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A novel endo-β-1,4-xylanase from *Pediococcus acidilactici* GC25; purification, characterization and application in clarification of fruit juices

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ABSTRACT

A novel extracellular xylanase was purified and characterized from *Pediococcus acidilactici* GC25 (GenBank number: MF289522). The purification was 4.6-fold with a yield of 43.61% through acetone precipitation, Q-Sepharose, and CM-Sepharose ion change chromatography. The molecular weight of the enzyme was 48.15 kDa, and the optimum pH and temperature were 7.0 and 40 °C, respectively. The maximum activity was observed between 20 and 50 °C. Although it was active within a wide pH range (pH 2.0–9.0), it retained over 85% of its activity after 24 h incubation; and over 70% of its activity after 168 h incubation in neutral and alkaline pH. It was observed that the enzyme showed high stability with K^+, Ba^{2+}, Cd^{2+}, Co^{2+}, Sr^{2+}, Mg^{2+}, Ca^{2+}, Al^{3+}, Zn^{2+}, and Ni^{2+} ions. The K_m and V_{max} for the xylanase were 3.10 mg mL^{-1} and 4.66 U/mg protein, respectively. It was determined that treatment of different fruit juices with *P. acidilactici* GC25 xylanase improved the clarification. The highest increase in the reducing sugar amount and decrease in the turbidity was 24.47 ± 1.08 and 21.22 ± 0.58 for peach juice at 0.15 U/mL enzyme concentration. These results showed that the xylanase purified from *P. acidilactici* GC25 may have a wide potential in biotechnological processes of the food and baking industry.
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1. Introduction

Fermented sausages are the meat products with the highest production and consumption rates due to their pleasant textures, distinctive taste, and high nutritional values. The most important factor in the ripening, development, and quality of fermented sausages is the microbial activity, and the most significant role at this stage is undertaken by the species that are the members of the *Micrococcus*, *Pediococcus*, *Staphylococcus* and *Lactobacillus* genera [1,2]. Lactic Acid Bacteria (LAB) are the Gram-positive and facultative anaerobic microorganisms in the shape of a bar or coccus, which do not form endospore. They are resistant to acids and are homo and hetero-fermentative [1,3]. Except for some, they are generally nonpathogenic, and play important roles in the ripening, production, and endurance of food products when added (or sometimes they exist naturally) to especially meat and fishery products (sausage, fish, etc.), grain products (bread, boza, ogi etc.), dairy products (yogurt, cheese, kefir etc.), wine and vegetables (cabbage and cucumber, pickle, etc.) [4,5]. *Lactobacillus*, *Paralactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus*, *Streptococcus*, *Weisella*, *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Oenococcus*, *Sporolactobacillus*, *Tetragenococcus*, *Vagococcus*, and *Bifidobacterium* belong to this group [5–7]. LAB are widely used in various biotechnological processes due to their wide enzyme profiles [5,8]. Xylanase [β-1,4-D-endoxylanase (EC 3.2.1.8)], one of these enzymes, hydrolyzes the β-1,4 glycoside bonds of xylan, and decompose it to D-xylose units. In this way, they increase the bio-efficacy of hemicellulosic substances [9]. Xylan is the second most common polysaccharides in the nature, and constitutes the main component of plant cell walls and hemicelluloses of some algae, and one-third of the renewable organic carbon sources in the whole world [10–12]. Xylan has a very complex and heterogeneous structure consisting of the connection of xylose residues with β-1,4-glycosidic bonds. For this reason, xylan hydrolysis is performed by a synergic effect of a wide enzyme group, which is called the xylanolytic enzyme system with a wide variety. β-1,4-endoxylanase, β-xylosidase, α-L-arabinofuranosidases, α-glucuronidase, acetyl xylan esterase, and phenolic acid (Ferulic and p-Coumaric acid) esterase enzymes are in this group [10,13]. The most important hydrolase among these enzymes is the β-1,4-endoxylanase, which decomposes the xylan backbone randomly to release xylo-

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oligomers. Xylanase has a wide application area in biotechnological and industrial processes, and is used commonly in general applications like the production of liquid or gas fuels, some solvents and sugar groups; and in single-cell protein production, in the conversion of lignocellulosic materials, and in the conversion of agricultural wastes into fermentative products, and in feed, textile, paper and pulp industry [14,15]. Xylanase has several applications especially in various sectors of the food industry. For example, in bread production, in clarifying fruit juices and in the beer industry. In addition, xylooligosaccharides, which are obtained with the enzymatic hydrolysis of xylan, are important components for the development of new functional foods due to their prebiotic effects [10]. Industrial production of xylanolytic enzymes is based on microbial biosynthesis.

Microbial xylanase is an important catalyst preferred for xylan hydrolysis due to high specificity, mild reaction conditions, negligible substrate losses and by-products [15–17]. In addition, successful applications in industrial processes have made the microorganisms that can produce xylanolytic enzymes become a focus of interest in recent years [18]. Xylanase enzyme is produced by various microorganisms in the bacteria, yeast and fungus group. Previously, although fungi were considered as potential microorganisms for production of xylanase due to their high activity; restrictions on mass production and industrial applications have placed bacterial xylanases as a challenging competitor against fungi in the industrial area [19,20]. Bacterial xylanases are more advantageous in biotechnological applications than fungal xylanases [21]. While the optimum pH for bacterial xylanases is in the neutral or alkaline range, the fungus is in the acidic range for xylanases. The low pH requirement for the growth of fungi and the production of xylanases requires additional steps in subsequent down-stream processes which make the fungi xylanases less attractive. In addition, fungi xylanases are produced simultaneously with cellulase which increases the length of down-stream processes; in bacteria, xylanase is usually produced by itself, thereby lowering the processing time [22]. Therefore; in-depth studies on the biology and biochemistry of bacterial xylanases will provide a better field for the investigation or application of high-throughput xylanases for effective use in industrial areas [21].

Previous studies have revealed that xylanase enzyme that is purified from various bacteria and characterized has extremely varying enzymatic properties [23]. This has led scientists to conduct studies on isolation of microorganisms from new sources, and purification and characterization of the enzyme that has biotechnological importance. So far, scientists have conducted several studies to explore the potential usage areas of these enzymes [24]. In this study, the *Pediococcus acidilactici* GC 25 bacterium was isolated from fermented sausage sample, characterized by using phenotypic and genotypic methods, and the purification and characterization of the xylanase enzyme produced by this bacterium in an extracellular way, and the applicability of it in clarification of some fruit juices were investigated.

2. Materials and methods

2.1. Isolation of lactic acid bacteria (LAB)

The sausage sample was purchased from a market in Erzincan. The sausage samples (25 g) was added to 225 mL sterile saline peptone water for microbiological analysis and was homogenized in a stomacher for 1 min (Laboratory Blender Stomacher 400, Seward Medical, London, UK). Afterward, the decimal dilutions of the homogenate were prepared. 0.1 mL sample of appropriate dilutions were plated onto MRS (DeMan, Rogosa and Sharpe) agar plates and were incubated at 30 °C for 24–48 h [1]. Colonies that were different from each other were selected randomly and pure cultures were prepared in MRS agar medium and kept at Nutrient Broth (NB) medium with 15% glycerol at −86 °C until the xylanase pre-enzyme activity determination studies were made.

2.2. Analyzing xylanolytic activity of the isolated LAB

In order to define the isolate with the highest xylanase enzyme activity, solid xylan medium in petri dish [25] was prepared and autoclaved. Each isolate that was animated from the stock was transferred to xylan plates, and incubated at 30 °C for 3–4 days; and 0.1% Congo Red stain was added to the plates after taking them out from incubator and were kept for 15 min. Then, 1 M NaCl salt solution was added to the plates, and washed for 15 min. After this process, zone formation was observed and the isolate GC25 with highest zone formation was used as test isolate for xylanase purification.

2.3. Phenotypic and genotypic characterization of xylanolytic isolate

After the xylanolytic activity stage, the isolate GC25, which gave the widest zone, was selected, and the characterization stage was started. A microscope was used to isolate the cell morphology. The isolate was gram-stained and tested for catalase production. Firstly, it was identified based on the phenotypic properties such as carbon dioxide production from glucose growth at different temperatures (15–45 °C) and pH (4–9) as well as the ability to grow in different concentrations of sodium chloride (2–6.5%) in MRS broth. The API 50 CHL test strips (Bio Merieux, Inc., Marcy l'Etoile, France) [1] was used to determine the sugar fermentation patterns of the test isolate. The method modified by Adiguzel and Atasever [1] was used to extract the total genomic DNA of the test isolate (GC25). The 16S rRNA region, which was important in terms of bacterial systematics, was amplified using 27F (5′-AGAG TTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) primers, and then, it was cloned into *E. coli* JM101 strain with the pGEM-T Easy Cloning Vector (Promega, Southampton, UK) according to the instructions of the manufacturer. After the cloning, the plasmid isolation was performed by selecting the colonies that gave the positive result, and the sequence analysis was made by the Macrogen Company (Holland). The 16S rRNA obtained was compared with the other bacterial series in the GenBank and EzTaxon (http://blast.ncbi.nlm.nih. gov/blast.cgi and http://www.eztaxon.org), the similarity rate between them was determined and GenBank accession number was received. A phylogenetic tree was created through the Neighbor-Joining Technique by considering the result of the study. For this, the MEGA 4.0 Software Package was used [5,27].

2.4. Xylanase production conditions

250 mL Erlenmeyer Flask containing 50 mL of enzyme production medium was used to produce the enzyme. The composition was composed of 2.5% oatmeal, 0.5% yeast extract, 0.5% peptone, 1% K_2HPO_4, 0.025% $MgSO_4 \cdot 7H_2O$, 0.1% NaCl, and 1% inoculum, at pH 7.0 [28]. The fermentation process was performed on an orbital shaker at 150 rpm and at 37 °C for 24 h. The biomass was separated by centrifugation at 13,000 ×*g* for 20 min at 4 °C after the fermentation, and the supernatant was used as the extracellular enzyme source and was stored at 4 °C for further purification steps.

2.5. Xylanase enzyme purification

The culture supernatant containing the xylanase enzyme was precipitated with 1:1 cold acetone. The acetone that was kept at −20 °C was added to the homogenate with Pasteur pipette drop-by-drop in a slow manner. During this process, a magnetic mixer was used and the process was performed at +4 °C. With the addition of the whole of the acetone, the mixture was kept at −20 °C for 30–60 min. Then, it was centrifuged at 13,000 ×*g* for 30 min. The precipitation was kept at room temperature in an open container to remove the acetone. The precipitation was dissolved with 20 mM pH 8.0 Tris-HCl buffer solution in a minimum volume. In order to remove the substances that were not dissolved in the precipitation, the centrifuging was repeated, and a clear

enzyme solution was obtained. The enzyme solution that was obtained as a result of the precipitation process with acetone was dialyzed in 20 mM pH 8.0 Tris-HCl buffer for 24 h and loaded to Q-Sepharose column that was balanced with 20 mM pH 8.0 Tris-HCl buffer. The bonded xylanase was eluted with 0–500 mM NaCl (0.5 mL/min) gradient that was prepared in the same buffer. Active fractions were collected, and dialyzed with 20 mM pH 8.0 Tris-HCl buffer and were then loaded to CM-Sepharose column that was balanced with the same buffer. After the elusion, the fractions showing high xylanase activity were dialyzed [29].

2.6. Enzyme assay

Xylanase activity was determined by using the dinitrosalicylic acid (DNS) method [30]. Then, 0.3 mL 50 mM Tris-HCl buffer with 7.0 pH and 0.2 mL of 5% birchwood xylan solution, which were prepared in the same buffer, were added to the 0.5 mL enzyme solution; and the reaction solution was incubated for 15 min at 37 °C. As the blank one, the reaction mixture, to which 50 mM pH 7.0 Tris-HCl buffer solution was added, was used instead of enzyme solution. After the incubation, 1 mL DNS solution was added to the reaction mixtures to stop the reaction, and the mixture was kept at 90 °C for 5 min, and then was measured by comparing absorbance blank at 540 nm in a spectrophotometric way (Shimadzu Uv-1600 Uv-visible Spectrophotometer, Japan). One unit (U) xylanase activity was defined as the enzyme amount that liberated 1 μmol xylose and equivalent sugar in 1 min under optimal conditions.

2.7. Protein determination

The protein concentration was determined according to the Lowry method in which bovine serum albumin (BSA) was used as a standard [31].

2.8. Electrophoresis and zymogram

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli [32] by using 5% stacking gel and 10% separation gel (Mini-PROTEAN Tetra Cell Unit Bio-Rad, USA). Protein bands were determined with Coomassie Brilliant Blue R250 staining. As protein marker standard, the Bio-Rad Precision Plus Protein™ Unstained was used.

For zymogram analysis, the samples were heated at 35 °C for 5 min and loaded to 10% SDS-PAGE gel. After the electrophoresis application, the gel that was taken out from the tank was kept at 2.5% TritonX-100 for 30 min, and after shaking, for another 30 min. After this process, it was kept at 50 mM pH 7.0 sodium phosphate buffer that included gel in 1% xylan substrate for 30 min at 40 °C and was stained with 1% Congo-red for 15 min. The gel was then washed with 1 M NaCl solution until the band was visible. In order to receive the latest image, the gel was treated with 5% acetic acid [29].

2.9. Effect of pH on xylanase activity and stability

In order to determine the pH value in which the purified xylanase showed the best activity, the activity determination was performed at 37 °C by using buffers at various pH values. Glycine-HCl buffer (pH 2.0–3.0), citrate buffer (pH 3.0–6.0), phosphate buffer (pH 6.0–8.0), and Tris-HCl buffer (pH 8.0–9.0) were used in the present study. The pH value in which the highest activity was observed in drawing the graphical view of the pH profile of the enzyme was accepted as 100, and the other results were compared according to it as a relative activity (%).

For the purpose of examining the pH stability of xylanase, the pure enzyme eluate was mixed with buffers between pH 2.0–9.0 at a ratio of 1:1 and was incubated at 4 °C for 24 and 168 h. The remaining activity (%) was determined at optimum pH and temperature [5].

2.10. Effect of temperature on xylanase activity and stability

In order to examine the effect of the temperature on xylanase activity, activity determination was performed at different temperature values (10–80 °C) at optimum pH [5]. The optimum temperature value for xylanase enzyme was determined from the temperature-relative activity (%) graphics by using the obtained data.

For the purpose of examining the thermal stability of the enzyme, it was incubated at 40, 50 and 60 °C for 120 min. Activity determination was performed every half hour at optimum pH and temperature. The remaining activities were computed by comparing the activity value of the enzyme to which no pre-incubation was applied and which was computed under optimum conditions, and then the time-remaining activity (%) graphics were drawn [5].

2.11. Determination of K_m and V_{max}

To determine the Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) values of the xylanase enzyme, the enzymatic activity was measured in different concentrations of Birchwood xylan substrate (0.15–8.00 mg mL^{-1}). The activity was performed under optimum conditions by using the standard activity method. The kinetic values of the enzyme were measured with Lineweaver-Burk graphics [33].

2.12. Effect of metal ions on xylanase activity

In order to examine the effect of the metal ion on the enzyme activity, the purified xylanase was incubated at room temperature for 1 h with metal ions (K^+, Ba^{2+}, Cd^{2+}, Co^{2+}, Sr^{2+}, Mg^{2+}, Ca^{2+}, Al^{3+}, Zn^{2+}, and Ni^{2+}) at 5 mM final concentration At the end of this time, the measurements were performed with the standard activity assay method at optimum conditions. The activity of the enzyme without any metal ions was accepted as 100%, and the activity of those that included metal ions was determined as the remaining activity [34].

2.13. Application of GC25 xylanase in fruit juice clarification

The skins of the fruits (orange, pomegranate, apricot, peach, apple, grape, and kiwi) that would be used in the study were peeled off, and the seeds were removed. The juices were obtained by using a fruit juice extractor. These juices were centrifuged at 4500 rpm for 30 min. The supernatant was used in enzymatic experiments [35]. Then, 0.05, 0.10, 0.15 U/mL enzyme was treated with the fruit juice and was incubated at 40 °C. After 1 h, the reducing sugar in the samples was determined with the DNS method [30], and the optical density at 660 nm was examined. The results were compared with a controlled study that did not include enzymes [2].

2.14. Statistical analysis

The measurements made to determine the xylanase activity were repeated three times, and the results were given as mean ± standard deviation. The Anova Test was applied using SPSS 21.0 Program. The differences (if any) between the groups were investigated by using the Student-Newman-Keuls (SNK) Test.

3. Results and discussion

3.1. Identification of xylanolytic bacteria

Following the isolation of LAB from sausage sample, ten isolates were determined (data not shown) according to morphological and colony differences on Petri surface. Later, the xylanase production abilities of these isolates were determined; and GC25 test isolate with significant zone formation on Petri plate was chosen to study. The GC25 test strain that was isolated in this study was identified after various morphologic, physiologic

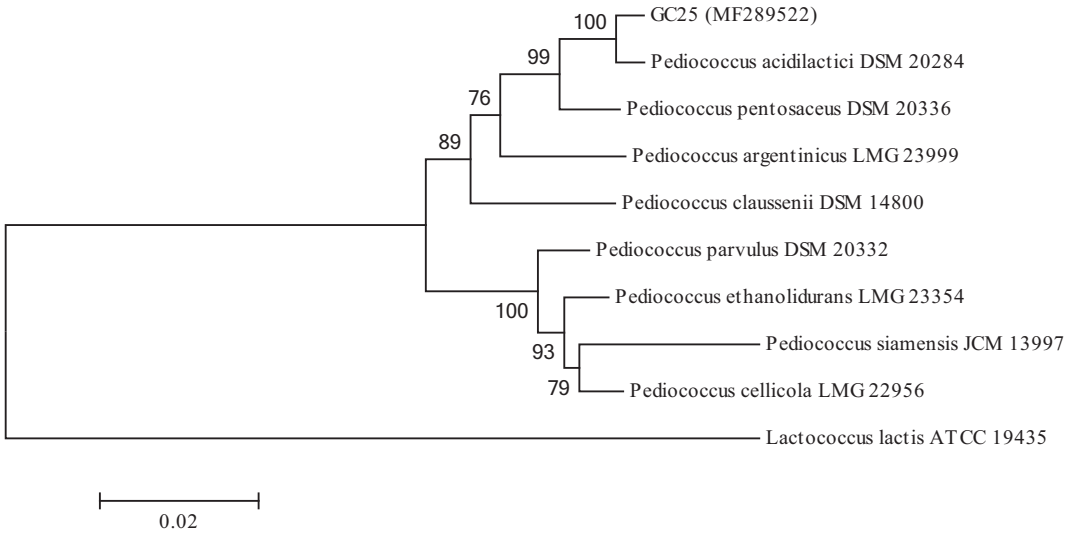


Fig. 1. On the basis of 16S rRNA gene sequence data of the Lactic Acid Bacteria (isolated from Turkish fermented sausage), neighbor-joining phylogenetic tree (*Lactococcus lactis* ATCC 19435 was used as out-group). Bootstrap values, which are based on 1000 replications, are listed as percentages at branching points. Only bootstrap values >50% are shown at nodes. The scale bar represents 2% divergence.

and biochemical tests. The results showed that the test isolate was Gram-positive, facultative anaerob, catalase negative, had coccus cell morphology, and reproduced at 30–45 °C in pH 5.5–7.0 and 2–6% salt concentrations. Then, it was determined that this isolate was *Pediococcus acidilactici* after the analysis by using the API CH-50 test system. It was also determined that these data complied with the literature data [36–39].

3.2. Evolutionary relationships of the isolate

According to 16S rRNA sequencing, one of the molecular techniques, the test isolate was determined to have nearly 1538 nucleotides. Then, in consequence of the blast study performed by using the database included in the GenBank and EzTaxon, it was determined that this isolate resembled *P. acidilactici* GC25 (GenBank number: MF289522) at a high rate (99%). In terms of the 16S rRNA gene sequences of *P. acidilactici* isolate, a similar value (>98%) was obtained, as specified before by Mandal [38] and Nadaroglu [2], who verified that the similarity of *P. acidilactici* strains was higher than 98%.

The evolutionary relationship of the test strain (and 9 other closely related species) was determined by using the Neighbor-Joining Method. The tree was constructed by using the *p*-distance of nucleotide difference (Fig. 1) [40]. The replicate tree percentage in which the associated taxa are clustered together in the bootstrap test (1000 replicates) is shown next to the branches [41]. Since those evolutionary distances were used to infer the phylogenetic tree, the tree is drawn to scale with branch lengths in the same unit. By eliminating the positions containing gaps and missing data, a total of 1418 positions were obtained in the final dataset.

3.3. Purification of the xylanase from P. acidilactici GC25

After acetone precipitation of xylanase enzyme from *P. acidilactici* GC25, it was purified by using Q-Sepharose and CM-Sepharose chromatography. The purification profile of xylanase enzyme is given in Table 1. Xylanase was purified 2.14-fold with 70.43% recovery in the step where Q-Sepharose column was used.

The active fractions taken from the Q-Sepharose column were loaded to the CM-Sepharose column. After the final purification step, the xylanase enzyme was purified 4.60 folds with 43.61% yield. In recent studies, xylanase was purified from *Streptomyces griseorubens* LH-3 and *Thermoascus aurantiacu* at 4.2 and 5.27 folds with 21.6% and 36.5% yield, respectively [42,43]. Silva et al. [44] reported 5.3 purification folds and 12% recovery for the xylanase isolated from *Trichoderma inhamatum*. The protein yield obtained in this study for the purification of xylanase from *Pediococcus acidilactici* GC25 is higher than the values reported for the studies mentioned above. The SDS-PAGE analysis of the purified GC25 xylanase was observed to be a single-band on the gel. The molecular weight of the xylanase was calculated as ~48.15 kDa by transferring the relative mobilities (Rf) of each protein to the graphic in correspondence to the logarithm of the molecule weights of the standard proteins (Fig. 2A). The molecular weights of the xylanase defined with SDS-PAGE and obtained from different sources vary according to the source from which the enzyme is isolated [24,25,45]. The endoxylanase obtained from bacteria and fungi exhibit the single-subunit protein structures whose molecular weights vary between 8.5 and 85 kDa [46]. In the zymogram analysis that was stained with Congo-red, the existence of a clear band that is responsible for the xylanase activity (Fig. 2B) was observed.

3.4. Effect of pH on xylanase enzyme activity and stability

The enzyme activity assay performed between pH 2.0–9.0 showed that the optimum pH value of the *P. acidilactici* GC25 xylanase was 7.0. It was found that the enzyme was ≥60% active in all pH values; however, it was more active in neutral and alkaline pH values (Fig. 3a). It was also reported that the optimum pH value of most of the bacterial xylanase was between 5.0 and 8.0 [47]. For example, the optimum pH of the purified xylanases from *Bacillus tequilensis* [47] and *Streptomyces* sp.

Table 1
Purification procedure of xylanase from *P. acidilactici* GC25.

Purification steps	Total activity (U)	Total protein (mg)	Specific activity (U/ mg)	Purification fold	Yield (%)
Crude extract	42.35×10^3	612	69.19 ± 0.57	1	100
Acetone precipitation	40.67×10^3	358	113.60 ± 2.53	1.64	96.03
Q- Sepharose	29.85×10^3	201	148.57 ± 3.06	2.14	70.48
CM- Sepharose	18.47×10^3	58	318.45 ± 5.05	4.60	43.61

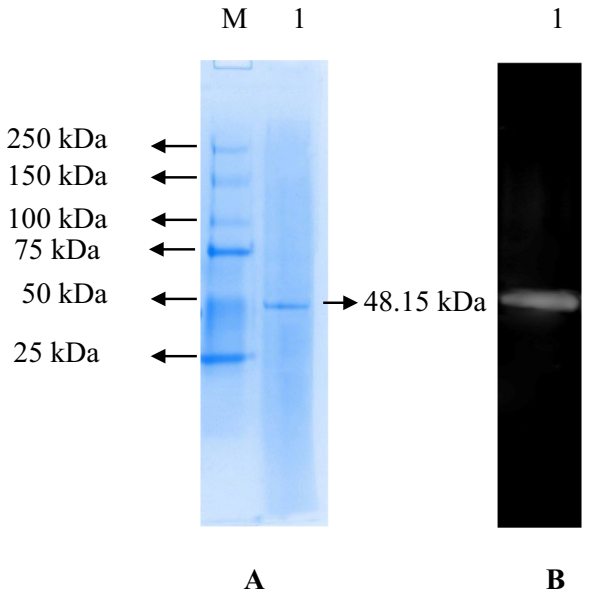


Fig. 2. SDS-PAGE profile and zymogram analysis of the purified xylanase from *P. acidilactici* GC25. A. SDS-PAGE analysis. Proteins were stained with Coomassie brilliant blue R-250. Lane M: Standard protein molecular mass markers, Lane 1: Purified xylanase; B. Lane 1: Zymogram of the purified xylanase.

SWU10 [14] were 7.0, and 5.0, respectively. In many areas of biotechnological applications, bacterial xylanases are considered to be more advantageous than fungi xylanases and are therefore preferred due to their activity and high stability in the alkaline range [21].

The studies conducted to reveal the pH stability of the enzyme showed that the enzyme stayed active within a wide pH range after incubating for 24 h in different pH values (pH 2.0–9.0). As it is given in Fig. 3b, it was also determined that after 168-h incubation, the enzyme preserved its activity over 70% in alkaline pH values and in values that were close to neutral, and as the acidity (pH 2.0–7.0) increased, the activity decreased. It was observed that the *P. acidilactici* GC25 xylanase preserved over 85% of its activity within a wide pH range (pH 2.0–9.0) after 24-h incubation. The pH stability of the *Thielaviopsis basicola* xylanase was examined, and it was determined that after 5-h incubation at pH 5.0 and 8.0, the enzyme activity decreased at a rate of 10% and 15%, respectively [24]. In the *B. tequilensis* xylanase, on the other hand, it was observed that it preserved its activity at a rate of 80% after 10-h incubation in acidic and usual pH values; and lost 60% of its activity in basic pH values [47].

Xylanase of *P. acidilactici* GC25 exhibited stability over a wide pH range. It showed that it could be a suitable candidate for use in industrial applications that required stability and activity in acidic environment such as maceration and purification of fruit juices and wines and in other applications that required stability and activity in neutral/alkaline environment [48]. As a result; *P. acidilactici* GC25 xylanase enzyme is of great importance due to its higher activity and stability at alkaline pH values as well as its extremely high tolerance within a wide range of pH in terms of its potential industrial applications [49].

3.5. Effect of temperature on xylanase enzyme activity and stability

The optimum temperature of *P. acidilactici* GC25 xylanase was found to be 40 °C (Fig. 3c). Similarly, the optimum temperatures of *Penicillium* sp. CGMCC 1669, *Streptomyces* sp. SWU10, *Aspergillus niger* DFR-5 xylanase was reported as 40 °C [14,26,50]. In addition, the optimum temperature value of *B. tequilensis* xylanase was reported as 35 °C [47]. Since preparing dough and proofing steps in which the xylanase that shows its enzyme activity mostly in low and medium-level occur generally under 35 °C, it is considered that GC25 xylanase may be highly suitable for use in the baking industry [51].

In order to examine the thermal stability of the enzyme, it was incubated at 40, 50, 60 °C for 120 min. The remaining activities were determined by using the standard xylanase activity method. When the thermal stability profile of the enzyme was examined, it was determined that it preserved its enzymatic activity over 80% after 2 h at 40 and 50 °C. However, at 60 °C, it was observed that the activity decreased in time (Fig. 3d). It was reported that the activity of the *Penicillium* sp. CGMCC 1669 xylanase decreased at a fast pace in incubation at 50 °C and the xylanase activity was lost completely after 30 min [26].

3.6. Kinetic parameters of xylanase

The K_m and V_{max} values of the purified GC25 xylanase were determined as 3.10 mg mL^{-1} and 4.66 U/mg protein, respectively. The K_m values of the *Bacillus* sp. NTU-06 and *Staphylococcus* sp. SG-13 xylanase showed similarities with the K_m values of the GC25 xylanase and were determined as 3.45 and 4.00 mg mL^{-1}, respectively [51,52]. The K_m value determined for GC25 xylanase was lower than the values determined in the literature for *Myceliophthora thermophila* BF1-7, *Trichoderma inhamatum*, *Neosartorya tatenoi* KKU-CLB-3-2-4-1, *A. niger* DFR-5 xylanase [44,50,53,54].

3.7. The effect of metal ions on activity

The metal ion stability of the *P. acidilactici* GC25 xylanase was examined in the existence of 5 mM K^+, Ba^{2+}, Cd^{2+}, Co^{2+}, Sr^{2+}, Mg^{2+}, Ca^{2+}, Al^{3+}, Zn^{2+}, and Ni^{2+} ions, and the results are given in Fig. 4. After 1 h of incubation, it was determined that *P. acidilactici* GC25 xylanase was not affected much by the metal ions, and preserved >80% of its enzymatic activity in the existence of all metal ions in question. Similarly, it was reported in the literature that the Ca^{2+}, Mg^{2+}, Ni^{2+}, Co^{2+}, K^+, and Zn^{2+} ions do not have significant effects on the xylanase activity [55–57]. On the other hand, there are also several other studies reporting that the enzyme is inhibited in the presence of Zn^{2+}, Co^{2+}, K^+ and Ba^{2+} [17,18,24]. Productions at industrial scale are run in big reactors, and there are several reaction stages that involve various metal ions [24]. For this reason, it is considered that the GC25 xylanase enzyme preserving its activity and stability in the presence of the metal ions mentioned in the study is an important advantage in using it in industrial processes.

3.8. Clarification of fruit juice with P. acidilactici GC25 xylanase

Enzymatic processes are used widely in fruit juice extraction and for clarification. Fruit juice clarified with enzymatic methods results in a decrease in viscosity and cluster formation. This facilitates the separation of suspended solids with centrifuging and filtration methods. As a result, it provides the fruit juice with higher clarification and more concentrated aroma and color [57].

The polymeric carbohydrates in fruit juices cause that it gains a hazy color and increase the viscosity. When the fruit juice is treated with xylanase, reducing carbohydrate units are revealed. The resulting reducing sugar amount is the indicator for the decomposition of the polymeric carbohydrate by the enzyme [35]. As it is given in Table 2, a decrease was observed in the 660 nm absorbance of the fruit juices with the increasing enzyme concentration. The decrease in the absorbance is more in the peach juice. It was determined that the treatment of orange and pomegranate juices with enzymes in different concentrations increased the reducing sugar amount at a rate of maximum 5.60 ± 1.08% in the orange juice, 3.45 ± 0.18% in pomegranate juice, 11.35 ± 0.88% in apricot juice, 21.22 ± 0.58% in peach juice, 9.25 ± 1.18% in apple juice, 6.10 ± 0.94% in grape juice and 9.89 ± 0.72% in kiwi juice. With the increasing enzyme concentration, decreases were observed in the 660 nm optical intensities of the fruit juices. The highest decrease in the absorbance in the 15 U/mL enzyme concentration was measured

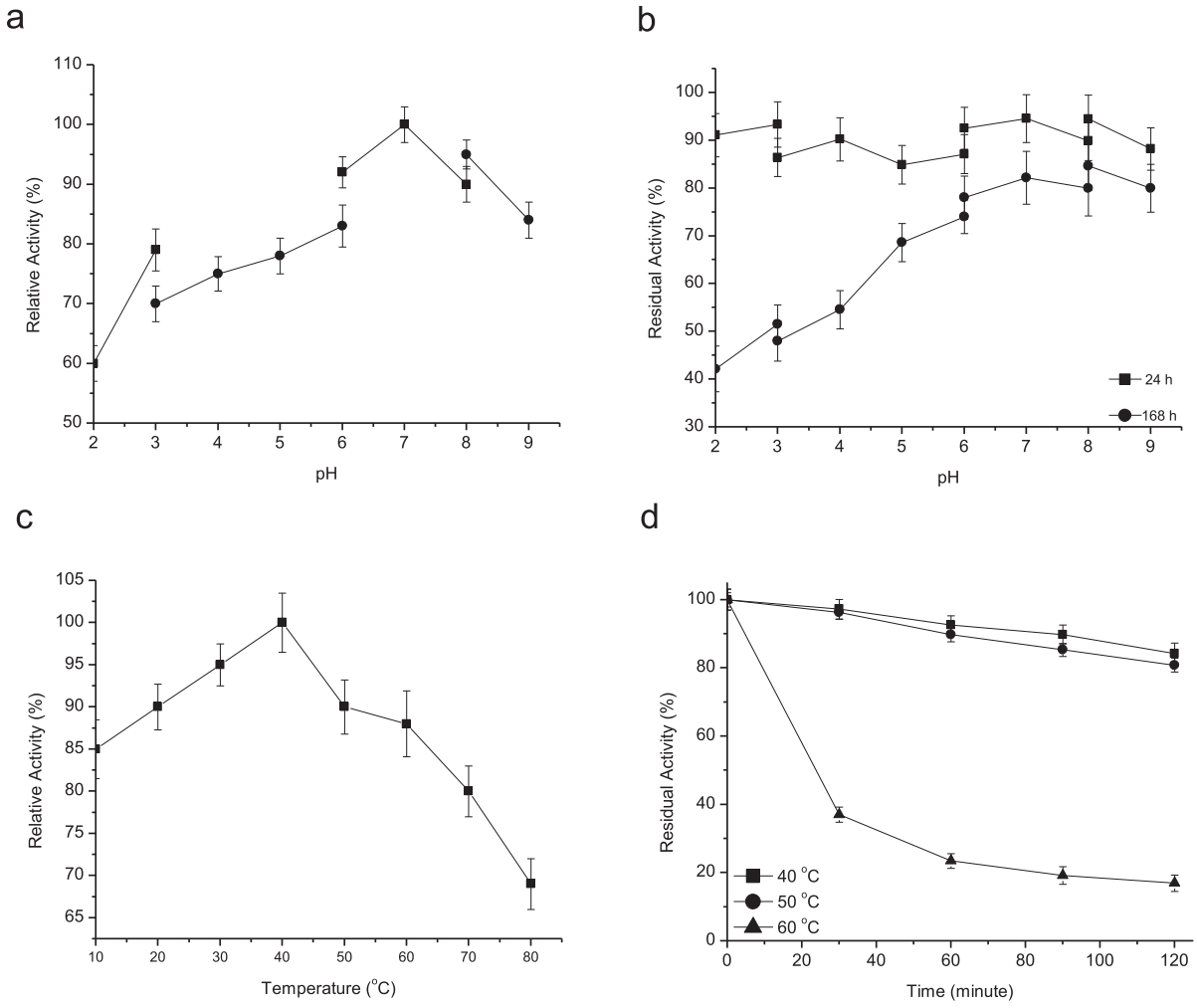


Fig. 3. Effect of pH and temperature on the purified xylanase from *P. acidilactici* GC25. (a) Effect of pH on enzyme activity, (b) pH stability of the xylanase, ■ residual activity after 24 h incubation, ● residual activity after 168 h incubation, (c) Effect of temperature on enzyme activity, (d) Thermostability of the xylanase, ■ residual activities at 40 °C, ● residual activities at 50 °C, ▲ residual activities at 60 °C.

as 24.47 ± 1.08% in peach juice. The lowest decrease in absorbance was obtained as 10.83 ± 0.47% in grape juice at the same enzyme concentration. The significant differences between fruit juices and enzyme concentration groups were shown in Table 2 according to the Student-Newman-Keuls (SNK) Test with significant levels ($p < 0.01$ and $p < 0.05$). It was determined that the treatment of orange, pomegranate, apricot, peach, apple, grape and kiwi juices with the *P. acidilactici* GC25 xylanase increased the reducing sugar and decreased the turbidity. The reducing sugar increased more in case of fruit juices that contained higher hemicellulosic content. Nagar et al. [58] reported similar correlation for the fruit juice clarification properties of *Bacillus pumilus* SV-85S Xylanase. There is also a correlation between clarification and the pH of fruit juices. The clarification increased as the pH of fruit juices increased. The increase in reducing sugar content was higher in orange juice when compared with apple juice. This result was similar to the samples treated with *Sclerotinia sclerotiorum* S2. The treatment of fruit juices with *P. acidilactici* GC25 xylanase was significant after one hour incubation at low enzyme concentration (0.05 U/mL, 0.10 U/mL, 0.15 U/mL). For *Streptomyces* sp., *B. licheniformis* P11, *Sclerotinia sclerotiorum* S2 xylanase, significant results were obtained after longer incubation periods at higher enzyme concentration [59–61]. The clarification potential of the xylanase is reported to be better than that reported for *Streptomyces* sp. xylanase in case of apple juice [61,62]. Cakmak and Ertunga [35] reported that the addition of *Geobacillus* sp. TF16 xylanase to apple juice increased the reducing sugar content by 3% after 60 min incubation at 50 °C. In this study, *P. acidilactici* GC25 xylanase caused more increase in reducing sugar content after 30 min incubation at 40 °C. Clarification process may affect the nutritive value of fruit juices [63]. Especially vitamin C is highly affected by temperature. For this reason, fruit juice clarification potential of GC25 xylanase at mild conditions is a desirable feature.

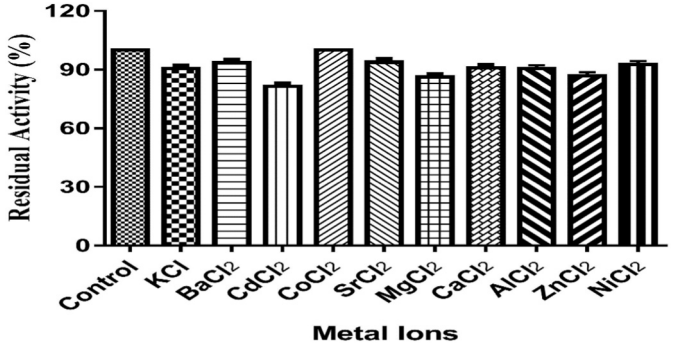


Fig. 4. Effect of various metal ions on *P. acidilactici* GC25 xylanase.

Table 2
Clarification of some fruit juice by using xylanase from *P. acidilactici* GC25.

Fruit juice	% decrease in absorbance at 660 nm			% increase in the reducing sugar amount		
	0.05 (U/mL)	0.10 (U/mL)	0.15 (U/mL)	0.5 (U/mL)	0.10 (U/mL)	0.15 (U/mL)
Orange	7.00 ± 0.52*	14.00 ± 1.24*	17.66 ± 1.32*	3.33 ± 0.54**	4.55 ± 0.26**	5.60 ± 1.08**
Pomegranate	4.22 ± 0.32*	7.44 ± 0.88*	11.26 ± 1.62*	2.08 ± 0.18*	2.94 ± 0.32*	3.45 ± 0.18*
Apricot	8.89 ± 0.61*	11.66 ± 0.98*	15.32 ± 1.35*	4.56 ± 0.63*	7.82 ± 0.41*	11.35 ± 0.88*
Peach	12.89 ± 1.11*	18.67 ± 1.64*	24.47 ± 1.08*	9.74 ± 0.85*	15.24 ± 0.84*	21.22 ± 0.58*
Apple	6.66 ± 0.85*	10.25 ± 1.02*	13.98 ± 1.74*	3.84 ± 0.64*	5.25 ± 1.35*	9.25 ± 1.18*
Grape	3.52 ± 0.46*	5.88 ± 0.63*	10.83 ± 0.47*	2.08 ± 0.21*	3.87 ± 0.42*	6.10 ± 0.94*
Kiwi	4.78 ± 0.45*	9.06 ± 0.74*	14.77 ± 0.94*	3.88 ± 0.73*	5.96 ± 0.12*	9.89 ± 0.72*

* $p < 0.01$.
** $p < 0.05$.

4. Conclusion

In this study, lactic acid bacteria were isolated from sausage samples that were purchased from a local market in Erzincan and screened to determine the producing capacities of xylanase, which was an industrially important enzyme. Ten morphologically different isolates were determined and *P. acidilactici* GC25 had the highest and most significant xylanase zone formation Petri assays. The xylanase enzyme produced by *P. acidilactici* GC25 was purified and characterized biochemically. Although there are studies in the literature with respect to *P. acidilactici* species and the identification of the isolates belonging to this species, this study is the first study of production, purification, biochemical characterization and evaluation of biotechnological aspects of a new xylanase produced by the newly isolated strain *Pediococcus acidilactici* GC25. It was determined that the GC25 xylanase enzyme preserved most of its activity in a wide pH range (2.0–9.0) and in the presence of various metal ions. The enzyme showed maximum activity within 20–50 °C. With this characteristic, it is possible to claim that GC25 xylanase is a suitable enzyme for the baking industry where the xylanase that shows activity at low and medium-level temperature is preferred. Beside this, in the clarification of peach and apricot juices, the increase of reducing sugar by *P. acidilactici* GC25 xylanase and the reduction in turbidity show that this enzyme may be used in clarification of the fruit juice and reducing the haze. For this reason, it has been considered that the xylanase purified has great advantages for baking and fruit juice industry, and maybe is a candidate for commercial practices.

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Conflict of interest

There is no conflict of interest to be declared.

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