



A novel approach for the waste industry: Investigating the impact of synthetic acetovanillone derivatives on thermophilic recombinant xylanase and its enzymatic effects

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Abstract

In this study, acetovanillone derivative compounds (1–7) were synthesized, and their structures were analyzed using nuclear magnetic resonance (^1H NMR— ^{13}C NMR) and Fourier-transform infrared (FTIR) methods. The xylanase gene within the scope of the study was cloned using recombinant DNA techniques. The purified recombinant protein was subjected to comprehensive characterization, including assessment of pH stability, temperature dependency, and substrate specificity. The optimal temperature and pH for the enzyme were determined to be 72 °C and 6.0, respectively. Investigations were done into how the synthetic compounds affected the xylanase enzyme. Especially compound 2 increased the activity at concentrations of 0.1, 0.3, 0.5 and 0.7 mM by 176.9, 153.25, 146.22 and 115.41%, respectively, compared to the positive control. It has also been observed that the effect on activity decreases with increasing concentration. Compound 3, on the contrary, increased the activity at high concentrations (0.7 and 1 mM) by 147.30 and 153.93%, respectively. Considering the positive sample (100%), CaCl_2 shows 70.38% activity at 0.1 mM, 68.04% at 0.3 mM, 79.96% at 0.5 mM, 61.99% at 0.7 mM, and 85.59% at 1 mM. These results show that CaCl_2 causes a slight decrease in enzyme activity. ZnCl_2 , the zinc compound, and CuCl_2 , the copper compound, showed a similar decrease in activity. It was determined that these different reactions showed different relative activity of the metal ions at varying concentrations for the recombinant xylanase enzyme.

Keywords Xylanase · Cloning · Waste industry · Acetovanillone · Synthesis

1 Introduction

With the increasing world population, there is a growing demand for products and resources from the environment. The utilization of raw materials, bioconversion of agro-industrial residues, and the environmental issues associated with the combustion of fossil fuels have prompted us to shift towards renewable and sustainable resources [1]. At this point, the term Green chemistry comes to the forefront by gaining more importance for the researchers [2]. Enzymes, which are biological catalysts, can play active

roles in living and non-living (in vitro) environments. In all these conditions, they serve as biochemical catalysis. When analyzed according to their usage areas, enzymes protect both the environment and people's living spaces from many toxic substances and the damages of solvents used in their removal [3, 4].

Biochemically processed products have become a widely favored technology for producing various goods, contributing to regional development and adding value [5]. The utilization of enzymes in industrial processes has sparked extensive research in the field of enzymology [1, 6–8].

Food, feed, paper industry, agricultural waste treatment, and utilization processes are among the applications of hydrolysis methods used during bioethanol conversion to xylan, a high-value-added commodity [9]. A significant portion of the enzymes employed in industrial applications are sourced from microorganisms, primarily bacteria. Bacterial organisms are favored for enzyme production due to their capability to be cultured in large quantities within a short timeframe, as well as their amenability to genetic

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manipulation for enhancing enzyme production. Consequently, researchers have extensively investigated and documented the properties and applications of various crucial enzymes, such as protease, lipase, xylanase, chitinase, cellulase, lactase, catalase, pullulanase, pectinase, amylase, among others, particularly those isolated from diverse bacterial strains [10].

The enzymes find widespread applications in industries such as bread-making, textile processing, detergent formulation, animal feed production, and pulp manufacturing. In the industrial sector, the pre-treatment of xylanases, which is an integral step in numerous industrial processes, typically involves operations conducted at high temperatures. Consequently, enzymes intended for use in such applications must possess robustness and resilience to withstand extreme variations in temperature and pH conditions prevalent in these environments [5, 9]. Xylans can generally be obtained from lignocellulosic agricultural products and various forest products [11]. In addition, the non-wood lignocellulosic parts of agricultural products have been the focus of many researchers worldwide. Considering all these, the recovery of these lignocellulosic materials through various biochemical processes, either biochemically or through enzymes, in a way that contributes to the economy has become remarkable [1, 12, 13].

Lignocellulosic biomass is very difficult to degrade due to its structure. In this process, this resistance to degradation and especially in the processes before being converted into refinery-oriented fuel needs to go through many preliminary steps [14–16]. Looking at the literature, many pre-treatment methods can be found for these processes [2, 17]. Pretreatment in the hydrolysis of lignocellulosic biomass can sometimes offer advantages and sometimes disadvantages. One of the biggest disadvantages is that it is expensive [18]. When the issues to be considered in pre-treatments are examined, the structure of cellulose fibers, the reduction of the inhibitor effect that may occur, and the elimination of high energy costs [19].

As a society that constantly consumes, we impose an increasing cost on nature. Considering all these, we have to turn to alternative resources. Microorganisms and the enzymes they produce are frequently used in many industrial fields. When microorganisms are examined, they show differences in terms of maintaining their vitality, growth, and development in varying temperature parameter ranges. Microbial organisms exhibit variances in their viability, proliferation, and maturation across diverse temperature regimes. Microbes that withstand and adapt to these specific conditions are classified into distinct categories such as psychrophiles, mesophiles, thermophiles, and hyperthermophiles. Generally, thermophilic bacteria are frequently encountered within soil,

hot springs, and volcanic habitats. These bacteria possess the ability to metabolize the same substrates as their mesophilic counterparts. Thermophilic bacteria are further classified into four groups based on their thermal preferences: moderate thermophiles (50–60 °C), extreme thermophiles (65–80 °C), hyperthermophiles (80–110 °C), and hyperthermophiles (110 °C and above) [20, 21]. In hot springs, soil, thermal industrial operations, and volcanic areas, thermophilic bacteria are frequently present [22]. Thermophilic bacteria, which grow well at temperatures of 60 °C and above, have stable enzymes due to their adaptation to their environment [23].

It is seen that commercial enzymes, which have a very important position in the global market, target a market of 10.5 billion dollars by 2024 [24]. In industry, mesophilic enzymes, which are generally preferred, are widely used in some research areas. These enzymes can lead to various errors such as low viscosity, substrate solubility problems, degradation at high temperatures, and contamination [9, 25]. Mesophilic enzymes are not well suited for enzymatic hydrolysis at high temperatures due to poor catalytic activity and stability under certain conditions. This requires the identification of suitable thermophilic enzymes that exhibit enhanced stability and activity at high temperatures [26]. In addition, enzymes that can operate at high temperatures are more suitable for pre-treatments. This eliminates the need for cooling stages in enzymatic processes and eliminates additional costs. The stability of thermophilic enzymes at high temperatures and their important contribution to the growth of the bioeconomy draw a lot of attention from the industrial sector [21].

The chemical and biochemical impacts of phenolic compounds on enzymes are seen when the xylanase enzyme is studied. On the xylanase enzyme, the impacts of phenolic substances like tannic acid, ferulic acid, vanillin, p-coumaric acid, and gallic acid were studied [27]. Acetovanillone is a ketone derivative of vanillin and is structurally similar to vanillin and ferulic acid. The effects of acetovanillone and their synthetic derivatives on the xylanase enzyme have not been studied before. Acetovanillone can be easily derivatized from position 5 according to the Mannich Reaction and the corresponding phenolic Mannich bases can be obtained. Phenolic Mannich bases are suitable for application in many fields such as paint, some industrially important cosmetic products, analytical reagents, refining products, petroleum processing, synthetic products, fabric, leather, and paper [28]. Acetovanillone is a good phenolic starting material for Mannich Reaction in order to produce the new phenolic Mannich base derivatives.

The aim of this study is to investigate the effects of acetovanillone and its phenolic Mannich base derivatives, which have not been studied before, on xylanase enzyme.

2 Experimental

2.1 Materials and methods

2.1.1 Biological materials

The bacteria and xylanase enzyme to be used in the study were produced recombinantly from *Bacillus subtilis* (BTX12) (Accession number, GenBank: MH101292.1), (<https://www.ncbi.nlm.nih.gov/>) bacteria isolated from thermal springs in Diyadin; Kopru Cermigi (Agri) [5].

2.2 Synthesis

The chemicals and solvents used in this research were bought from different suppliers and used directly. The WRS-2A melting point apparatus uses a microprocessor to calculate melting points, and its results are inaccurate. The FTIR spectra of the compounds were recorded with a BRUKER FTIR ALPHA-P spectrophotometer. NMR spectra were recorded on a Bruker spectrometer (400 MHz). Chemical shifts in deuterated dimethylsulfoxide (DMSO- d_6) were recorded as in ppm relative to tetramethylsilane (TMS) (δ 0.00 singlet).

2.2.1 Synthesis of compounds 2–4

The compounds were synthesized by a method designed by a group of researchers (Fig. 1) [29].

4-Acetyl-2-methoxyphenyl furan-2-carboxylate(2): Beige solid, yield 98%, mp: 133–135 °C. FTIR (cm^{-1}): ν_{max} 3143, 3088, 2978, 2930, 1741, 1682, 1596, 1286, 1167, 1089,

1011, 808. ^1H NMR (400 MHz, DMSO) δ 8.12 (s, 1H, ArH), 7.66 (dt, $J=5.4, 1.8$ Hz, 2H, ArH), 7.60 (d, $J=3.6$ Hz, 1H, ArH), 7.41 (d, $J=8.1$ Hz, 1H, ArH), 6.82 (dd, $J=3.6, 1.7$ Hz, 1H, ArH), 3.87 (s, 3H, OCH_3), 2.63 (s, 3H, CH_3). ^{13}C NMR (100 MHz, DMSO, APT) δ 196.9, 155.3, 151.1, 148.8, 142.5, 142.5, 136.1, 123.2, 121.7, 120.6, 112.8, 111.8, 56.0, 26.7.

4-Acetyl-2-methoxyphenyl thiophen-2-carboxylate(3): White solid, yield 99%, mp: 130–132 °C. FTIR (cm^{-1}): ν_{max} 3072, 2974, 2934, 1719, 1682, 1595, 1499, 1283, 1164, 1020, 810. ^1H NMR (400 MHz, DMSO) δ 8.08 (dd, $J=5.1, 4.4$ Hz, 2H, ArH), 7.73–7.58 (m, 2H, ArH), 7.42 (d, $J=8.1$ Hz, 1H, ArH), 7.32 (dd, $J=5.0, 3.8$ Hz, 1H, ArH), 3.86 (s, 3H, OCH_3), 2.63 (s, 3H, CH_3). ^{13}C NMR (100 MHz, DMSO, APT) δ 196.9, 159.1, 151.1, 142.8, 136.0, 135.5, 135.5, 131.1, 128.8, 123.3, 121.7, 111.8, 56.1, 26.7.

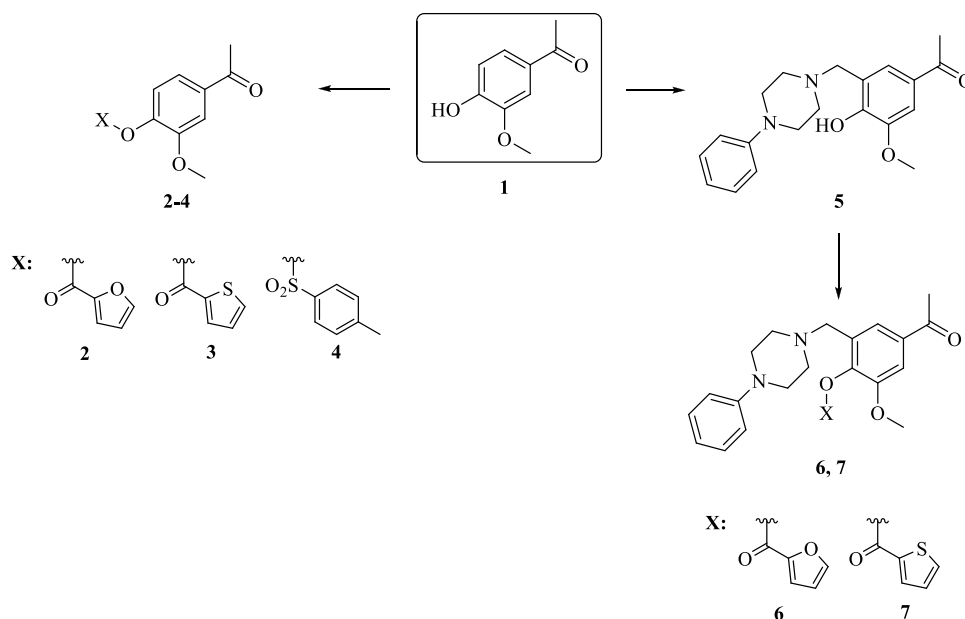
4-Acetyl-2-methoxyphenyl -4-methylbenzenesulfonate(4): Beige solid, yield 97%, mp: 88–90 °C. FTIR (cm^{-1}): ν_{max} 3143, 3071, 2945, 1741, 1682, 1596, 1498, 1389, 1285, 1166, 1088, 808. ^1H NMR (400 MHz, DMSO) δ 7.35 (dt, $J=4.8, 2.0$ Hz, 2H, ArH), 7.59 (dd, $J=8.4, 2.0$ Hz, 1H, ArH), 7.50 (d, $J=2.0$ Hz, 1H, ArH), 7.47 (d, $J=8.0$ Hz, 1H, ArH), 7.24 (d, $J=8.3$ Hz, 1H, ArH), 3.60 (s, 3H, OCH_3), 2.58 (s, 3H, CH_3), 2.43 (s, 3H, PhCH_3). ^{13}C NMR (100 MHz, DMSO, APT) δ 196.8, 151.4, 145.8, 141.1, 136.6, 132.1, 129.9, 128.2, 123.5, 121.4, 112.3, 55.8, 26.7, 21.1.

2.2.2 Synthesis of compound 5

By using a technique they developed, the researchers were able to synthesize the compounds (Fig. 1) [30].

4-Hydroxy-3-methoxy-5-(4-phenylpiperazine -1-yl)methyl acetophenone(5): Brown solid, yield 89%, mp: 167–169 °C.

Fig. 1 Synthetic route for preparation of compounds 2–7



FTIR (cm^{-1}): ν_{max} 3070, 3051, 2989, 2921, 1683, 1595, 1489, 1262, 1163, 822. ^1H NMR (400 MHz, DMSO) δ 7.49 (s, 1H, ArH), 7.41 (s, 1H, ArH), 7.21 (d, $J=8.7$, 2H, ArH), 6.93 (d, $J=7.9$ Hz, 2H, ArH), 6.79 (t, $J=7.3$ Hz, 1H, ArH), 3.84 (s, 3H, OCH_3), 3.75 (s, 2H, NCH_2Ph), 3.16 (t, $J=4.8$ Hz, 4H, CH_2NCH_2), 2.62 (t, $J=4.8$ Hz, 4H, CH_2NCH_2), 2.51 (s, 3H, CH_3). ^{13}C NMR (100 MHz, DMSO, APT) δ 196.1, 151.2, 150.8, 147.3, 128.9, 128.0, 123.3, 122.0, 119.0, 115.5, 110.2, 58.0, 55.7, 52.1, 48.2, 26.2.

2.2.3 Synthesis of compounds 6 and 7

Compounds were synthesized according to method reported previously (Fig. 1) [31].

4-Acetyl -2-methoxy -6-[(4-phenylpiperazine -1-yl) methyl]phenyl furan-2-carboxylate (6): White solid, yield % 95, mp: 161–163 °C. FTIR (cm^{-1}): ν_{max} 3137, 2965, 2822, 1744, 1677, 1598, 1503, 1293, 1174, 1092, 755. ^1H NMR (400 MHz, DMSO) δ 8.07 (s, 1H, ArH), 7.63 (d, $J=8.0$ Hz, 2H, ArH), 7.57 (d, $J=3.5$ Hz, 1H, ArH), 7.16 (t, $J=7.9$ Hz, 2H, ArH), 6.82 (d, $J=8.1$ Hz, 2H, ArH), 6.79 (dd, $J=3.5$, 1.7 Hz, 1H, ArH), 6.74 (t, $J=7.2$ Hz, 1H, ArH), 3.86 (s, 3H, OCH_3), 2.86 (m, 4H, CH_2NCH_2), 2.63 (s, 3H, CH_3), 2.39 (t, $J=4.6$ Hz, 4H, CH_2NCH_2). ^{13}C NMR (100 MHz, DMSO, APT) δ 197.1, 155.0, 151.8, 150.9, 148.4, 143.1, 141.8, 135.2, 132.0, 128.8, 122.8, 119.8, 118.7, 115.2, 112.7, 111.1, 57.2, 56.2, 52.3, 47.8, 26.8.

4-Acetyl -2-methoxy -6-[(4-phenylpiperazine -1-yl) methyl]phenyl thiophene-2-carboxylate (7): White solid, yield 97%, mp: 158–160 °C. FTIR (cm^{-1}): ν_{max} 3090, 2961, 2824, 1724, 1677, 1594, 1499, 1272, 1169, 1087, 753. ^1H NMR (400 MHz, DMSO) δ 8.03 (d, $J=8.0$ Hz, 2H, ArH), 7.63 (d, $J=11.4$ Hz, 2H, ArH), 7.29 (t, $J=4.4$ Hz, 1H, ArH), 7.16 (t, $J=7.8$ Hz, 2H, ArH), 6.81 (d, $J=8.0$ Hz, 2H, ArH), 6.73 (t, $J=7.2$ Hz, 1H, ArH), 3.87 (s, 3H, OCH_3), 2.84 (m, 4H, CH_2NCH_2), 2.63 (s, 3H, CH_3), 2.38 (m, 4H, CH_2NCH_2). ^{13}C NMR (100 MHz, DMSO, APT) δ 197.1, 158.7, 151.9, 150.9, 142.2, 135.1, 134.9, 134.9, 131.9, 128.8, 128.7, 122.8, 118.7, 115.2, 111.2, 57.4, 56.2, 52.4, 47.8, 26.8.

2.3 Enzyme studies

2.3.1 Enzyme activity

Xylanase activity was determined spectrophotometrically by DNS method with beech xylan as substrate [32]. The enzyme solution was added to the test tube with 1% xylan solution. When the solution reached the ideal temperature for enzyme action, DNS reagent was added, and the mixture was then incubated for 5 min. The products obtained were measured by spectrophotometric method at 540 nm wavelength. The amount of enzyme releasing 1 micromole of reducing sugar equivalent to xylose per minute under the

assay conditions outlined was used to define one unit of xylanase activity [33].

2.3.2 Isolation of genomic DNA

By modifying Sambrook's traditional phenol, chloroform, and isoamyl alcohol technique, DNA was isolated [34]. The isolates were kept at -80 °C for future research [35]. The isolate preferred in the study was isolated in previous studies and sequence analysis was performed by service procurement (<https://www.bmlabosis.com/>) [5].

2.3.3 Production of recombinant xylanase

In order to determine the similarities of the 1,4- β -endo xylanase gene, which will be cloned and produced as a recombinant protein within the scope of the study, with the genes in other organisms and to determine the primer DNA sequences to be used in cloning studies carried out in order to realize heterologous protein expression in *E. coli*, a database such as NCBI Genbank was used (<https://www.ncbi.nlm.nih.gov/>). *Bacillus* species were selected for the determination of candidate genes since it was aimed to produce 1,4- β -endo xylanase enzyme recombinantly. The protein and DNA sequences xylanase in the genome of the organism were accessed from the NCBI database.

The design of primer sequences was conducted for the PCR amplification of the identified candidate gene. Initially, the candidate gene sequences underwent restriction cutting analysis using Webcutter software. Restriction enzymes that do not cleave the gene region were then identified. The enzymes found in the multiple cloning region of the pET 16b vector were added to the primer sequences and primer sequences were designed (F-xylanase GTCGAGATGAAA AAATTACTTGTTGTCTTA and R-xylanase AAGCTTTCA AACAAGGAAATATATCTCCAAA).

2.3.4 Cloning of xylanase protein genes

The identified genes were amplified by PCR and PCR amplicons were transferred into carrier vectors and then into *E. coli*. Using gDNA as a template, the amplification of candidate genes was carried out using the primers determined. PCR products were analyzed in 1% agarose gel and DNA bands of the desired size belonging to the relevant genes were cut from the gel and purified [9]. The amplicons obtained were transferred into pGEMT-Easy vector by TA cloning and then into *E. coli* DH5- α host by heat shock. The plasmid DNAs purified from the positive clones were purified by cutting reaction with XbaI and KpnI enzymes and appropriate restriction enzymes with recognition sequences on the primer DNAs. The plasmid DNAs identified as a result of restriction analysis were checked by sequence analysis and positive clones were identified.

2.3.5 Heterolog production and purification of xylanase enzyme

To purify the enzymes from the positive clones obtained through molecular cloning, the clones were incubated in LB medium supplemented with ampicillin (0.1 mg/ml) at 37 °C until reaching an OD₆₀₀ value of 0.6–0.8. Subsequently, the clones were induced with IPTG for 3 h to overexpress the protein. In the purification step, 6X-His end-modified proteins were obtained by nickel affinity method [36]. The molecular weight of the protein purified by SDS-PAGE was determined.

2.3.6 The effect of pH and temperature factors on the stability of enzymes.

Citrate buffer (0.2 M citric acid and 0.2 M Na₂HPO₄·7H₂O and distilled water) was used to determine the activity of the enzyme between pH 4.0 and 5.5 at different temperature ranges of 35 and 115 °C. Sodium-Phosphate buffer (0.2 M NaH₂PO₄ and 0.2 M Na₂HPO₄·7H₂O and distilled water) was used to determine the activity between pH 6.0 and 8.0. Glycine–NaOH buffer (0.2 M Glycine with 0.2 M NaOH and distilled water) was used to determine the activity between pH 8.5–10.0. The 0.5 ml combination of substrate and enzyme was allowed to incubate for one hour. The combination was then given 1.5 ml of DNS, and it was incubated for 5 min in a boiling water bath. Enzyme activity measurements were performed at different pH ranges in each prepared buffer [32]. Spectrophotometric measurements were determined at 550 nm wavelength. Enzyme activity was accepted as 100 for thermal stability. This percentage was calculated as relative activity (%). When the enzyme's temperature stability was tested, it was incubated in sodium-phosphate buffer at 40, 50, 60, 70, and 80 °C for 1, 2, and 3 days, respectively.

2.3.7 K_m and V_{max}

K_m and V_{max} values of recombinant xylanase enzyme were measured with different substrate concentrations of substrate (0.5, 1, 1.5, 2, 2.5, 2.5, 3, 3.5, and 4 mM xylan). The Lineweaver-Burk graphs were used to determine the kinetic value [37].

2.3.8 Effects of synthesized compounds and metal ions on enzyme activity

Solutions comprising SDS, EDTA, HgCl₂, CaCl₂, CuCl₂, and ZnCl₂ were used to examine the effects of synthesized acetovanillone derivatives and metal ions on the activity. The optimum pH and temperature

values, 50 µl of 0.1, 0.3, 0.5, 0.7 and 1 mM samples, 50 µl of xylanase enzyme and 100 µl of DNS were prepared for activity test. Enzyme and substrate were pre-incubated at 50 °C for 5 min. The samples were then cooled at room temperature and 100 µl DNS was added. Following that, the samples were incubated for 5 min in a heated (boiling) water bath. At a wavelength of 540 nm, measurements were calculated following incubation [9].

3 Result and discussion

3.1 Chemistry

In this study, six acetovanillone derivatives (**2–6**) were synthesized starting from acetovanillone according to Schotten Baumann and Mannich Reactions (Fig. 1). The targeted compounds were characterized in terms of their structures by FTIR, ¹H NMR and ¹³C NMR spectroscopic methods. The support material file contains the compound spectra.

When the FTIR spectra were examined, the compounds' aromatic and aliphatic C–H stretching bands were observed in 3143–3070 and 2989–2965 cm^{−1}, respectively. C=O stretching bands of the ester and ketone moieties appeared at 1744–1719 and 1683–1677 cm^{−1}, respectively [31]. For compound **4**, asymmetrical and symmetrical S=O stretching bands of SO₂ group were seen at 1389 and 1166 cm^{−1} [38]. The characteristic features and values of the bands obtained from the literature are quite compatible with the structures.

When compared to their chemical surroundings, aromatic proton bands from the compounds' ¹H NMR spectra can be seen at δ 8.12–6.74 ppm as doublets, triplets, and multiplets. For compound **5**, the aminomethyl proton (N–CH 2) peaks were observed as a singlet at δ 3.75 ppm [30]. For compounds **5–7**, peaks of the piperazine protons were resonated at δ 3.16–2.28 ppm. Methoxy and methyl protons were seen at δ 3.87–3.60 and δ 2.63–2.51 ppm, respectively. Integration, chemical shifts and divisions are quite consistent with the structure and literature.

In the ¹³C NMR spectra of the target compounds, characteristic peaks of the C=O carbons of the ketone moiety resonated at δ 197.1–196.1 ppm and the C=O carbons of the ester moieties were observed at δ 159.1–155.0 ppm. Peaks of the aromatic carbons were seen at δ 151.9–110.2 ppm. For compounds **5–7**, peaks of the aminomethyl carbons appeared at δ 58.0–57.2 ppm [30]. Integration, chemical shifts and divisions are quite consistent with the structure and literature.

3.2 Cloning of the xylanase gene

For the production of xylanase gene from *B. subtilis*, PCR amplification of the xylanase gene region from gDNAs, insert ligation, transformation, plasmid isolation, determination of whether it carries an insert with restriction enzymes, and completion of cloning by sequence analysis of the insert were performed respectively. In this context, firstly, xylanase gene regions were amplified by PCR using the xylanase gene primers designed by bioinformatic analyses from gDNAs (Fig. 2a and b).

The enzyme purification process involved the use of agarose with Nickel NTA beads, which exhibit His affinity, and Ni-NTA beads with 6×His histidine attachments. Initially, the enzyme was filtered and then purified using kit solutions. The purification efficiency was assessed by analyzing the enzymes purified with Ni-NTA beads. Subsequently, the purified samples were loaded onto the gel at different time points (1st, 2nd, and 3rd hours) based on their induction

by IPTG (Fig. 3a). The SDS-PAGE result was verified by a Western blot analysis using an anti-His antibody, and the purified protein was recognized as xylanase from *B. subtilis* (Fig. 3b).

4 Enzyme kinetics

In the measurement of purified xylanase enzyme at varying pH ranges, it was determined that the optimum pH value was 6.0 (Fig. 4a). The optimum temperature for enzyme activity was found to be 72 °C, according to experiments carried out at the ideal pH (pH 6.0) and different temperature factors (37 and 115 °C) (Fig. 4b).

As a result of the activity tests of the produced enzyme at different substrate concentrations, the optimum enzyme activity was measured as 1846 U/mg at 3.5% concentration for the recombinant enzyme (Fig. 5a). V_{max} and K_m values of the enzyme were determined using 1% oat spelt xylan

Fig. 2 **a** 1% agarose gel image of PCR amplification result of xylanase gene (M:Marker, Dnr Bio-imaging Systems Mini-Lumi), **b**. 1% agarose gel purification result of xylanase gene, **X**. *Bacillus subtilis*, **M**. Lambda DNA/EcoRI/Hind III Marker

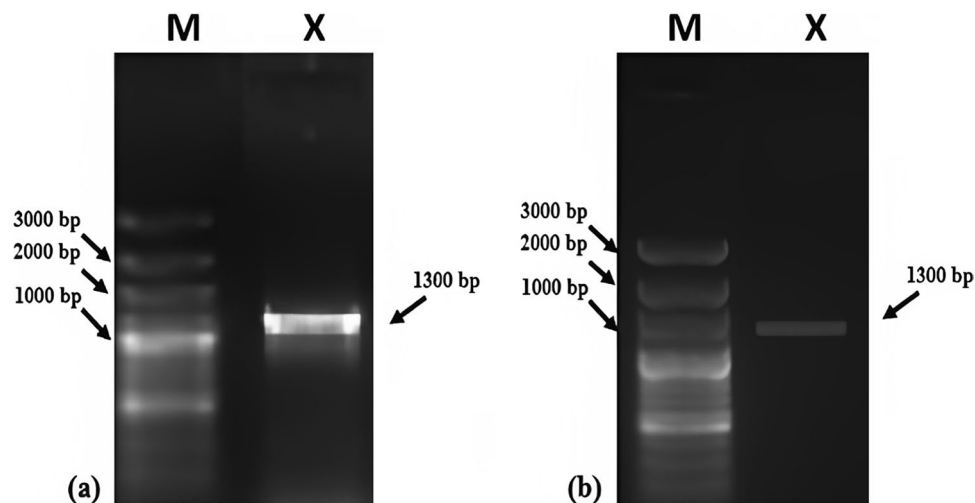
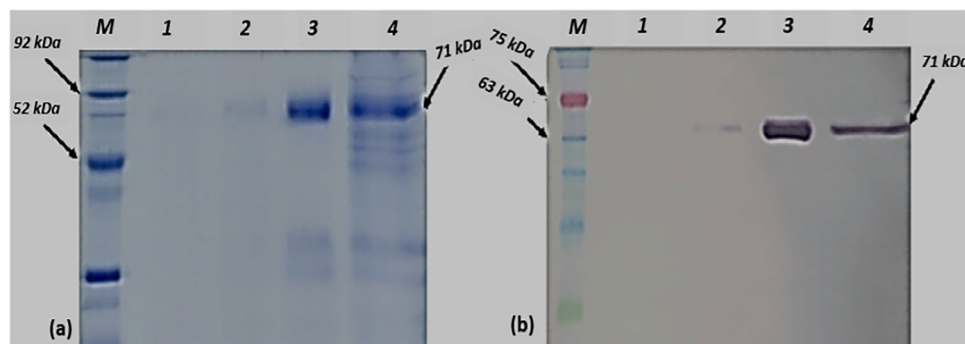
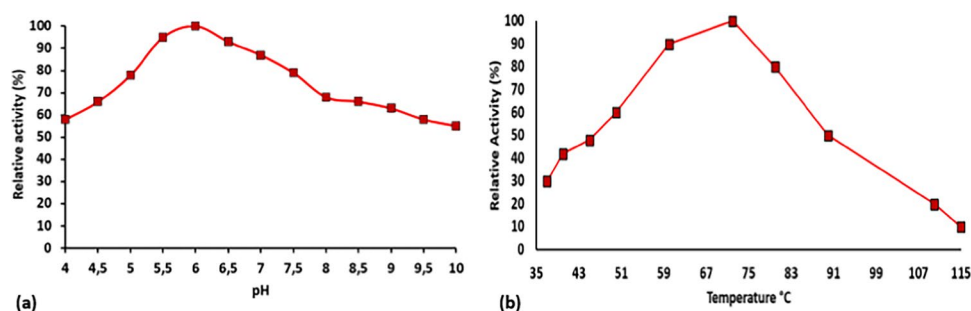


Fig. 3 **a** Image of SDS gel resulting from Ni-NTA purification. **M**: Marker (SDS-PAGE molecular weight standards Broad Range (Bio-RAD, Cat. no: 161–0317)), **1**: 1 h induced, **2**: 2 h induced, **3**: 3 h induced, and **4**: Total protein, **b**. Western blot analysis of recom-

nant xylanase protein. **M**. Marker (Kaleidoscope pretained standards BIORAD), **1**: 1 h induced, **2**: 2 h induced, **3**: 3 h induced, and **4**: Total protein Western blot images with antihistidine and secondary antibody

Fig. 4 **a** The optimal pH was found to be 6.0 after enzyme function at various pH levels was assessed. **b.** The temperature range between 55 and 85 °C (optimum 72 °C) was discovered when the enzyme's optimal temperature action was investigated



substrate. V_{max} and K_m values were measured by Lineweaver Burke equation. The recombinant enzyme's V_{max} value was found to be 27.47 (EU/mL. min.) and K_m value to be 59.9 (mM) (Fig. 5b).

4.1 Effect of the synthesised compounds and metal ions on activity

The obtained data were analyzed separately for each concentration, considering the enzyme substrate solution as 100%. The effects of acetovanillone derivatives and different metal ions on the enzyme activity were compared, and graphical results were generated (Fig. 6a, b, c, d, and e). The results indicated that the starting compound, acetovanillone (1), exhibited a decrease in xylanase enzyme activity at all concentrations. Conversely, the O-acyl derivatives of acetovanillone (2 and 3) significantly increased the activity. Specifically, compound 2 increased the activity by 176.9%, 153.25%, 146.22%, and 115.41% at concentrations of 0.1, 0.3, 0.5, and 0.7 mM, respectively, compared to the positive control. Furthermore, it was observed that the impact on activity diminished as the concentration increased. In contrast, compound 3 exhibited an increase in activity at high concentrations (0.7 and 1 mM) by 147.30% and 153.93%, respectively. The

O-sulfonyl derivative of acetovanillone decreased the activity at all concentrations.

The activity of the C-Mannich base derivative of acetovanillone, designated as compound number 5, showed a significant decrease of at least 97% at all concentrations. Likewise, the O-acyl derivatives of compound 5 exhibited a substantial decrease in activity of at least 99% across all concentrations. These findings suggest that phenolic Mannich bases and their derivatives act as inhibitors of xylanase activity. On the other hand, the presence of thiophene and particularly O-acyl acetovanillones containing a furan ring resulted in a significant increase in enzyme activity. The furan ring appears to be particularly important to the action. These findings demonstrate that CaCl_2 , albeit to a lesser degree, also results in a decrease in the enzyme's activity. ZnCl_2 , the zinc compound, and CuCl_2 , the copper compound, showed a similar decrease in activity. These different responses were found to show the different relative activity of metal ions at varying concentrations for the recombinant xylanase enzyme. It has been suggested that this may be because the metal ions impacted both the catalytic and non-catalytic xylan binding sites of xylanase, which are both involved in the hydrolysis of the substrate [39].

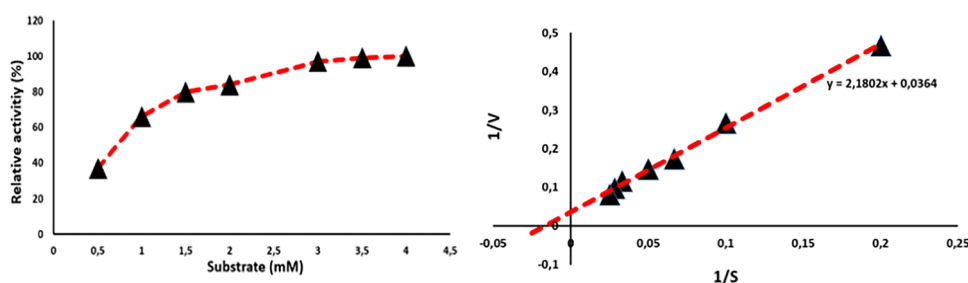


Fig. 5 **a** In the activity tests performed on the generated enzyme across various substrate concentrations, the recombinant enzyme exhibited an optimal activity of 1846 U/mg at a concentration of 3.5%. **b.** The determination of V_{max} and K_m values for the enzyme

was carried out utilizing a 1% oat spelt xylan substrate, employing the Lineweaver Burke equation (as stated in test b). The recombinant enzyme demonstrated a V_{max} value of 27.47 (EU/mL. min.) and a K_m value of 59.9 (mM)

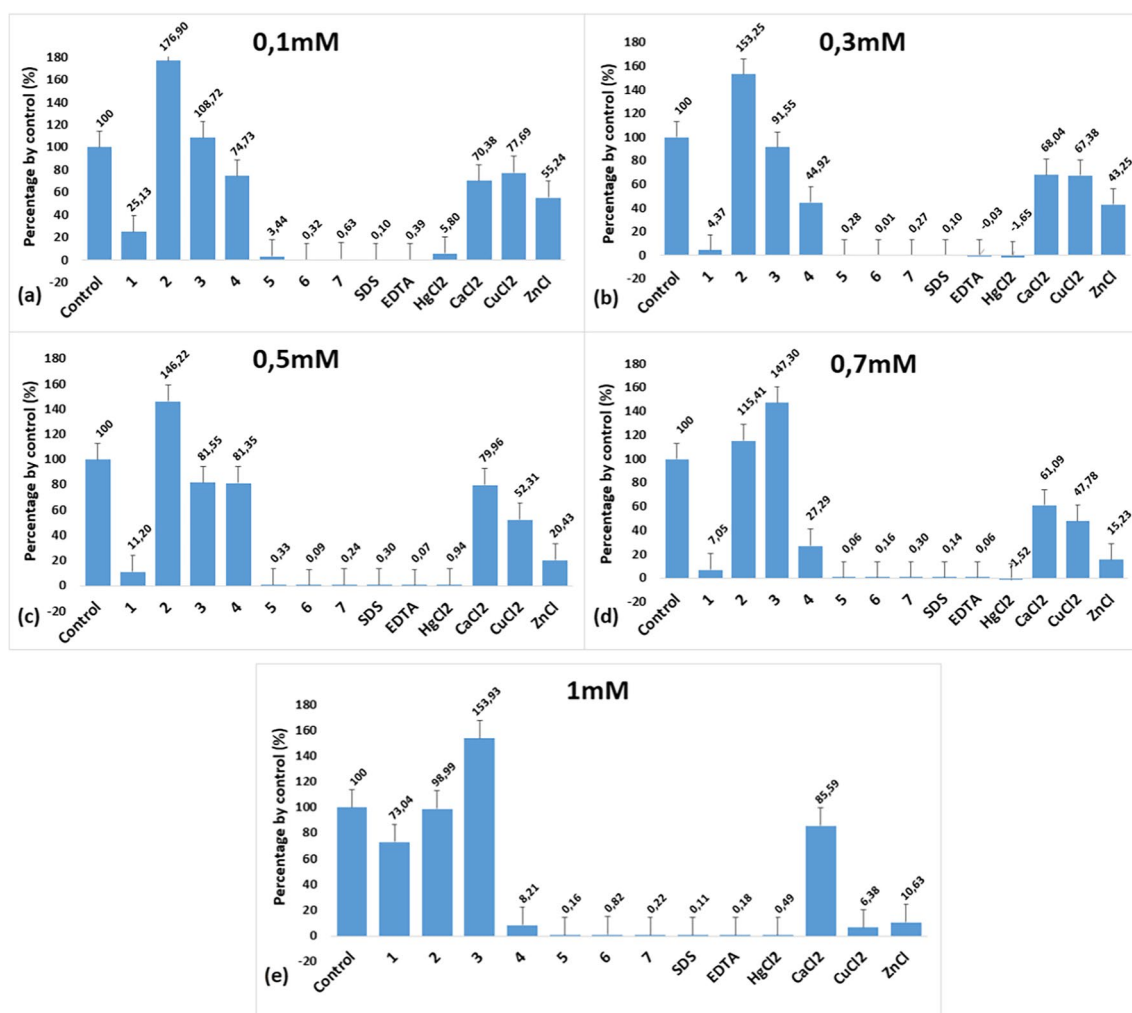


Fig. 6 Xylanase enzyme activity results of the compounds in 1% substrate (Birchwood Xylane) solution at concentrations of **a.** 0.1 mM, **b.** 0.3 mM, **c.** 0.5 mM, **d.** 0.7 mM and **e.** 1 mM

5 Conclusions

With the widespread use of enzymes, chemical catalysts used in industry have decreased and environmental damage has decreased significantly. Enzymes that are ecological alternatives have become preferred. As different and new types of thermophilic enzymes are researched, they will be used as biocatalysts in more processes. The preference for enzymes will provide benefits to both the environment and economies in the long term.

The commercial availability of recombinant xylanase enzymes is quite common. In this context, the activity results to be obtained in industrial and biotechnological studies that prefer activation or inhibition of the enzyme will further facilitate the availability of the enzyme. This will pave the way for the commercial production of an enzyme that will be used more preferably. The countries in the world have their own production circles in enzyme

production and sell these products in the world market. For this reason, it is of great importance that an enzyme has a low cost of use in industry and industry, can be used repeatedly, has high activity, and can show activity under extreme conditions [9, 21]. It is important to discover new inhibitors and activators of this enzyme. This study, it was aimed to produce the enzyme recombinantly. The impacts of some phenolic compounds on the enzyme's activity were then investigated. The potential inhibitors and activators of the enzyme were determined in vitro. The results obtained from the study showed that compounds 2 and 3 activate the xylanase enzyme effectively. On the other hand, the other acetovanillon derivatives and the metal ions decreased the activity. Since compounds 2 and 3 activate xylanase, they stand out as compounds with potential use in the waste industry. Moreover, the starting materials used to obtain these compounds are easily available and inexpensive compounds. The reaction required

to obtain the compounds is one step and does not require harsh conditions.

The selection of bacteria for this research was based on their favorable characteristics, specifically their ability to produce highly stable enzymes capable of functioning under harsh environmental conditions and high temperatures, owing to their thermophilic nature. In light of these advantages, the study focused on xylanase, an enzyme highly sought-after in the industry. The objective was to obtain xylanases with enhanced efficiency and commercial viability.

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Author contributions Orhan Ulucay: conducted the experiments methodology, formal analysis, writing—original draft, writing—review and editing. Feyzi Sinan Tokali: conducted the experiments, conceptualization and editing.

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Data availability All data used and analyzed in the current study will be available upon request.

Declarations

Ethical approval Not applicable.

Competing interests The authors declare no competing interests.

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