International Journal of Biological Macromolecules 119 (2018) 1017–1026

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: http://www.elsevier.com/locate/ijbiomac

New xylanolytic enzyme from *Geobacillus galactosidasius* BS61 from a geothermal resource in Turkey

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ARTICLE INFO

Article history:
Received 9 May 2018
Received in revised form 24 July 2018
Accepted 25 July 2018
Available online 27 July 2018

Keywords:
Thermophilic
16S rRNA sequencing
BOX-PCR
Cloning
Geobacillus galactosidasius BS61
Xylanase

ABSTRACT

In this study, isolation, conventional and molecular characterizations of ten thermophilic bacteria from Rize/Ayder were carried out. Xylanase from *Geobacillus galactosidasius* BS61 (GenBank number: KX447660) was purified by acetone precipitation, Diethylaminoethyl-cellulose and Sephadex G-100 chromatographies. The xylanase of *G. galactosidasius* BS61 in clarifying fruit juice was also investigated. Enzyme was purified 29.80-fold with 75.18% yield; and molecular weight was determined as 78.15 kDa. The optimum temperature of xylanase was 60 °C. The enzyme activity was maintained fully after 24 h and over 50% after 168 h at pH 4.0–10.0, while optimum pH was 7.0. K_m and V_{max} for beech wood xylan were measured as 3.18 mg mL^{-1}, 123 U mg $protein^{-1}$. In addition, Ca^{2+}, Na^{+}, Al^{3+}, Zn^{2+}, Cd^{2+}, Mg^{2+}, Ni^{2+}, Cu^{2+} had decreasing effect on enzyme activity, while enzyme activity had been protected against anions, especially HSO^{3-} and HPO_4^{2-} stimulated enzyme activity. Xylanase applications (with 15 U/mL enzyme activity) in orange and pomegranate juices were increased; and the sugar and turbidity amounts were reduced 17.36% ± 1.18 and 30.52 ± 1.23, respectively. These results indicated that the xylanase of *G. galactosidasius* BS61 has biotechnological potential in juice clarification due to its stability against metal ions, chemicals and high pH-values.

1. Introduction

Microbial life on Earth has existed about 3–3.7 million years before the formation of high-structured organisms and their precipitation in the evolution process, adapting to different ecosystems [1]. Especially microorganisms can be easily adapted to extreme conditions, so they are not only in air, soil, lakes and seas, they have been isolated from a wide range of sites from ice, volcanoes and geothermal wells, alkaline media and high acidic media [1]. Thermophilic bacteria isolations are noted in recent times [2] due to their enzymes because of thermophiles could keep their catalytic activities at high temperatures and wide pH range [3]. This makes the investigation of enzymes from thermophilic organisms to be used in industrial applications a current topic.

Geobacillus spp., which has recently attracted attention, can also survive at extreme conditions. *Geobacillus* genus, which has been separated from *Bacillus* genus in 2001 and re-classified, consists of Gram positive, rod-shaped, spore-forming members with aerobic and anaerobic respiration [4]. The ability of *Geobacillus* spp. to metabolize five and six carbon sugars, starch and xylan attracts the attention to use them in biotechnological applications requiring biomass transformation, such as hot composting [5–7]. Besides isolation from composting materials, *Geobacillus* spp. has been isolated from high temperature oil fields [8], corroded fields [9] and hot springs [10].

In the identification of thermophilic microorganisms, molecular techniques viz., 16S rRNA sequencing, BOX-PCR, GTG-PCR, etc., have been improved because of long time and high workload requirements, need for experienced researchers and open to different interpretations of results of conventional methods [11]. The separation of the genus *Geobacillus* from the *Bacillus* group with studies based on 16S rRNA sequence analysis is one of its important consequences. Some other genius separated from *Bacillus* are *Aeribacillus*, *Alicyclobacillus*, *Amphibacillus*, *Aneurinibacillus*, *Anoxybacillus*, *Brevibacillus*, *Coprobacillus*, *Filobacillus*, *Geobacillus*, *Gracilibacillus*, *Halobacillus*, *Jeotgalibacillus*, *Marinibacillus*, *Paenibacillus*, *Sulfobacillus*, *Salibacillus*, *Thermobacillus*, *Ureibacillus* and *Virgibacillus* [12, 13].

Microbial diversity has led scientists to research the potential of new thermophilic isolates and their ability to produce thermophilic enzymes (protease, lipase, amylase, pectinase, DNA polymerase, xylanase, chitinase and cellulase) [14, 15]. Among these enzymes, xylanase is a

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https://doi.org/10.1016/j.ijbiomac.2018.07.166
0141-8130/

glucosidase which catalyses β 1,4 D xylosydic bonds in xylan. This enzyme is produced in mediums where microorganisms can directly or indirectly reach the xylan. Thermophilic microorganisms with xylanase production ability are primarily isolated from hot springs [16–19] and these enzymes, which break down the xylan, are preferred especially in biotechnologically available areas such as paper and paper pulp, pharmaceutical, feed and food industries (bread, clarification of fruit juices) [16, 17]. For instance, the increase in the consumption of natural fruit juices improved the efforts to increase the quality of the fruit juices, because the presence of starch, pectin and hemicellulose-like polysaccharides is a parameter that lowers the quality of fruit juice. In order to eliminate this problem, xylanase has been used as a solution to clarify fruit juices [17]. Due to specificity, moderate reaction conditions and low toxicity, it is more important and valuable to use enzymes like xylanases than chemical agents to produce high quality products [16, 17].

In the beginning of the study, isolation of thermophilic bacteria from Rize/Ayder hot spring and conventional and molecular identifications of these bacteria were carried out. Selection of bacteria, purification and characterization of xylanase, research on potential of using this enzyme in biotechnological processes such as juice clarification were also investigated.

2. Materials and methods

2.1. Sampling and identification of microorganisms

The water and sludge samples from Rize/Ayder were collected and the samples were transferred to Tryptic Soy Agar (TSA) medium (casein peptone (15 g L^{-1}), sodium chloride (5 g L^{-1}), soya peptone (5 g L^{-1}) and agar (15 g L^{-1})) in order to incubate (Thermo Scientific Heratherm, the USA) at 55 °C for 24 h. The growing colonies were spread on the TSA to obtain pure cultures. The pure and single colonies were stored in the Nutrient Broth (NB) with 15% glycerol content at −86 °C for further studies [20], and these colonies were subsequently referred to as "isolates."

The test isolates were subjected to conventional tests. The pH (Mettler Toledo) and temperature range for bacterial growth were tested between pH 4.0–10.0 and 30–80 °C with 1 °C interval. The NaCl requirement for growth was also tested in NB medium containing 2.0, 3.0, 4.0, 5.0, 8.0, 10.0% (w/v) NaCl. Cell and colony morphology (Leica ICC50 HD light microscope), Gram reactions and the presence of catalase, oxidase, and nitrate reactions were also investigated by the method of Prescott et al. (2002) [21].

2.1.1. DNA extraction from test isolates

The isolates were first stimulated by transferring to TSA medium from stock cultures. A single colony from stimulating medium was selected and inoculated to Tryptic Soy Broth (TSB) at 55 °C 150 rpm. After the incubation period, genomic DNA isolation was carried out according to Promega Wizard^{R} DNA purification kit and was stored at +4 °C until use [20].

2.1.2. Gene amplification, cloning and sequencing for phylogenetic studies

The 16S rRNA, evolutionary gene of prokaryotic ribosome of total DNA of the isolates was amplified by Polymerase Chain Reaction (PCR) with the forward primer UNI16S-L: (5′ ATTCTAGAGTTTGATC ATGGCTCA 3′) corresponded to positions 11 to 26 of *Escherichia coli* 16S rRNA, and the reverse primer UNI16S-R: (5′ ATGGTACCGTGTGA CGGGCGG TGTGTA 3′) corresponded to the complement of positions 1411 to 1393 of *E. coli* 16S rRNA [22] The amplified fragments were cloned to a vector system (pGEM-T, Promega, the UK) and the clones were sequenced (Macrogen, Amsterdam, the Netherlands). The results of 16S rRNA gene sequencing were analysed using the GenBank and EzTaxon (http://blast.ncbi.nlm.nih.gov/blast.cgi and http://www.eztaxon.org/) servers [23, 24]. Considering the results of the study, a phylogenetic tree was formed via the neighbour-joining method by using the software package MEGA 4.0 [24, 25].

2.1.3. Genomic fingerprinting

The isolates were subjected to PCR with universal primer of BOX elements (BOX A1R 5′ CTACGGCAAGGCGACGCTGACG 3′) to obtain genomic fingerprinting [26]. To obtain PCR products, 50 ng of purified DNA was used as a template in a 30 μL reaction mixture. 27 μL of reaction cocktail was prepared as follows: specific Gitschier Buffer 5 μL, Dimethyl sulphoxide 2.5 μL (100%, 20×), dNTPs (10 mM) 1.25 μL, bovine serum albumin 1.25 μL (20 mg/mL), primer (5 μM) 3.0 μL, Taq polymerase (250 U) 0.3 μL, water 13.7 μL. A negative control (no DNA) was included in each PCR assay. PCR reactions were performed with a 3 × 32-well ProFlex™ PCR System, using the following conditions: an initial denaturation at 94 °C for 7 min; 30 cycles consisting of 94 °C for 30 s and annealing at 45 °C for 1 min with BOX primer, extension at 65 °C for 8 min and a final polymerization at 65 °C for 15 min before cooling at 4 °C.

The PCR products (27 μL) were mixed with 3 μL gel loading buffer (6×) and subjected to gel electrophoresis in Tris-Acetate-EDTA (TAE) buffer at 100 V for 110 min. After the separation of amplification products by gel, the fragments were stained with ethidium bromide solution (2 μL Etbr/100 mL 1× TAE buffer). The amplified DNA product was monitored by using the Quantum Vilber Lourmat Gel Documentation System (Australia).

2.2. Biotechnological potentials of isolates

Amylase assay was performed at 55 °C for two days in a medium which contained $Na_2HPO_4.7H_2O$ (6 mg mL^{-1}), KH_2PO_4 (3 mg mL^{-1}), NaCl (0.5 mg mL^{-1}), NH_4Cl (1 mg mL^{-1}), $MgSO_4.7H_2O$ (0.24 mg mL^{-1}), $CaCl_2.2H_2O$ (0.24 mg mL^{-1}), peptone (3 mg mL^{-1}), starch (10 mg mL^{-1}) and agar (15 mg mL^{-1}). The amylase activity was screened by Lugol's solution [24].

A medium containing carboxymethylcellulose (10 mg mL^{-1}), peptone (5 mg mL^{-1}), yeast (5 mg mL^{-1}), KH_2PO_4 (1 mg mL^{-1}), $MgSO_4.7H_2O$ (0.2 mg mL^{-1}), NaCl (10 mg mL^{-1}) and agar (15 mg mL^{-1}) was used for cellulose assay. Incubation was carried out at 55 °C for 2 days, and after the incubation, the Petri dishes were stained by Congo-Red Dye [15].

Test isolates were inoculated on tributyrin agar medium which contained 1% tributyrin (glycerol tributyrate) and were incubated at 55 °C. Formation of hydrolysis zone around the culture on Petri dish was controlled for 2 days, and the strains with transparent and the highest zone formation (lipolytic activity) were determined as lipase producers [27].

Twenty-four-hour bacterium culture in NB was propagated in Skimmed Milk Agar medium at 55 °C for 24–48 h to assay the proteolytic activities of the test isolates. The plates were evaluated according to the zone formations, and the observation of a halo zone indicated positive protease activity [28].

2.3. Purification and characterization of enzyme

2.3.1. Microorganism and xylanase production

In order to determinate xylanase-producing bacteria, the isolates in stock culture were first inoculated to TSA and then xylan-containing solid medium in which enzyme production was obtained. The growth was at 55 °C and for four days [29]. Xylan-containing Petri dishes were treated with 1% Congo red for 15 min. and then washed with 1 M NaCl for 25 min. Presence of zone was called xylanase-positive and the other was xylanase-negative [30]. The isolate with its most significant zone was inoculated in liquid medium containing 0.12% $NaNO_3$, 0.3% KH_2PO_4, 0.6% K_2HPO_4, 0.005% $CaCl_2$, 0.001% $MgSO_4$, 0.0001% $ZnSO_4$ and 1% (Xylan or oat bran) with minor modifications [31]. The growth medium, which was shaken at 55 °C 150 rpm for four days, was centrifuged at 13000 rpm for 30 min. at the end of the incubation.

The precipitate was removed and supernatant was used as 'crude enzyme'.

2.3.2. Purification

All purification steps were at +4 °C. Precipitation of xylanase was made by treatment of crude enzyme with acetone at a ratio of 1:1 (v/v). Crude enzyme and acetone mixture was kept at cold for 1 h and centrifuged at 10000 rpm +4 °C for 30 min. The precipitate, which was standing on air at +4 °C to evaporate the acetone, was diluted with 20 mM pH 8.0 Tris-HCl buffer. It was then applied to Diethylaminoethyl-cellulose (DEAE-Cellulose) column equilibrated with 20 mM pH 8.0 Tris-HCl buffer. The column was washed with 20 mM pH 8.0 Tris-HCl buffer in order to remove the components not connected to the column resin. The components that were connected to the column were eluted with 0–500 mM NaCl gradient (0.5 mL min^{-1}). Active fractions were collected, concentrated and dialyzed with 20 mM pH 8.0 Tris-HCl buffer. After dialysis, crude enzyme was loaded to Sephadex G-100 column. Elution was repeated as before. Active fractions were dialysed with the same buffer [32].

2.3.3. Determination of xylanase activity

Xylanase activity was measured by Dinitrosalicylic acid (DNS) method, spectrophotometrically. 500 μL of 1% solubilised beech wood xylan (prepared in buffer solution) and 500 μL of enzyme solution containing 2.50 mg mL^{-1} purified xylanase was mixed and incubated at 50 °C for 20 min. After the incubation, the reaction was stopped with the addition of DNS at a rate of 1:1 (v/v) and boiled at 90 °C for 5 min. The amount of reducing sugar that was released after cooling was determined by measuring the absorbance at 540 nm wavelength against the blot in the spectrophotometer [33]. Xylose was used as a standard. A xylanase enzyme unit was defined as the amount of enzyme that released 1 μmol of xylose equivalents per minute in reaction conditions. All assays were triplicated.

2.3.4. Protein determination

Protein concentration of purified enzyme at all purification and characterization steps was determined by Lowry method [34]. The standard was Bovine Serum Albumin.

2.3.5. Electrophoresis and zymogram

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was carried out by using 5% stacking gel and 8% separation gel [35] (Mini-PROTEAN Tetra Cell Unit Bio-Rad, the USA). The bands were made visible by staining with Coomassie Brilliant Blue R250. Using this gel, molecular weight of the protein molecule was calculated using Bio-Rad Precision Plus Protein ™ Unstained.

The purified enzyme samples were heated at 45 °C for 5 min for zymogram analysis and loaded to 8% SDS-PAGE gel. After electrophoresis, the gel was hold on 2.5% TritonX-100 for half an hour and with 50 mM pH 7.0 sodium phosphate buffer for half an hour, in order. The reaction between enzyme and substrate took place in 50 mM pH 7.0 sodium phosphate buffer at 55 °C for 15–30 min. The expected reaction was achieved by using 0.1% Congo red for 15 min and washing with 1 M NaCl until colour change. For a clearer visualization of the activity band, the gel was immersed in 0.5% acetic acid solution and photographed [32].

2.3.6. Substrate specificity

Xylans from beech wood, birch wood, oat bran and carboxymethylcellulose (CMC) was used as different substrates. A 5% solution of each substrate was prepared and activity assays were carried out at standard conditions. Catalysis effect of xylanase against indicated substrates was expressed as % relative activity. The value at which the highest activity was observed was accepted as 100% [36].

2.3.7. Effect of pH and temperature on xylanase activity

The effect of pH was determined by measuring xylanase activity with different buffers at 50 mM concentration viz. sodium acetate buffer for pH 4.0–5.0, sodium phosphate buffer for pH 6.0–7.0, Tris-HCl buffer for pH 8.0–9.0 and Glycine-NaOH buffer for pH 10.0 by mixing at a rate of 1:1. In order to examine the effect of temperature on xylanase activity, enzyme activity assays were carried out at 30–90 °C with 10 °C intervals [37]. Activity values were expressed as relative (%) activity and the highest activity was considered as 100%. Optimum temperature and pH values were determined from the temperature-relative activity (%) and pH-relative activity plots were plotted using the obtained data [38].

2.3.8. Effect of pH and temperature on xylanase stability

Purified enzyme eluate was mixed with buffers at a rate of 1:1, as mentioned before. The mixtures were incubated at 4 °C for 24 and 168 h and then xylanase activities were measured to examine the pH stability of enzyme [38]. Temperature stability was also examined and purified enzyme was kept at 4, 40, 60 and 80 °C for 30, 60, 90 and 120 min. The enzyme concentration was 2.50 mg mL^{-1}. Xylanase activity measurements were done with half an hour interval. The remaining activities in the pH and thermal stability processes were calculated by comparing the activity value determined at the optimum conditions of the enzyme without any pre-treatment. Thus, time-remaining activity (%) plot was drawn.

2.3.9. Kinetics parameters

To establish maximum velocity (V_{max}) and Michaelis-Menten (K_m) constant of purified xylanase, reaction mixtures with final concentrations of 0.1–10.0 mg mL^{-1} beech wood xylan at 10 different levels were prepared. Enzyme activities were measured and K_m and V_{max} values were calculated by Lineweaver-Burk plot [39].

2.3.10. Effects of some metal ions and chemicals on the enzyme activity

The changes of isolated and purified xylanase in the presence of cations viz. Na^+, Zn^{2+}, Ca^{2+}, Mn^{2+}, Cu^{2+}, Mg^{2+}, Ni^{2+}, Cd^{2+}, Al^{3+} and anions viz. CN^-, HSO_3^{2-}, SO_4^{2-}, CO_3^{2-}, HCO_3^-, HPO_4^{2-}, $B_4O_7^{2-}$ were examined. Final concentrations of ions were fixed at 5 mM in reaction mixture for activity assay. EDTA, β-mercaptoethanol and sulphonamide with final concentration of 5 mM were investigated to determine whether they had any effect on xylanase activity. Enzyme activity of reaction mixture without metal ions and chemical agents was accepted as 100% and other activities were calculated as % remaining activity [40].

2.4. Application of xylanase in fruit juice clarification

In the study, orange, pomegranate, apricot, peach, apple, grape and kiwi fruits which were taken from the local market were peeled and fruit juices were obtained by squeezing manually. These fruit juices were centrifuged at 5000 rpm for 30 min. and the obtained supernatant

Table 1
Comparison of 16S rRNA gene sequences of isolates with those in GenBank.

Isolate code	Closest phylogenetic relative	Accession number	Identity	Number of nucleotides
BS11	*Bacillus licheniformis*	KX447652	%99	1425 bp
BS12	*Bacillus licheniformis*	KX447653	%99	1425 bp
BS13	*Bacillus licheniformis*	KX447654	%99	1420 bp
BS14	*Bacillus licheniformis*	KX447655	%99	1425 bp
BS15	*Bacillus licheniformis*	KX447661	%99	1511 bp
BS16	*Bacillus licheniformis*	KX447656	%99	1425 bp
BS17	*Brevibacillus brevis*	KX447657	%99	1411 bp
BS18	*Aeribacillus pallidus*	KX447658	%99	1515 bp
BS19	*Bacillus thermoamylovorans*	KX447659	%99	1426 bp
BS61	***Geobacillus galactosidasius***	**KX447660**	**%99**	**1516 bp**

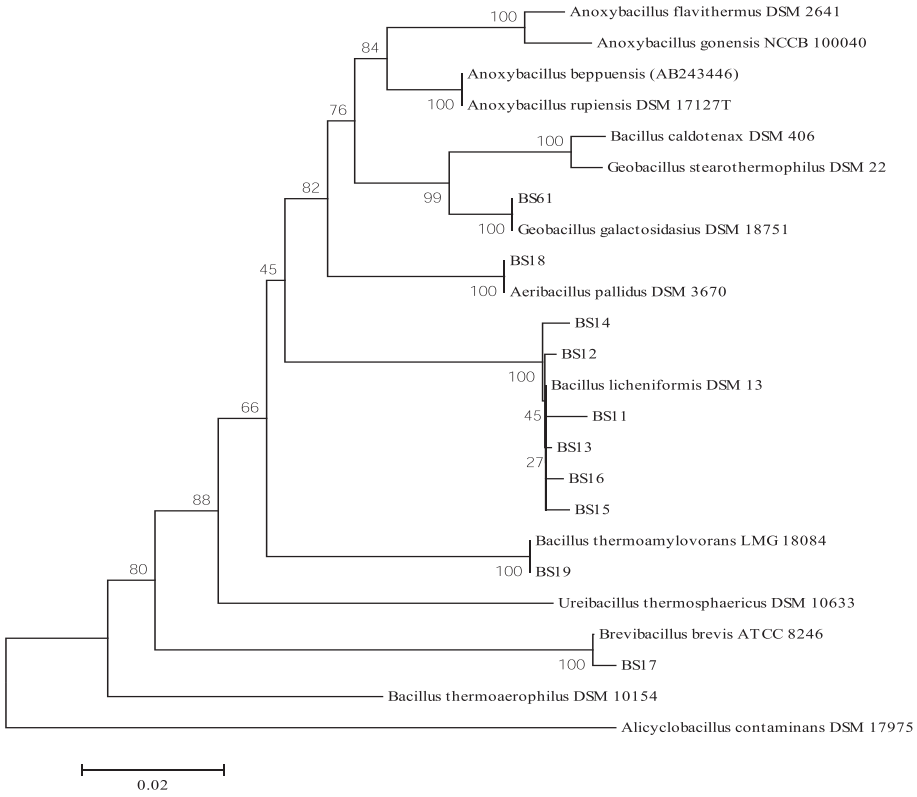

Fig. 1. Neighbour-joining phylogenetic tree on the basis of 16S rRNA gene sequence data of isolates. *Alicyclobacillus contaminans* DSM 17975 was used as out-group. Bootstrap values based on 1000 replications are listed as percentages at branching points. Only bootstrap values >50% are shown at nodes. The scale bar represented 2% divergence.

was used in enzymatic trials [16]. Purified enzyme at a concentration of 0.5, 0.10, and 0.15 U/ mL was firstly incubated with each fruit juice at 60 °C for 20 min. The reducing sugar in samples was measured by DNS method and optical densities were examined at 660 nm were examined. Results were compared with the control which had no enzyme [41].

2.5. Statistical analysis

Measurements were triplicated in all assays. The results were evaluated in SPSS 21.0 with ANOVA/Student-Newman-Keuls (SNK) test and the results were given as mean ± standard deviation. Significant differences were calculated in 95% confidence intervals. The results were considered statistically significant when p values were <0.05 and 0.01.

3. Results

3.1. Conventional and molecular aspects of results

Identification of ten thermophilic isolates from water and sludge samples of Rize-Ayder was carried out at species level by molecular and conventional methods. For this purpose, genomic DNA was isolated firstly. Then the 16S rRNA region, which was evolutionarily conserved, was amplified using universal primers; and the bands of fragments were identified at approximately 1500 bp. The sequence of isolates was compared with 16S rRNA sequences of other bacteria in the GenBank and the nucleotides were analysed using BLAST and EzTaxon. BS11, BS12, BS13, BS14, BS15 and BS16 isolates were similar to

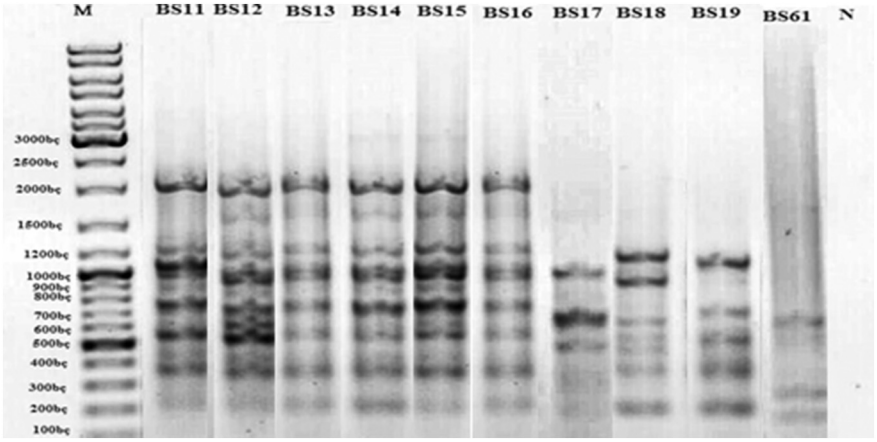

Fig. 2. BOX-PCR gel image of isolates. N; negative control; M) molecular marker (10 kb).

Table 2
Morphological, physiological and biochemical test results of isolates.

Isolate code	Gram	Endospore	Cell morphology	Salt concentration (%)	pH	Temperature (°C)	Oxidase	Catalase	Nitrate	Amylase	Protease	Xylanase	Cellulase
BS11	+	+	Rod-shape	2–8	6.5–9.5	30–60	+	+	+	+	+	+	+
BS12	+	+	Rod-shape	2–8	6.5–9.5	30–60	+	+	+	+	+	+	+
BS13	+	+	Rod-shape	2–8	6.5–9.5	30–60	+	+	+	+	+	+	+
BS14	+	+	Rod-shape	2–8	6.5–9.5	30–60	+	+	+	+	+	+	+
BS15	+	+	Rod-shape	2–8	6.5–9.5	30–60	+	+	+	+	+	+	+
BS16	+	+	Rod-shape	2–8	6.5–9.5	30–60	+	+	+	+	+	+	+
BS17	+	+	Rod-shape	2–8	6.5–8.5	40–60	+	+	+	−	+	+	−
BS18	+	+	Rod-shape	2–10	7.0–8.0	30–70	+	+	+	−	−	−	−
BS19	+	−	Rod-shape	2–6	5.5–8.5	45–60	+	+	+	+	−	+	+
BS61	+	+	Rod-shape	2–4	6.0–8.0	50–60	+	+	+	+	−	+	+

B. licheniformis, BS17 to *B. brevis*, BS18 to *A. pallidus*, BS19 to *B. thermoamylovorans* and BS61 to *G. galactosidasius*, at a rate of 99%. 16S rRNA sequence of isolates is given in Table 1 and phylogenetic tree is drawn for 16S rRNA genes of isolates (Fig. 1). To define the genetic polymorphisms in species, BOX-PCR was carried out (Fig. 2). BS61 had six bands with 200–1600 bp and BS17 and BS19 had six bans with 200–1000 bp, while BS18 eight bands with 200–1200 bp, BS16 and BS13 eight bands with 200-2000 bp, BS11 and BS14 nine bands with 200–2000 bp and 200–3000 bp, BS12 and BS15 eleven bands with 200–2000 and 200-3,000 bp.

Some tests were applied to determine the morphological, physiological and biochemical characteristics of isolates or strains (Table 2). As a result, it was determined that all strains were Gram positive, endospore-forming except BS19, facultative and rod-shaped. Salt concentration, pH and temperature requirements of the strains were determined for optimum microbial growth. BS11–BS17 need optimum concentration of NaCl between 2 and 8% while BS18 needs 2–10%, BS19 needs 2–6% and BS61 needs 2–4%. Optimum pH of the growth medium for each strain was as follows;; pH 6.5–9.5 for BS11–BS16, pH 6.5–8.5 for BS17, pH 7.0–8.0 for BS18, pH 5.5–8.5 for BS19 and pH 6.0–8.0 for BS61. Temperature tolerance was variable; however, BS61 was between 50 and 60 °C while BS11–BS16 was between 30 and 60 °C, BS17 was between 40 and 60 °C, BS18 was between 30 and 70 °C and BS19 was between 45 and 60 °C.

As a result of the tests performed to determine the biochemical characteristics of the strains, it was seen that all of the isolates were positive for oxidase, catalase and nitrate (Table 2). Potential of industrially important enzyme production was also investigated. Eight of the strains were amylase positive (except BS17 and BS18), seven were protease positive (except BS18, BS19 and BS61), nine were xylanase positive

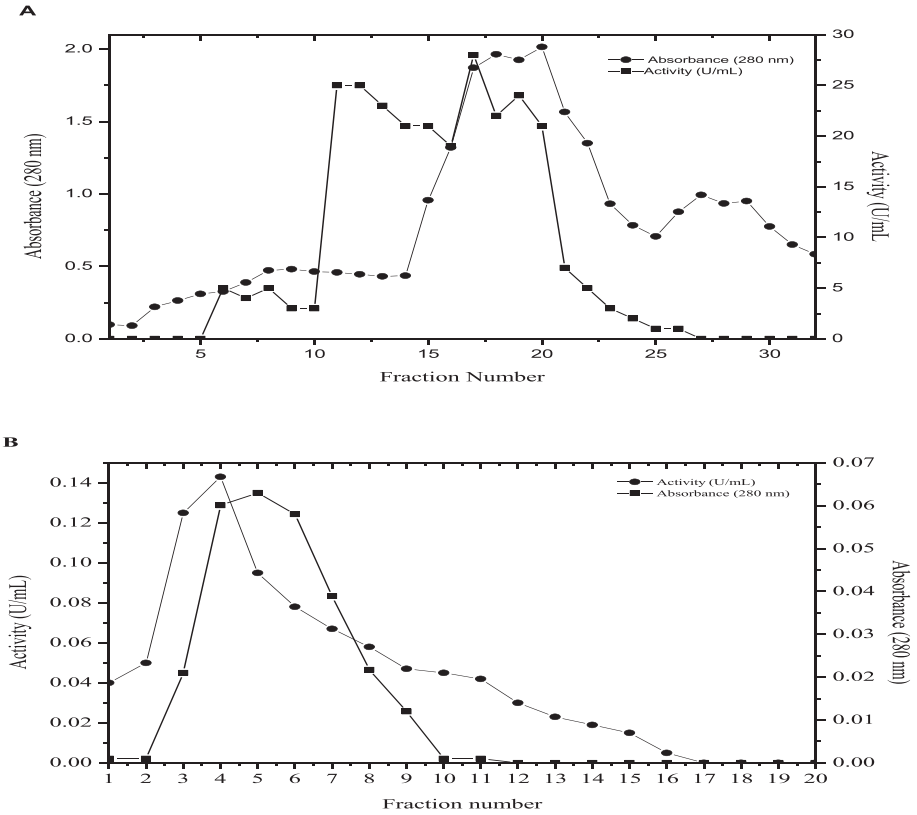

Fig. 3. Purification of xylanase from *G. galactosidasius* BS61: A) DEAE-cellulose ion exchange column chromatography, B) Sephadex G-100 gel filtration column chromatography.

Table 3
Purification procedure summary of the xylanase of *G. galactosidasius* BS61.

Sample	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1})	% Yield	Purification fold
Crude extract	21,780	539.24	40.39 ± 1.05	100	1
Acetone precipitation	16,375	92.25	177.51 ± 1.85	75.18	4.39
DEAE-cellulose	12,675	45.58	278.08 ± 3.56	58.19	6.88
Sephadex G-100	7679	6.38	1203.70 ± 15.96	35.26	29.80

(except BS18) and nine were cellulase positive (except BS17 and BS18). Following molecular characterization of thermophilic bacteria, enzyme studies continued with *G. galactosidasius* BS61, among xylanase producers, with the most significant enzyme zone.

3.2. Production and purification of xylanase

G. galactosidasius BS61 was grown at 55 °C for four days in mediums, which contained xylan or oat bran as carbon source. After incubation, activity assay was carried out in supernatant (crude enzyme) of culture. Insignificant difference was observed in enzyme production when xylan or oat bran was used as the carbon source, and therefore, in this study, we used oat bran as carbon source in the medium prepared for xylanase production.

Crude enzyme from *G. galactosidasius* BS61 was subjected to acetone precipitation followed by ion exchange and gel filtration chromatographies (Fig. 3). Xylanase was purified with a yield of 58.19% and 6.88-fold in DEAE-cellulose column (Fig. 3A) and active fractions were combined for loading to Sephadex G-100 column (Fig. 3B). After elution, enzyme activity was determined for fractions which had the highest specific activity and 35.26% yield with 29.80-fold purification of xylanase were determined (Table 3). Purification procedure was verified with a single and homogeneous band corresponding to 78.15 kDa in SDS-PAGE (Fig. 4-1). The zymogram study performed for xylanase from *G. galactosidasius* BS61 revealed the presence of a clear band that confirmed the purified enzyme was a xylanase (Fig. 4-2).

3.3. Biochemical properties of xylanase enzyme

3.3.1. Effect of pH and temperature on the activity and stability of BS61 xylanase

Optimum pH of xylanase from *G. galactosidasius* BS61, was determined with seven different buffers (pH 4.0–5.0 sodium acetate, pH 6.0–7.0 sodium phosphate, pH 8.0–9.0 Tris-HCl, pH 10.0 Glycine-NaOH) in activity reaction mixture and optimum value for pH was 7.0. When the % relative activity-pH graph was examined, the activity was observed above 60% at pH values of 6.0 and 7.5 (Fig. 5A). This showed the enzyme activity was higher and close to neutral pH. On the other hand, purified xylanase from *G. galactosidasius* BS61 had optimum temperature at 60 °C and the xylanase enzyme was active over a wide range of temperature from 30 to 90 °C (Fig. 5B).

The pH 4.0–10.0 range was studied for pH stability (Fig. 6A). It was observed that, xylanase activity was completely protected at all pH values after 24 h while the activity was found to be over 50% at all pH levels after 168 h. For studying thermal stability of purified xylanase, enzyme was incubated at 4, 40, 60 and 80 °C for 30, 60, 90 and 120 min. At the end of the incubation period, the remaining activity (%)-time graph was drawn (Fig. 6B). When the thermal stability profile of the enzyme was examined, the activity was completely preserved at the end of 120 min. at 4 °C. Xylanase activity was also nearly preserved at 40 °C for 90 min while the activity decreased by 10% at the end of 120 min. The activity was 50% of initial at optimum temperature point for 120 min. However, the enzyme rapidly lost activity at 80 °C.

3.3.2. Substrate specificity and kinetic parameters of xylanase

The xylanase from *G. galactosidasius* BS61 had the highest catalytic activity against beech wood xylan while xylan from birch wood, oat bran and carboxymethylcellulose (CMC) were other substrates (Table 4). Apart from beech wood, a very high activity was observed in oat bran and then in the birch wood. It was determined that the purified xylanase from *G. galactosidasius* BS61 was not capable of CMC cleavage. In order to examine the effect of the change in substrate concentration on the enzymatic activity of xylanase, reactions with different concentrations of beech wood xylan were carried out at optimum conditions. In this study, activity determinations were performed with the concentration of beech wood xylan ranging between 0.1 and 10.0 mg mL^{-1}. The K_m and V_{max} values were 3.18 mg mL^{-1} and 123 U mg $protein^{-1}$, respectively.

3.3.3. Effects of various metal ions and chemicals on xylanase activity

The behaviour of xylanase which was produced by *G. galactosidasius* BS61, in the presence of certain cations and anions was also studied. The enzyme activity was inhibited by Ca^{2+}, Mn^{2+}, Na^{+}, Mg^{2+}, Al^{3+}, Cd^{2+}, Ni^{2+}, Zn^{2+} and Cu^{2+} at a final concentration of 5 mM (Table 5). The highest inhibition was observed in the presence of Mn^{2+} (78.42%) and the minimum inhibition was in the presence of Ca^{2+} (1.89%) in all cations studied. Purified xylanase was also observed in anionic reactions with a final concentration of 5 mM for each anion (Table 5). Xylanase activity was decreased in the presence of CN^{-}, SO_4^{2-}, CO_3^{2-}, HCO_3^{-} and $B_4O_7^{2-}$ (>70%). HSO_3^{-} and HPO_4^{2-} anions stimulated the activity 24.78% and 24.22%, respectively.

β-Mercaptoethanol, Sulphonamide and EDTA were studied to understand their effects on xylanase activity. The highest inhibition was observed in the presence of EDTA (59.37%) and then Sulphonamide (3.8%) and β-mercaptoethanol (2.38%), respectively (Table 6).

3.4. Clarification of fruit juice with BS61 xylanase

One of the most abundant polysaccharide on the cell wall of fruit is xylan. Therefore, in this study, the ability of xylanase from *G. galactosidasius* BS61 to hydrolyse xylan in juice was studied. The decrease in the optical densities of fruit juices at 660 nm was observed with increasing enzyme concentrations (Table 7). The decrease in optical density with 0.15 U/ mL xylanase was measured as 39.74% ± 1.04,

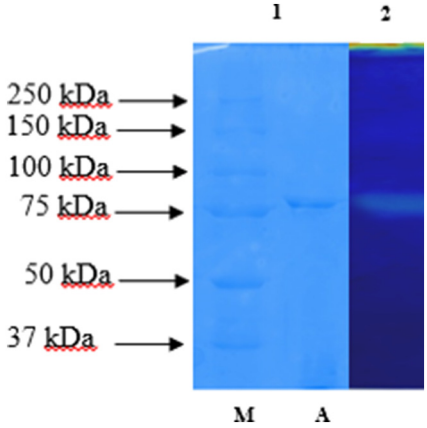

Fig. 4. SDS-PAGE and zymogram profiles of the xylanase from *G. galactosidasius* BS61. 1) SDS-PAGE analysis, M: protein marker (Bio-Rad: (10–250 kDa), A) purified xylanase; 2) zymogram analysis.

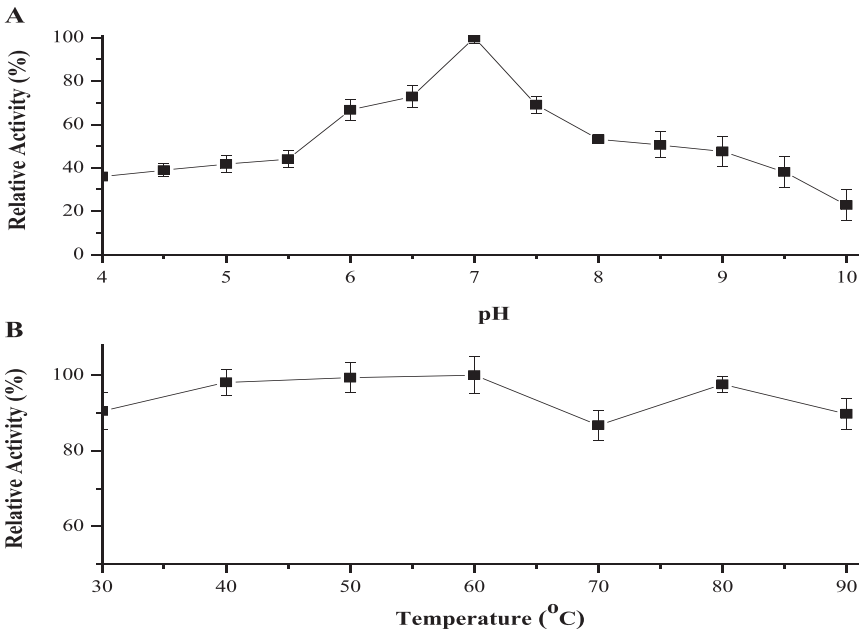

Fig. 5. Influence of A) pH and B) temperature on xylanase from *G. galactosidasius* BS61.

30.52% ± 1.23 and 22.25% ± 0.99 for peach, orange and apricot, respectively. As shown in Table 7, the absorbance (at 660 nm) decrease of orange, pomegranate, apricot, peach, apple, grape and kiwi juices with increasing enzyme concentration was noticed together with increase in reducing sugar amounts. Treatment of apricot and peach juices with increasing concentrations of enzyme resulted in a significant increase in reducing sugars of both fruit juices, with a slightly higher amount of reducing sugar in the peach juice. The increase in the concentration of reducing sugar in the peach juice treated with 0.5, 0.10 and 0.15 U mL^{-1} enzyme was 9.02% ± 0.45, 39.52% ± 0.28 and 45.88% ± 0.42, respectively. The increase in the amount of reducing sugar was found to be at most 36.27% ± 0.97 in apricot juice. The increase in the amount of reducing sugar in other fruit juices remained below 20%. Xylanase was effective at the lowest level in the kiwi juice. The increase

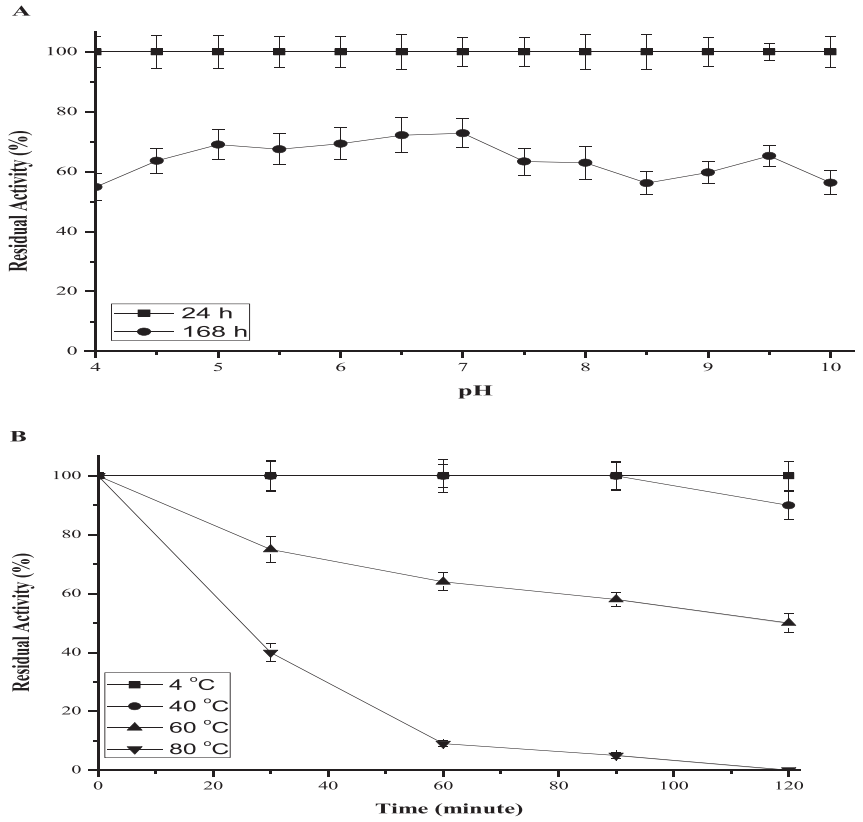

Fig. 6. A) The pH stability B) thermal stability of xylanase from *G. galactosidasius* BS61.

in the amount of reducing sugar in kiwi juice was 4.68% ± 0.20 at the maximum, and the decrease in the optical density was 9.71% ± 0.96 at the maximum.

4. Discussion

Molecular and conventional identifications with basic biochemical parameters were examined and *G. galactosidasius* BS61 were isolated from Rize/Ayder hot spring in Turkey. Meintanis et al. (2008) [42] and Adiguzel et al. (2009) [43] concluded genomic fingerprinting (BOX-PCR) and sequence analysis of 16S rDNA were powerful molecular techniques not only for estimating genetic relatedness, but also for identification and characterization of thermophilic bacterial strains at species/subspecies level. Savas et al. (2009) [44], Adiguzel et al. (2009) [43] and Yanmis et al. (2015) [24] determined that rep-PCR was quite successful for identification of *Brevibacillus*, *Ureibacillus*, *Aeribacillus*, *Geobacillus*, *Anoxybacillus* and *Bacillus* genus, at species and subspecies level. In this study, BOX-PCR among rep-PCR methods was highly successful in the molecular characterizations of test strains. As a result of 16S rRNA sequence analysis, test isolates were similar to *B. licheniformis*, *B. brevis*, *A. pallidus*, *B. thermoamylovorans*, and *G. galactosidasius* at a rate of 99% and Savas et al. (2009) [44] stated that these species were widely available in thermal sources of Turkey. Parallel to the literature, similar species have been identified as dominant flora in this study.

Nazina et al. (2001) [45] carried out 16S rRNA sequence analysis and they found that the test isolates belonged to *Geobacillus* or *Bacillus* genus at a rate of 99%. These *Bacillus* and *Geobacillus* strains were rod-shaped, gram and endospore positive at optimum growth temperatures 35–75 °C and pH 6.0–9.0 according to conventional test results. Poli et al. (2011) [7] determined the temperature and pH range for growth and optimum values were at 70 °C and pH 5.0–9.5 for *G. galactosidasius* sp. nov. from compost. Jiang et al. (2016) [46] studied on a deep-sea thermophile, *Geobacillus* sp., and reported that it showed the optimum response at 70 °C and pH 5.0. Wainø and Ingvorsen (2003) [47] produced β-xylanase from *Halorhabdus utahensis*, an extreme halophilic archaea and reported that it could be produced in a wide range of 0–30% NaCl. Bryanskaya et al. (2015) [10] isolated a novel thermophilic *Geobacillus* sp. in Russia and it was Gram positive, endospore forming, aerobic and grown optimum at 60–65 °C, pH 6.5–7.0 and in the presence of 1% NaCl while 5% NaCl was inhibitory concentration on growth. It was also oxidase negative and catalase positive.

The general information about high temperatures causing enzyme denaturation has been destroyed for several years. Thermophiles are microorganisms that can survive in different localization and depth of geothermal sources where the temperature becomes hotter. Due to the microbial diversity of different geothermal resources, enzymes of these thermophiles are also varied according to the microbial diversity [48]. Almost all of the isolates in this study were producers of amylase, protease, xylanase and cellulase enzymes. These enzymes were industrially important and the importance was more significant when the producers could grow at high thermal values.

Xylanase from *G. galactosidasius* BS61 purified with DEAE-cellulose and Sephadex G-100 columns and molecular weight estimated about 78.15 kDa by SDS-PAGE. After purification steps, the xylanase purified and the specific activity reached to 1203.70 ± 15.96 U mg protein^{-1} with 29.80-fold purification and 35.26% enzyme recovery. In a recent study the GH11 xylanase from *Aspergillus tamarii* Kita purified 7.43-fold with 36.72% yield [49]. The xylanase from *Streptomyces griseorubens* LH-3 was reported to be purified 4-fold with 21.6% enzyme yield [50]. Sanghi et al. (2010) [51] tried to determine molecular weight of cellulase-free xylanase from *Bacillus subtilis* ASH and calculated as 23 kDa with a single band. Gallardo et al. (2004) [52] studied the molecular weight of recombinant xylanase from *Bacillus* sp. BP-7 and it was 24 kDa.

Xylans from beech wood, birch wood, oat bran and carboxymethylcellulose (CMC) were used as different substrates. The xylanases, which do not fragment the CMC, selectively hydrolyse the hemicellulose components; in this way, the cellulose that contributes to the production of quality paper is slightly damaged. In terms of behaviour against various substrates, inability to hydrolyse CMC suggests that xylanase from *G. galactosidasius* BS61 is a suitable biocatalyst with the desired properties in the paper industry [18].

The specific activity, purification fold or enzyme recovery after purification vary depending on materials used in purification or organism used as xylanase producing materials. For this reason, different enzyme yield or purification fold is reported for xylanase from different species [49, 50, 53, 54].

The optimum temperature value of xylanase from *G. galactosidasius* BS61 was 60 °C, and the enzyme retained nearly 50% of its activity after 120 min incubation at optimum temperature. The optimum pH was 7.0. The enzyme activity was fully conserved at pH 4.0–10.0 after 24 h incubation. Bhalla et al. (2015) [55] determined the optimum pH of xylanase from *Geobacillus* WSUCF as 70 °C. Bin et al. (2012) [56] reported that xylanase from *Geobacillus* sp. TC-W7 was optimum at 75 °C while Gerasimova and Kuisiene [57] reported the optimum temperature of xylanase from *G. thermodenitrificans* JK1 was also 70 °C. Optimum temperatures of xylanases from *Geobacillus* sp. TF16 [16] and *Geobacillus* sp. WSUCF1 [3] were reported to be 50 °C and 70 °C, respectively; and it was found that the activity fell rapidly below and above the optimum temperature [3, 16]. Xylanase from *Geobacillus* sp. TF16 protected 53% of its activity at 65 °C for 40 min [16]. Capacious temperature range of *G. galactosidasius* BS61 xylanase has a distinctive feature among xylanase. As a result of our literature search, we did not find a temperature profile similar to xylanase which we have investigated. A similar temperature profile was reported for a protease from a *B. subtilis* strain by Uttatree and Charoenpanich [58].

The pH stabilities of xylanases from *G. stearothermophilus* and *Geobacillus* sp. TF16 were less than *G. galactosidasius* BS61's, because their xylanase activities increased at the end of 24 hour-incubation

Table 5
Influence of some metal ions on xylanase of *G. galactosidasius* BS61.

Cation (5 mM)	Remaining activity (%)	Anion (5 mM)	Remaining activity (%)
Control	100 (%)	Control	100 (%)
Ca^{2+}	98.11 ± 2.65	HSO_3^-	124,78 ± 5.24
Mn^{2+}	21,58 ± 0.56	CN^-	79.17 ± 2.74
Na^+	93.68 ± 2.95	SO_4^{2-}	85.39 ± 3.02
Mg^{2+}	78.22 ± 3.88	CO_3^{2-}	78.98 ± 3.52
Al^{3+}	89.72 ± 3.54	HCO_3^{2-}	96.32 ± 4.03
Cd^{2+}	86.61 ± 3.22	HPO_4^{2-}	124.22 ± 4.99
Ni^{2+}	76.53 ± 3.85	$B_4O_7^{2-}$	78.32 ± 3.66
Zn^{2+}	88.21 ± 2.96		
Cu^{2+}	71.06 ± 2.75		

Table 4
Substrate specificity of xylanase of *G. galactosidasius* BS61.

Substrate	Relative activity (%)
Birchwood xylan	93.23 ± 1.95
Beechwood xylan	100
Oat bran	98.52 ± 1.45
Carboxymethyl cellulose	0.00 ± 0.00

Table 6
Influence of some chemicals on xylanase of *G. galactosidasius* BS61.

Inhibitor (5 mM)	Remaining activity (%)
Control	100
β-Mercaptoethanol	97.62 ± 1.30
Sulphonamide	96.20 ± 0.81
EDTA	40. 63 ± 2.08

Table 7
Clarification of some fruit juice by using xylanase of *G. galactosidasius* BS61. (* $p < 0.01$; ** $p < 0.05$).

Fruit	Decrease in absorbance at 660 nm (%)			Increase in reducing sugar (%)		
	0.05 (U/mL)	0.10 (U/mL)	0.15 (U/mL)	0.05 (U/mL)	0.10 (U/mL)	0.15 (U/mL)
Orange	10.61 ± 0.92*	21.46 ± 0.96*	30.52 ± 1.23*	5.58 ± 0.47**	11.08 ± 0.63**	17.36 ± 1.18**
Pomegranate	9.33 ± 1.22*	13.09 ± 1.08*	19.16 ± 1.29*	5.08 ± 0.35*	8.42 ± 0.83*	12.55 ± 1.28*
Apricot	12.66 ± 0.52*	17.95 ± 1.03*	22.25 ± 0.99*	9.02 ± 0.45*	22.45 ± 0.83*	36.27 ± 0.97*
Peach	16.05 ± 1.06*	29.42 ± 1.31*	39.74 ± 1.04*	24.74 ± 0.98*	39.52 ± 0.28*	45.88 ± 0.42*
Apple	8.95 ± 1.37*	12.47 ± 1.01*	19.01 ± 1.53*	6.67 ± 0.84*	8.89 ± 1.38*	11.20 ± 1.11*
Grape	5.55 ± 0.61*	9.08 ± 0.76*	13.87 ± 0.97*	3.86 ± 0.19*	4.89 ± 0.25*	10.54 ± 0.75*
Kiwi	4.11 ± 0.38*	6.57 ± 0.42*	9.71 ± 0.96*	1.22 ± 0.12*	3.03 ± 0.10*	4.68 ± 0.20*

[16, 59]. With these features, xylanase of *G. galactosidasius* BS61 is an enzyme that can be used in a wide range of pH and temperature-dependent industrial processes. Moreover, from this point of view, it is more advantageous than other xylanases in the literature. In addition, the enzyme exhibited a stability over a wide pH range and in the presence of metal ions. Inhibition of xylanase with Mn^{2+} was reported for *Bacillus* sp. strain K-1, *Bacillus halodurans* S7 and *Penicillium oxalicum* CZ1028 [60–62]. Conversely, there is also evidence that the same ion significantly enhanced xylanase activity [53, 63]. The coordination numbers, the coordination geometries in the compounds and the Lewis acid potentials of the metal ions may be different from each other. Therefore, metals may exhibit different properties from each other against proteins, and behaviours of proteins towards metal ions may differ [64].

EDTA caused 59.37% inhibition rate while β-mercaptoethanol (2.38% inhibition) and Sulphonamide (3.80% inhibition) did not significantly affect enzyme activity (Table 6). Due to the EDTA inhibition, it can be said that xylanase of *G. galactosidasius* BS61 requires a metal ion for its activity or it lowers the enzyme activity by causing the change of the enzyme conformation through EDTA. Cakmak and Ertunga [16] reported that the xylanase of *Geobacillus* sp. TF16 was inhibited >60% in the presence of 1 mM EDTA. *Penicillium* sp. UCP 1286 [65], *Myceliophthora thermophila* BF1–7 [54] and *Streptomyces thermovulgaris* TISTR1948 [53] was also inhibited at a rate of 5%, 5% and 15%, with 15% EDTA.

Kinetic parameters of *G. galactosidasius* BS61 xylanase were studied using xylan from beech wood, which was determined to be the best substrate for this enzyme. K_m and V_{max} values of *G. galactosidasius* BS61 xylanase were calculated as 3.18 mg mL^{-1} and 123 U mg $protein^{-1}$, respectively. The K_m and V_{max} of xylanases from *G. thermoleovorans* and *G. thermodenitrificans* TSAA1 were reported to be 2.6 mg mL^{-1} and 31.2.5 U mg^{-1}, and 0.625 mg/ mL and 555.5 U mg^{-1}, respectively [18, 19]. The xylanase of *Glaciecola mesophila* KMM 241 exhibited K_m value as 1.22 mg mL^{-1} and V_{max} value as 98.31 U mg^{-1} for beech wood xylan as substrate [66].

It was determined that the effect of xylanase on the pomegranate, apple, grape and kiwi juices were lower than that of peach, orange and apricot juices. Xylanase of *G. galactosidasius* BS61 that leads to an increase in the amount of reducing sugars and a decrease in turbidity has shown the potential to be used in apricot, peach and orange juice industries. The presence of hemicelluloses, starch, pectin and other cellulosic polysaccharides in crude fruit juices causes turbidity, and thus, an increase in optical density. The hydrolysis of these components ensures the clarity of the juice and the quality improvement [17]. In a study, xylanase from *Bacillus licheniformis* P11 (C) was reported to reduce the amount of reducing sugar by 10 and 5-fold, respectively after 24 hour treatment with orange and apple juices [67].

5. Conclusion

Today, the use of enzymes has come to the forefront as a more environmental-friendly and productive option for various practices; and this has inspired the researchers to discover new enzymes and enzyme groups that are resistant to extreme conditions. Thermophilic microorganisms have enzymes that are designed to function at high temperatures, which enable them to perform different physiological functions in changing physicochemical micro-environments. In the present study, a purification and characterization of a novel xylanase was performed from *G. galactosidasius* BS61 strain, which had the highest xylanase enzyme activity, among the obtained thermophilic isolates. It was concluded that the xylanase enzyme, which was purified from *G. galactosidasius* BS61 strain, could have the potential of being used in a wide variety of biotechnological fields mainly in purification of fruit juice because it has a good level activity at high temperatures.

Acknowledgements

This work was supported by the Research Development Centre of Ataturk University (Grant numbers are 2015-339 and FAD-2018-6352).

Conflict of interest

There is no conflict of interest to be declared.

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