-6 xylanase (DQ868502, 68530...69768) is the most thoroughly studied xylanase of geobacilli [1, 19]. Comparison of JKI gene with this xylanase gene revealed only 81.6% similarity. This difference was also reflected on the amino acid composition.

Cloning and expression of recombinant xylanase. In order to further characterize xylanase of *G. thermodenitrificans* JK1, it was subcloned into pET-28c(+) and expressed in *E. coli* BL21 (DE3). After induction with IPTG, lysate of recombinant *E. coli* was analysed by SDS-PAGE (Fig. 2). Lysate of *E. coli* BL21 (DE3) as well as those of uninduced recombinant *E. coli* and bacterium with empty vector were used as controls. His-tagged xylanase (~50.0 kDa) was observed only in the induced recombinant bacterium. After purification by affinity chromatography, a purified His-tagged xylanase was obtained (Fig. 2).

The activity of the recombinant protein was tested by zymogram analysis. Two active proteins were detected—~50 and ~45 kDa (Fig. 1b). Control lysates used for SDS-PAGE were also subjected to zymographic analysis. No active proteins were detected in these lysates (data not shown). Two active proteins in the sample of recombinant xylanase can be explained as co-purification of His-tagged xylanase and signal

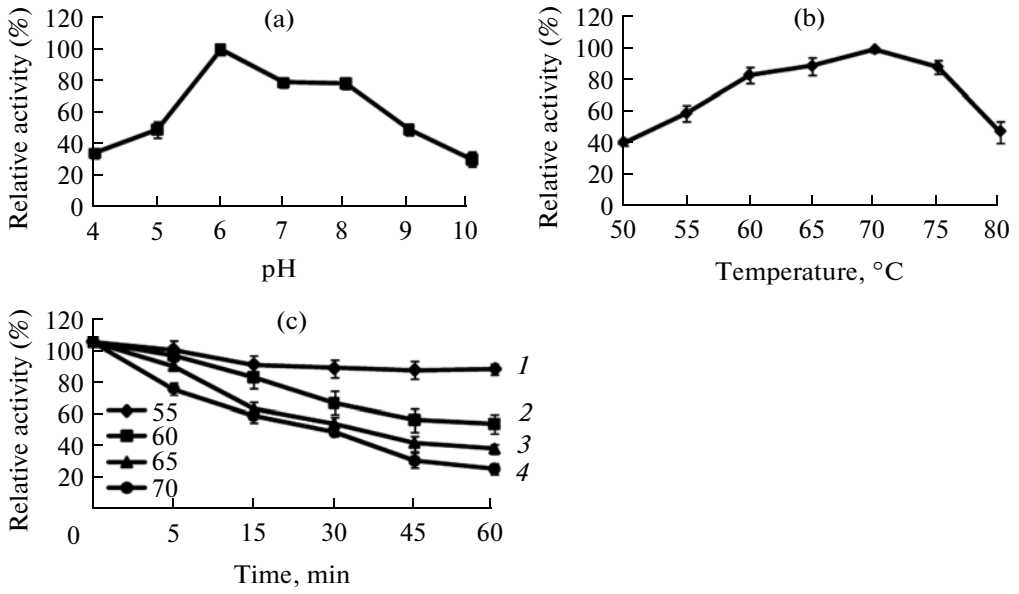

Fig. 3. Effects of pH (a) and temperature (b) on the recombinant xylanase activity and stability (c). (a) Effects of pH on the recombinant xylanase activity were assayed using different buffers at 60°C for 30 min. (b) The recombinant xylanase was assayed at various temperatures ranging from 50 to 80°C for 30 min under pH 6.0 in order to determine the effects of temperature. (c) Effect of temperature on recombinant xylanase stability. The residual enzymatic activities of recombinant xylanase were monitored after incubation at 55 (*1*), 60 (*2*), 65 (*3*) and 70°C (*4*) for 5, 15, 30, 45 and 60 min at pH 6.0.

peptide-free xylanase. Further analysis of signal peptide of *G. thermodenitrificans* JKI xylanase showed that this peptide is recognized by both gram-positive and gram-negative bacteria. Cleavage of the signal peptide from the recombinant protein in *E. coli* removes N-terminal His-tag. Although the amount of this signal peptide-free xylanase is very small (Fig. 1b), it is more active than recombinant protein and, consequently, appears as a clear band of ~45 kDa on zymogram.

Effect of metal ions (1 mM) on recombinant xylanase of *Geobacillus thermodenitrificans* JK1

Chemical	Xylanase activity, %
None	100.0
NaCl	96.8
KCl	95.1
$MgCl_2$	99.8
$CoCl_2$	99.3
$MnCl_2$	102.9
$CaCl_2$	98.5

Characterization of recombinant xylanase. In order to determine the characteristics of *G. thermodenitrificans* JK1 xylanase, the recombinant enzyme was analysed. Xylanase activity was assayed by measuring the released reducing sugars using DNS method. It should be noted that zymographic analysis was equally carried out in order to detect the differences between the recombinant xylanase and the signal peptide-free xylanase that had been present in a very small amount in the sample of recombinant protein. No detectable differences were observed in the behaviour of these two xylanases (results not shown). Consequently, we present here the results obtained using the DNS method as a more accurate one.

The xylanase was active in a broad range of temperature and pH. The highest activity of recombinant xylanase was detected at pH 6.0, and approximately 80% of the activity was retained at pH 7.0–8.0 (Fig. 3a). Optimum temperature was determined to be 70°C with the 90% of activity at 65 and 75°C (Fig. 3b). Recombinant xylanase was cellulase-free—no activity was detected using avicel and CM-cellulose as substrates.

The effects of different metal ions on the recombinant xylanase are shown in table. The metal ions Na^+, K^+, Ca^{2+}, and Co^{2+} showed partial inhibition of the activity. Mn^{2+} had a slight stimulating effect on the enzymatic activity.

Recombinant xylanase was thermostable over the temperature range of 55–70°C. It presented the high-

est stability after incubation at 55°C for 60 min showing 84% residual activity. 50% residual activity was found after incubation at 60°C for 60 min as well as at 65 and 70°C for 30 min (Fig. 3c).

DISCUSSION

Almost all known xylanases of geobacilli belong to the GH10 family (www.cazy.org [20]). Xylanase of *G. thermodenitrificans* JK1 was also found to belong to this family. It was ~45 kDa in size, while xylanolytic enzymes of *G. thermodenitrificans,* partially characterized by Canakci et al. [7], were only 30 kDa in size. Biochemically characterized xylanases of other geobacilli were 35 kDa [7], 36 kDa [6] and 40 kDa [4]. 43 kDa xylanase of *G. stearothermophilus* T-6 was the most similar in size [3]. But JK1 xylanase differed from the latter in pH optimum (pH 6.0 for JK1 and pH 6.5 for T-6) and temperature optimum (70°C for JK1 and 75°C for T-6)—both pH and temperature optimums of JK1 were lower than of *G. stearothermophilus* T-6. Two of the wild type strains of *G. thermodenitrificans* [7] also had 70°C temperature optimum, but pH optimums for these strains were 7.0 and 8.0.

The lack of xylanase activity during the log phase has been reported for some microorganisms [21]. In contrast, *G. thermodenitrificans* JK1 secreted 45 kDa xylanase during this phase. No xylanolytic activity was detected during the growth of *G. thermodenitrificans* JK1 on xylose, although xylanase production in *Geobacillus* is usually induced by this monosaccharide [2–4, 19]. JK1 xylanase is cellulase-free and this contrasts with *G. stearothermophilus* T-6 xylanase possessing negligible endo-1,4-β-glucanase activity [3].

The effect of different metal ions on JK1 xylanase activity was unique among all the characterized xylanases of geobacilli [2, 3, 6]. Xylanases of the other geobacilli were inhibited by Mn^{2+} or it had no effect on the activity, while xylanase of JK1 was slightly stimulated by these ions. Monovalent cations Na^+ and K^+ partially inhibited JK1 xylanase but stimulated xylanases of the other geobacilli.

Some amino acids (T187, N194, K195, I200, S212, D363; amino acid numbering is according to Zhang et al. [1]) suggested to affect the thermostability of xylanases of geobacilli, were identified in JK1 xylanase sequence. Results of the thermostability experiments showed that xylanase of JK1 had a quite low thermostability when compared with the respective enzymes of other geobacilli. It showed 50% residual activity after incubation at 60°C for 1 h, while the activity of xylanase of *G. stearothermophilus* T-6 was not affected after incubation at 65°C for 10 h [3]. Xylanase of *Geobacillus* sp. MT-1 was also more thermostable than the xylanase of JK1 exhibiting stability at 55 and 60°C for at least 3 h [6].

To our knowledge, this study is the first report about cloning and extensive characterization of the xylanase of *G. thermodenitrificans*. Our results showed that this is a novel cellulase-free xylanase of geobacilli possessing a few unique characteristics including quite low thermostability.

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