TÜBİTAK

Turkish Journal of Biology

http://journals.tubitak.gov.tr/biology/

Research Article

Turk J Biol
(2014) 38: 586-592
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doi:10.3906/biy-1403-76

Characterization of a novel xylose isomerase from *Anoxybacillus gonensis* G2^T

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Received: 20.03.2014 • **Accepted:** 08.05.2014 • **Published Online:** 05.09.2014 • **Printed:** 30.09.2014

Abstract: The xylA gene encoding xylose isomerase from *Anoxybacillus gonensis* G2^T has been cloned and successfully expressed in *E. coli*. Xylose isomerase was purified 10.98-fold by heat-shock and sequential column chromatography techniques to homogeneity, and the biochemical properties of the enzyme were characterized. The optimum temperature of the enzyme was 85 °C and maximum activity was observed at a pH of 6.5. Its *Km* and *Vmax* values were calculated as 25 ± 2 mM and 0.12958 ± 0.002 µmol/min/mg protein, respectively. The effects of various metal ions on the xylose isomerase were examined. Divalent cations Co^{2+}, Mg^{2+}, and Mn^{2+} were essential for xylose isomerase activity; however, bivalent metal ions (Ca^{2+}, Hg^{2+}, Ni^{2+}, Zn^{2+}, Fe^{2+}, and Cu^{2+}) showed inhibitory effects. This is the first report of characterization of the xylose isomerase of *Anoxybacillus* spp. According to results obtained from this study, xylose isomerase is a promising candidate for industrial applications in production of xylulose and ribose.

Key words: Xylose isomerase, *Anoxybacillus*, characterization, thermophilic

1. Introduction

Xylose isomerase (XI) (D-xylose ketol-isomerase E.C 5.3.1.5) catalyzes the isomerization of D-xylose into xylulose as the first step of xylose metabolism in many microorganisms (Wovcha et al., 1983). It is also responsible for catalyzing the isomerization of glucose to fructose in vitro, and is an important enzyme in the food industry, used in the production of high fructose corn syrup. This is the reason why XI is also known as glucose isomerase (Jensen and Rugh, 1987; De Raadt et al., 1994). The fact that the enzyme isomerizes xylose to xylulose means that it could be used industrially for producing ethanol from hemicellulose (Wang et al., 1980; Ertunga et al., 2007; Karaoglu et al., 2013). Xylose, one of the major fermentable sugars in nature, is, after glucose, the second most abundant sugar in lignocellulosic biomass. Efficient fermentation of xylose is a necessary step in developing economically viable processes for producing biofuels, such as ethanol, from biomass (Zeikus, 1996; Schenck, 2000). For this reason, many XI genes appropriate for industrial applications were transferred to *Saccharomyces cerevisiae* (Joo et al., 2005). The enzyme has been isolated from many microorganisms and is well studied (Chen, 1980; Schellenberg et al., 1984; Wilhelm and Hollenberg, 1985; Saari et al., 1987; Amore and Hollenberg, 1989; Kikuchi et al., 1990; Dekker et al., 1991). Besides its commercial importance, XI is also an ideal enzyme for studying structure–function relationships from an academic perspective (Ertunga et al., 2007; Karaoglu et al., 2013).

Very recently, a novel hot spring thermophile, *Anoxybacillus gonensis* G2^T, was isolated and characterized based on its biochemical, taxonomic, and genetic properties. *Anoxybacillus gonensis* G2^T is a xylanolytic, sporulating, gram-positive, rod-shaped, facultative anaerobe and moderately thermophilic bacterium that grows naturally at 55–60 °C in thermal springs in Gönen, Balıkesir, Turkey (Belduz et al., 2003). In this study, the *A. gonensis* G2^T *xylA* gene encoding for xylose isomerase was cloned and expressed in *E. coli* and the product of the *xylA* gene was characterized. We think that this study will be a guide for researchers conducting further research on XI in *Anoxybacillus* species.

2. Materials and methods

2.1. Substrates and chemicals

Chemicals used in this study were purchased commercially from Merck AG (Darmstadt, Germany), Sigma (St. Louis, MO, USA), Fluka Chemie AG (Buchs, Switzerland), Acumedia Manufacturers (Baltimore, MD, USA), and Aldrich-Chemie (Steinheim, Germany). The Wizard

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Genomic DNA Purification Kit, Wizard Plus SV Minipreps DNA Purification System, MagneHis Protein Purification System, *Taq* DNA Polymerase, dNTP, and all of the restriction enzymes were purchased from Promega (Madison, WI, USA). All chemicals were reagent grade and all solutions were made with distilled and deionized water.

2.2. Strains, vectors, and media

E. coli BL21 (DE3):pLysS, pET28(a–c)+ were supplied by Karadeniz Technical University, Molecular Biology Laboratory. *E. coli* containing recombinant plasmids were cultured according to the method described (Karaoglu et al., 2013).

2.3. Genomic DNA isolation

Genomic DNA isolation was performed using the Wizard Genomic DNA Purification Kit according to the manufacturer's directions.

2.4. Cloning and overexpression of *xyl*A gene

The *xyl*A gene was amplified by using the primers (Xyla_Ex_F1-Xyla_Ex_R1 and Xyla_Ex_F1-Xyla_Ex_R2) designed by Karaoglu et al. (2013). PCR reactions were performed according to the method described by Karaoglu et al. (2013). *E. coli* BL21 cells containing p*Ago*G2XI-his or p*Ago*G2XI were grown to an optimum density of about 0.6 at 600 nm. Overexpression of recombinant plasmids was induced by addition of 1 mM iso-propyl-β-D-thiogalactopyranoside (IPTG). After 4 h, cells were harvested by centrifugation at 10,000 rpm for 5 min. The cells were disrupted using a Sartorius Labsonic M sonicator at 0.6 cycle scale (80% amplitude). The cell debris was removed and the cell-free extract was assayed for xylose isomerase activity (Chen et al., 2014).

2.5. Activity assay for XI

The XI activity of the obtained cell extract was measured using the method described by Belfaquih et al. (2000). The extract was dissolved in a reaction mixture. The reaction was performed in a solution containing 10 mM $MnSO_4$, 1 mM $CoCl_2$, 0.2 M xylose, and 0.5 µg of the enzyme in 50 mM MOPS buffer (pH 6.5) at 85 °C for 30 min in 100 µL reaction volume. The reaction was stopped by the addition of 100 µL of perchloric acid, after which 40 µL of 1.5% cysteine hydrochloride, 40 µL of 0.12% carbosol, and 1.2 mL of 70% sulfuric acid were added. The reaction mixture was vortexed and incubated at room temperature for 30 min. The activity was determined spectrophotometrically at 545 nm absorbance for xylulose. One unit of activity was defined as the amount of enzyme that released 1 µmol of xylulose/min under the assay conditions described above.

2.6. Enzyme purification

2.6.1. Heat treatment

The crude extract was dissolved in a 50 mM MOPS (pH 7.0) buffer including 1 mM $MnSO_4$ and heated for 15 min at 75 °C. The soluble fraction was recovered after centrifugation at 14,800 rpm for 15 min.

2.6.2. Ion exchange chromatography

Supernatant obtained from thermal shock was loaded on a column (1.5 × 50 cm) of DEAE-Sepharose pre-equilibrated with 50 mM MOPS, pH 7.0, containing 1 mM $MnSO_4$. The column was washed with 250 mL of the same buffer at flow rate of 1 mL/min and eluted with a linear gradient of (0–0.5 M) NaCl in the same buffer. The active fractions were pooled and concentrated by ultrafiltration (Sartorius, 10,000 MWCO filters).

2.6.3. Hydrophobic interaction chromatography

A saturated ammonium sulfate solution was added to the enzyme solution to give a final concentration of 1.3 M. A column (0.75 × 20 cm) of phenyl-sepharose-6 (Sigma) had previously been equilibrated with 50 mM MOPS (pH 7.0) containing 1 mM $MnSO_4$ and 1.3 M $(NH_4)SO_4$. The column was washed with 100 mL of this buffer and eluted with a 100 mL linear gradient of 1.3–0 M $(NH_4)SO_4$ at a flow rate of 0.5 mL/min. The active fractions were pooled and concentrated by ultrafiltration and dialyzed against 50 mM MOPS (pH 7.0) containing 1 mM $MnSO_4$ overnight (Table 1).

2.6.4. Determination of protein concentration

Protein concentration was determined by Bradford's method (1976). Bovine serum albumin was used as the standard for the procedure (Bradford, 1976).

2.6.5. Determination of K_m and V_{max} values

The kinetic parameters V_{max} (µmol/min/mg) and K_m were determined from Michaelis–Menten plots of specific activities at various D-xylose concentrations varying between 2.5 mM and 100 mM (Sandalli et al., 2014).

2.7. Determination of the temperature effects on activity and stability

The effect of temperature on *Ago*G2XI activity was determined spectrophotometrically using D-xylose as the substrate. Activity assays were performed at various temperatures over the range of 25–100 °C by using the method described previously, and the results were expressed as relative activity (%) obtained at optimum temperature. The effect of temperature on *Ago*G2XI stability was determined by measuring the residual activity (%) after 30 min of pre-incubation at 40, 50, 60, 65, 70, and 75 °C. The percentage residual xylose isomerase activity was calculated compared to unincubated enzymes.

2.8. Determination of the pH effects on activity and stability

The optimum pH of the enzyme was measured at 85 °C and 545 nm by using buffer solutions of different pH values and measuring their relative activities (%). The following buffers (50 mM) were used: sodium acetate (pH 5.0–6.0), potassium phosphate (pH 6.0–7.0), Tris-HCl (pH 7.0–9.0), and glycine-NaOH (pH 9.0–10.0), and the results were expressed as relative activity (%).

In order to determine the pH stability of the enzyme, pre-incubation was performed at each pH value at room temperature for 60 min, and the residual activities were measured. The percentage residual xylose isomerase activity was calculated in comparison with unincubated enzyme.

2.9. Activator effects of some metal ions on XI activity

Bhosale et al. (1996) reported that bivalent metal ions (Co^{2+}, Mn^{2+}, and Mg^{2+}) are activators of xylose isomerases. The activator effects of various metal ions on *Ago*G2XI activity were tested at optimum reaction conditions. In the first step, the enzymes' metal ions were removed by dialysis; then the enzyme solution was pre-incubated with 0.1, 0.5, 1, 2, 4, 10, 20, and 50 mM of bivalent metal ions such as Co^{2+}, Mn^{2+}, and Mg^{2+} chloride salts for 15 min. The xylose isomerase activity of the enzyme without metal ions was defined as the 100% level. The residual activity (%) was assayed spectrophotometrically (Table 2).

2.10. Inhibitor effects of some metal ions on XI activity

The inhibitor effects of various metal ions on *Ago*G2XI activity were assayed at optimum reaction conditions. Following removal of the metal ions of the enzyme by dialysis, the enzyme solution was pre-incubated with 0.1, 1, 5, 10, and 20 mM of bivalent metal ions such as Cd^{2+}, Ca^{2+}, Hg^{2+}, Ni^{2+}, Zn^{2+}, Fe^{2+}, and Cu^{2+} chloride or sulfate salts for 15 min. Xylose isomerase activity of the enzyme without metal ions was defined as the 100% level. The residual activity (%) was assayed spectrophotometrically.

3. Results

The gene encoding XI was amplified with 2 different primer sets. Each of the amplified genes were cloned in pET28(a–c) and expressed in *E. coli* as described in Karaoglu et al. (2013). Due to the low activity of *Ago*G2XI-His, purification studies were initiated with *Ago*G2XI. All the purification steps were carried out at room temperature because the enzyme remains stable at room temperature for several hours. The effects of all purification steps on specific activity, fold purification, and yield are shown in Table 1. The SDS-PAGE view of the purified enzyme is given in Figures 1 and 2.

The *Ago*G2XI exhibited a simple Michaelis–Menten kinetics for D-xylose (Figure 3). Based on the Michaelis–Menten plots, K_m value was calculated to be 25 ± 2 mM and V_{max} value was calculated to be 0.12958 ± 0.002 µmol/min (Figure 4).

Table 1. Summary of AgoG2XI purification steps.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification yield
Cell extract	20.88	66.39	3.18	100	1
Heat treatment	4.624	63.67	13.77	95.89	4.330
DEAE-Sepharose	2.15	57.8	26.88	87.05	8.45
Phenyl-Sepharose 6 Fast Flow	1.39	48.56	34.94	73.14	10.98

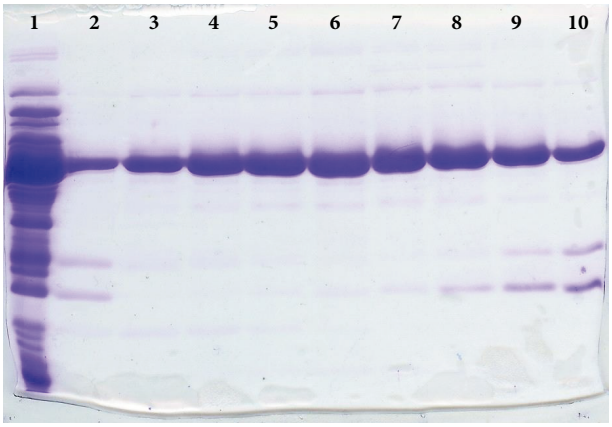

Figure 1. SDS-PAGE showing purified recombinant *Ago*G2XI enzyme; crude extract from *E. coli* BL21 DE3 expressing recombinant *Ago*G2XI enzyme 2, 3, 4, 5, 6, 7, 8, 9, 10; protein extract purified by ion-exchange column chromatography.

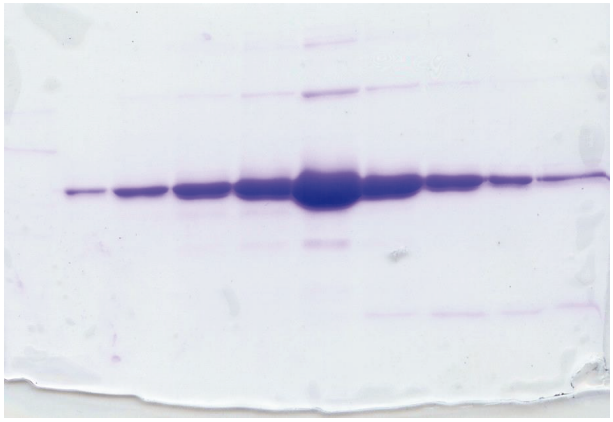

Figure 2. SDS-PAGE showing purified recombinant *Ago*G2XI enzyme obtained from ion-exchange column chromatography by hydrophobic column chromatography.

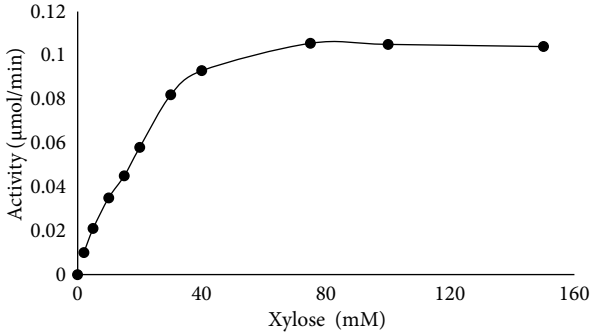

Figure 3. Michaelis–Menten model for *Ago*G2XI.

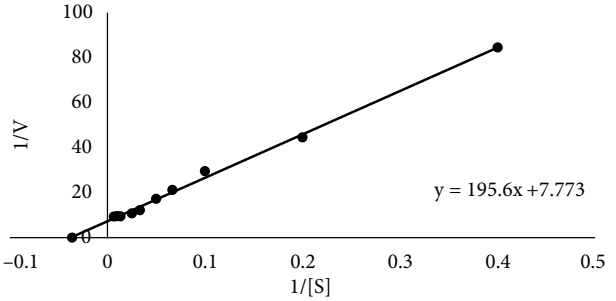

Figure 4. Lineweaver–Burk plot of *Ago*G2XI (K_m and V_{max} values were determined according to Lineweaver–Burk plot).

The optimum temperature for XI activity was 85 °C and the enzyme was active in the broad temperature range of 25–100 °C (Figure 5). At 55 °C, which is the optimal growing temperature of *Ago*G2, the enzyme barely lost any activity; it maintained 60% of its activity after 120 h at 80 °C; in applications at 85 °C, the optimum operating temperature of the enzyme, enzyme activity was reduced by 50% after 50 h; and that the enzyme lost all of its activity in a short time (4–6 h) at 95 and 90 °C (Figure 6). When assayed at various pH values at 85 °C, the purified enzyme exhibited optimum activity at a pH of 6.5 (Figure 7). The enzyme was highly active and stable in a broad pH range of 5.0–9.0 at 4 °C. The enzyme activity was reduced by half after 15 days at various pH values (Figure 8).

The effects of various metal ions known as activators on XI activity were determined at optimum conditions for the enzyme (85 °C, pH 6.5) by using xylose as the substrate. According to the results, Co^{2+}, Mg^{2+}, and Mn^{2+} ions, defined as bivalent metal ions, were required for XI activity. Moreover, the highest activity was observed with 5 mM Mn^{2+}. During the second part of the experiment, the effect of various combinations of Mg^{2+}, Co^{2+}, and Mn^{2+} ions at different concentrations on XI activity was examined. The enzyme exhibited the highest activity in the presence of 10 mM Mg^{2+} and 1 mM Co^{2+} (Figure 9). In addition, bivalent metal ions such as Cd^{2+}, Ca^{2+}, Hg^{2+}, Ni^{2+}, Zn^{2+}, Fe^{2+}, and Cu^{2+} inhibited XI activity, reducing *Ago*G2XI activity by 20% (Figure 10).

4. Discussion

In this study, the *Ago*G2XI gene was cloned to pET-28a(+) expression vector without HisTag tail and with HisTag tail at the C terminal. Since HisTag tail dramatically decreased the activity and expression level of *Ago*G2XI gene, the expression and purification steps were carried out with p*Ago*XI without HisTag.

In this study, enzyme activity was determined according to the revealed D-xylulose amount. When high amounts of D-xylulose were added to the reaction mixture to be analyzed, a large amount of colorful (violet) product was formed. The solution was diluted at least 30, and even 50 times so that the Lambert–Beer law was not violated after the enzyme reaction.

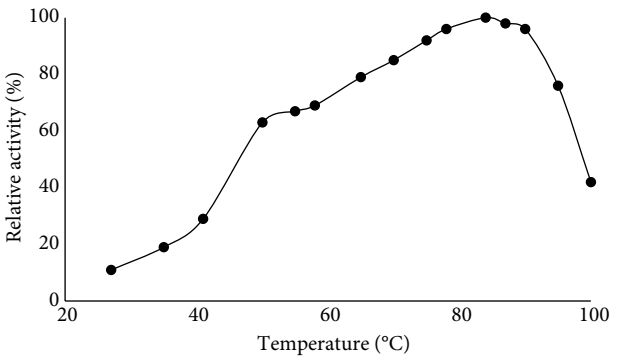

Figure 5. The effect of temperature on the activity of purified XI. The percentage relative enzyme activity was calculated compared to unincubated enzyme.

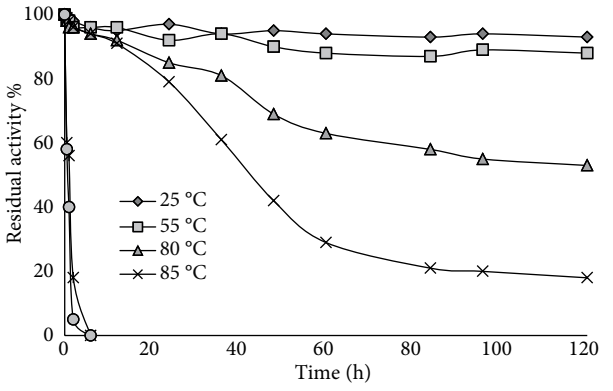

Figure 6. The effect of temperature on the stability of purified glucose isomerases. The percentage residual enzyme activity was calculated compared to unincubated enzyme.

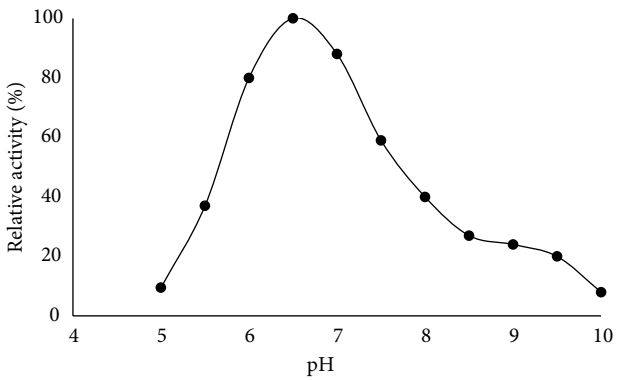

Figure 7. The effect of pH on the activity of purified glucose isomerases. The percentage relative enzyme activity was calculated compared to unincubated enzyme.

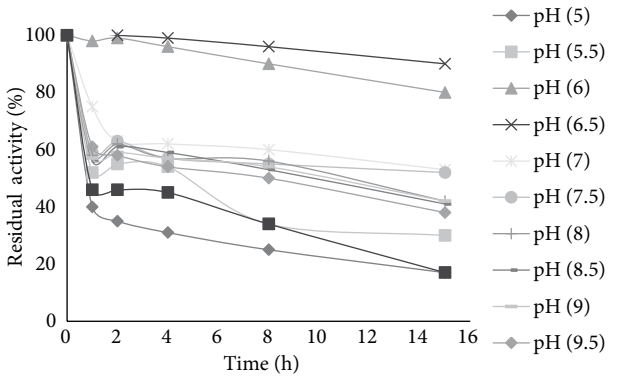

Figure 8. The effect of pH on the stability of purified glucose isomerases. The percentage residual enzyme activity was calculated compared to unincubated enzyme.

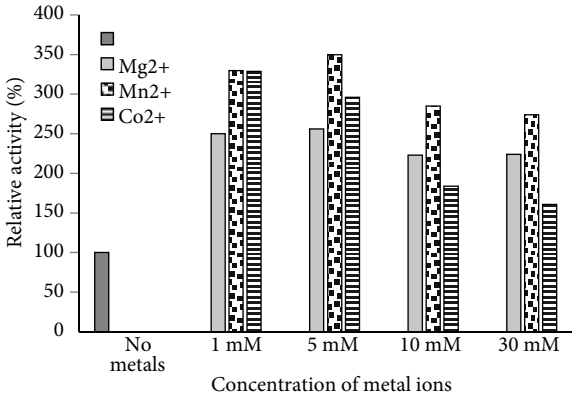

Figure 9. The activator effects of various metal ions on *Ago*G2XI activity.

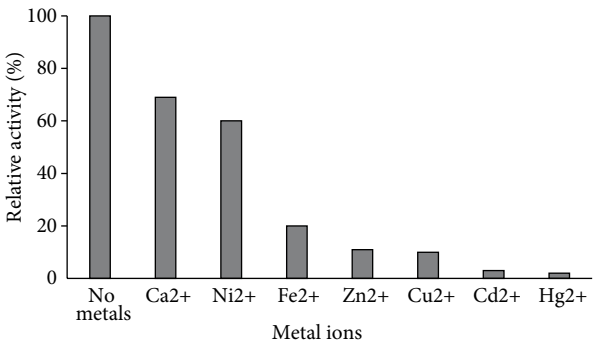

Figure 10. The inhibitor effects of various metal ions on *Ago*G2XI activity.

Table 2. The activator effects of various metal ion pairs on the XI activity of *Ago*G2XI.

Metal ion	% Activity
Without metals	100
Mg^{2+} (10 Mm)–Co^{2+} (1 Mm)	325
Mg^{2+} (30 Mm)–Co^{2+} (5 Mm)	203
Mn^{2+} (10 Mm)–Co^{2+} (1 Mm)	257
Mn^{2+} (30 Mm)–Co^{2+} (5 Mm)	287

Both the activity and the stability of the XI were tested in the range between pH 5.0 and 11.0. According to the results, the enzyme exhibited the highest activity around pH 6.5. These findings are in accordance with XIs isolated from *Thermoanaerobacterium* sp., *Actinoplanes missouriensis*, *Thermus aquaticus*, and *Lactobacillus brevis* (Bhosale et al., 1996; Kim et al., 2001; Karaoglu et al., 2013). However, the optimum activities of many XIs isolated from different microorganisms were reported at pHs higher than 6.5, ranging generally between pH 7.0 and 9.0 (Bhosale et al., 1996; Ertunga et al., 2007; Karaoglu et al., 2013). With this pH value, the enzyme operates in a much more acidic environment compared to many other microorganisms' XIs. To determine the enzyme stability at various pH values, the enzyme was incubated at each pH value at 4 °C for 15 days. It was observed that *Ago*G2XI was active and stable in a broad pH range, between 5 and 9, at 4 °C. The pH stability of XI is very important for the prediction of storage conditions. As XI does not lose its activity after being stored at different pH values, it can be stored in conditions with a broad range of pH for a long period.

*Ago*G2XI exhibited optimum activity at 85 °C. Most of the previously studied XIs have been reported to operate at an optimum temperature between 60 °C and 80 °C,

with a few exceptions (85 °C for XI of *Streptomyces* sp. and *Bacillus* sp., 95 °C for *Thermotoga neapolitana*) (Brown et al., 1993; Vieille et al., 1995; Bhosale et al., 1996; Ertunga et al., 2007; Karaoglu et al., 2013). Based on the literature, we propose that *Ago*G2XI is a highly thermophilic enzyme with an optimum operating temperature of 85 °C. To determine enzyme stability at different temperatures, the enzyme was incubated at 95, 90, 85, 80, and 55 °C and at room temperature. In the analyses of 85 °C, which is the optimum operating temperature of the enzyme, it was observed that enzyme activity was reduced to half after 50 h. At room temperature, however, the enzyme was found to maintain its stability. When compared with the literature (*Bacillus coagulans* XI loses 20% activity at a 60 min application of pH 9 and 50 °C; *Bacillus coagulans* XI is completely inactive after 60 min at pH 4 at 50 °C; *Bacillus* sp. XI is consistent at 80 °C for 10 min; *Bacillus* sp. XI loses 35% activity at 60 °C after 60 min; *Bifidobacterium adolescentis* XI loses 50% activity at pH 6 after 18 h and 8 °C), *Ago*G2XI is highly consistent as compared to other XIs (Liu et al., 1996; Lama et al., 2001).

XI is used industrially for producing ethanol from D-xylose. Despite low rates of fermentation in ethanol production from D-xylose and low rates of efficiency, studies focusing on transferring the XI gene into yeasts for providing simultaneous xylose isomerization and ethanol fermentation are increasing (Wang et al., 1980; Chiang et al., 1981; Gong et al., 1981; Chan et al., 1989; Bhosale et al., 1996). However, this kind of study is not suitable for *Ago*G2XI because the optimum operating temperature of the enzyme is 85 °C. However, it is reported that in order to produce ethanol the optimum temperature of *Thermus thermophilus* XI, whose optimum temperature is 90 °C, decreases to 60 °C with mutations and transfers to *Saccharomyces cerevisiae* (Lönn et al., 2003). The high optimum temperature of XI means that it is not suitable for ethanol production, since *Saccharomyces cerevisiae* cannot live at very high temperatures. This kind of study seems possible for *Ago*G2XI as well.

At the end of the kinetic studies, the K_m value of *Ago*G2XI was estimated to be 25 ± 2 mM. Comparisons with the XIs from different microorganisms (K_m value 1.1 mM for *Bacillus coagulans*, 2.25 mM for *Lactobacillus lactis*, 3.44 mM for *Thermus thermophilus*, 6.6 mM for *Bacillus* sp., 15 mM for *Thermus aquaticus*) have shown that this value is very high for the substrate D-xylose. Therefore, it can be stated that the value of *Ago*G2XI for D-xylose is generally lower than that for other identified XIs (Haki and Rakshit, 2003). Xylose can be fermented with a 2-step procedure by many types of yeasts, including *Saccharomyces cerevisiae*. In this procedure, which involves 2-step fermentation of xylose, xylose is initially isomerized with XI outside of the cell. More economical XI production is important for industrial utility. In this study, *Ago*G2XI is industrially important because transforming the XI enzyme into the *E. coli* BL21 (DE3) strain with pET28a(+) expression vector gives us the chance to produce the enzyme in large amounts. However, the K_m value of the enzyme against xylose must be reduced with mutations.

In the absence of bivalent metal ions such as Co^{2+}, Mn^{2+}, and Mg^{2+}, *Ago*G2XI lost more than 60% of its activity at the optimum temperature. However, in the presence of Co^{2+}, Mn^{2+}, and Mg^{2+}, the enzyme could retain its original activity for a long time. It was shown that these ions were very important for the stabilization of the multimeric structure, resulting in enzyme thermostability (Cha et al., 1994; Kim et al., 2001). The activity of *Ago*G2XI was more dependent on Mn^{2+} than Co^{2+} or Mg^{2+} at 85 °C. The maximum activity was observed when Mg^{2+} and Co^{2+} were both present in the reaction mixture. In addition, *Ago*G2XI was clearly inhibited by addition of Cd^{2+}, Ca^{2+}, Hg^{2+}, Ni^{2+}, Zn^{2+}, Fe^{2+}, and Cu^{2+} (Karaoglu et al., 2013). The activity and stability of the enzyme strictly depends on the presence of Mg^{2+}, Co^{2+}, and especially Mn^{2+}. On the other hand, the activity of the enzyme was inhibited by Cd^{2+}, Ca^{2+}, Hg^{2+}, Ni^{2+}, Zn^{2+}, Fe^{2+}, and Cu^{2+} ions.

This study, being the first to study XI among *Anoxybacillus* XIs, is also academically important because it can be a guide for XI enzyme studies with other bacteria. The results reported here are indicative of a new XI with desirable kinetics and stability parameters for the efficient production of xylulose and ribose on an industrial scale.

Acknowledgments

This study was financially supported by the Karadeniz Technical University Research Foundation (Grant no. 2003.111.04.6) and the Scientific and Technological Research Council of Turkey (TÜBİTAK) (Grant no. 104T472 and TBAG-AY/395 (104T380)).

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