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Expression and Characterization of Calcium- and Zinc-Tolerant Xylose Isomerase from *Anoxybacillus kamchatkensis* G10

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The enzyme xylose isomerase (E.C. 5.3.1.5, XI) is responsible for the conversion of an aldose to ketose, especially xylose to xylulose. Owing to the ability of XI to isomerize glucose to fructose, this enzyme is used in the food industry to prepare high-fructose corn syrup. Therefore, we studied the characteristics of XI from *Anoxybacillus kamchatkensis* G10, a thermophilic bacterium. First, the gene coding for XI (*xylA*) was inserted into the pET-21a(+) expression vector and the construct was transformed into the *Escherichia coli* competent cell BL21 (DE3). The expression of recombinant XI was induced in the absence of isopropyl-thio-β-galactopyranoside and purified using Ni-NTA affinity chromatography. The optimum temperature of recombinant XI was 80°C and measurement of the heat stability indicated that 55% of residual activity was maintained after 2 h incubation at 60°C. The optimum pH was found to be 7.5 in sodium phosphate buffer. Magnesium, manganese, and cobalt ions were found to increase the enzyme activity; manganese was the most effective. Additionally, recombinant XI was resistant to the presence of Ca^{2+} and Zn^{2+} ions. The kinetic properties, K_m and V_{max}, were calculated as 81.44 mM and 2.237 μmol/min/mg, respectively. Through redundancy analysis, XI of *A. kamchatkensis* G10 was classified into a family containing type II XIs produced by the genera *Geobacillus*, *Bacillus*, and *Thermotoga*. These results suggested that the thermostable nature of XI of *A. kamchatkensis* G10 may be advantageous in industrial applications and food processing.

Keywords: *Anoxybacillus*, xylose isomerase, calcium resistance, zinc resistance

Introduction

Xylose isomerase (E.C. 5.3.1.5, XI) is a member of the isomerase family that catalyzes the isomerization of D-xylose to D-xylulose. As this enzyme is also able to convert D-glucose to D-fructose, it is used in the food industry, especially for the production of high-fructose corn syrup, which is used as an artificial sweetener in soft drinks and foods [1]. At equal levels of sweetener, fructose is cheaper than sucrose and has a lower caloric value because of lower resorption. It is used in a wide range of food products, such as ice-cream, canned products, baking products, pickle, sauces, and meal products, because glucose and fructose have higher solubilities than sucrose and a reduced tendency to crystallize [2]. Since the characterization of the XI-coding gene (*xylA*) from *Escherichia coli* was first reported [3], *xylA* has been cloned from several microorganisms such as *Thermus thermophilus* [4], *Bacillus subtilis* [5], and *Thermoanaerobacterium* sp. [6]. After researchers understood the relation between the molecular structure of the enzyme and its catalytic properties, studies were performed to clone the *xylA* gene and enhance the industrial applications of the enzyme [7]. For industrial applications, the preferred characteristics of XI are thermostability, fast turnover speed, and high isomerization rate. A low optimal pH and resistance to inhibition by Ca^{2+} are also desirable.

Anoxybacillus kamchatkensis is a rod-shaped, facultatively anaerobic, gram-positive, thermophilic bacterium that was

first isolated from Kamchatka, Russia [8]. The *A. kamchatkensis* G10 used in this study was isolated from Indonesia after growth at temperatures between 55°C and 60°C and a pH range of 6.5–8.5 [9]. Several enzymes from *Anoxybacillus* sp. have been characterized, such as glucosidase [10], amylase [11], aldolase [12], esterase [13], proteinase [14], and xylanase [15]. In this study, the *xylA* gene from *A. kamchatkensis* G10 was cloned and XI was expressed in *E. coli*. The biochemical features of the recombinant XI were analyzed and compared with those of other industrial XIs. Furthermore, the correlation between the distances of sequence similarity and the variations in the optimal temperature and G+C ratio for 14 *xylA* genes were studied.

Materials and Methods

Strains, Enzymes, and Chemicals

E. coli DH5α and BL21 (DE3) were used for propagation of plasmids and expression of recombinant enzyme, respectively. Restriction endonucleases, *Pfu* DNA polymerase, nucleotides, T4 DNA ligase, and antibiotics were purchased from Thermo Scientific (USA). Xylose and xylulose were obtained from Sigma-Aldrich (USA).

Cloning, Expression, and Purification of the Recombinant Xylose Isomerase

The XI-coding gene from *A. kamchatkensis* G10 was demonstrated by draft-genome sequencing [9]. The *xylA* gene fragment with restriction enzyme engineering sites (NheI and BamHI) was obtained by PCR with a forward primer (5′-CGCGCTAGCATG GCGTATTTTGAAAACGTT-3′) and a reverse primer (5′-GTCGGATCCTTAGTGGTGGTGGTGGTGACGAGCTACAC AAACTTC-3′). The PCR product was digested, purified, and ligated into the pET-21a (+) expression vector (Novagen, USA). The generated plasmid, named pET-AnoxyXI, was transformed into *E. coli* BL21 (DE3) to express the recombinant enzyme. The bacterial colony was inoculated in Luria-Bertani medium containing 100 μg/ml ampicillin and incubated for 12 h at 37°C. The cell was corrected and resuspended in lysis buffer containing 50 mM sodium phosphate (pH 7.5), 0.5 M NaCl, and 25 mM imidazole. After sonication, the cell debris was removed by centrifugation at 4°C and the crude extract was loaded into a Ni-NTA packed column (Qiagen Inc., USA). After a wash step with 10 column volumes of lysis buffer, elution buffers containing 50 mM sodium phosphate (pH 7.5), 0.5 M NaCl, and serially diluted imidazole were used to elute the recombinant XI. The eluted fraction that contained recombinant XI was resolved by SDS-PAGE and the protein concentrations were measured by Bradford assay using bovine serum albumin as the reference standard [16]. The molecular mass of the native state was determined by gel filtration chromatography. A Superose 6 10/300 GL column (GE Healthcare, UK) was pre-equilibrated with buffer containing 50 mM sodium phosphate (pH 7.2), 150 mM NaCl, and 1 mM dithiothreitol. The eluent was monitored at 280 nm. The standard proteins used for molecular mass determination were ferritin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa), and ovalbumin (43 kDa).

Enzyme Activity Assay

The recombinant XI activity was assayed to measure the amount of xylulose formed in the reaction. A mixture of enzyme and reaction buffer, containing 200 mM xylose, 20 mM divalent metal cations, and 50 mM sodium phosphate (pH 7.5), was adjusted to 800 μl and incubated at 60°C for 30 min. The enzyme reaction was stopped by adding 200 μl of 0.5 M perchloric acid. The amount of xylulose was determined by using a cysteine-carbazole-sulfuric acid method [17]. One unit of XI activity was defined as the formation of 1 μmol xylulose per minute under the assay conditions. The experimental data were adjusted to the Michaelis-Menten equation to obtain the values of K_m and V_{max}.

Effects of Temperature and pH on the Enzymatic Activity

To measure the optimum temperature of the recombinant XI, the activity profile was analyzed at various temperatures (30°C to 100°C, in 10°C increments). In order to evaluate the thermal stability, the enzyme was preincubated at 60°C, 70°C, and 80°C for 2 h with a 0.5-h interval and the remaining activities were measured. The activities of the enzyme at different pH values were also evaluated through replacement of the enzyme reaction buffer with four buffer systems; namely, citrate-NaOH (pH 3.0–6.0), sodium phosphate (pH 6.0–8.0), HEPES-NaOH (pH 7.0–8.0), and glycine-NaOH (pH 8.0–10.0). The characteristics of the enzyme were presented as a relative percentage of the highest activity and expressed as an average of a minimum of three experiments.

Effects of Metal Ions on the Enzymatic Activity

To determine the effects of divalent metal cations on the recombinant XI activity, the purified enzyme was dialyzed overnight at 4°C in a dialysis buffer containing 50 mM sodium phosphate (pH 7.5), 0.5 M NaCl, 20 mM imidazole, and 10 mM EDTA. Overnight, the buffer was changed at least three times [18]. The enzymatic activity at 20 mM was evaluated in the presence of each of the following solutions: $MgCl_2$, $MnSO_4$, $CoSO_4$, $NiSO_4$, $CuSO_4$, $AlNH_4(SO_4)_2$, $CaCl_2$, $ZnSO_4$, and EDTA. All experiments were conducted in triplicate. The activities were compared with the enzyme incubated without metal cations under the same conditions.

Circular Dichroism Spectroscopy

Two-dimensional structures of the recombinant XI were measured with different divalent metal cations. The apoenzyme was prepared by the same procedure used to measure the effects of metal ions. The absorbance of the enzyme samples in four conditions, XI-Apo, XI-Ca^{2+}, XI-Zn^{2+}, and XI-Mn^{2+}, was analyzed

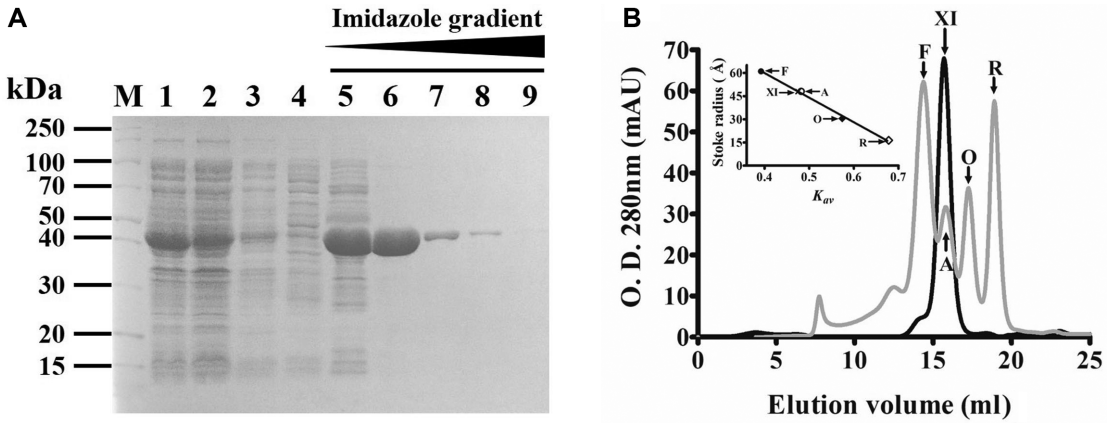

Fig. 1. Purification steps and size measurements of the recombinant xylose isomerase from *Anoxybacillus kamchatkensis* G10. (**A**) Separation of recombinant xylose isomerase by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Lane M, SDS-PAGE markers; lane 1, crude extract; lane 2, flow through; lane 3, washing I; lane 4, washing II; lane 5, 100 mM imidazole elution; lane 6, 150 mM imidazole elution buffer; lane 7, 200 mM imidazole elution buffer; lane 8, 300 mM imidazole elution buffer; and lane 9, 400 mM imidazole elution buffer. (**B**) In size-exclusion chromatography, the standards used were ferritin (440 kDa, 61.0 Å), aldolase (158 kDa, 48.1 Å), ovalbumin (43 kDa, 30.5 Å), and ribonuclease A (13.7 kDa, 16.4 Å).

between 190 and 260 nm in a 1-mm circular dichroism (CD) spectroscopy cuvette by using a J-1500 CD spectrophotometer (JASCO, Japan). The measurement interval was 0.5 nm and the bandwidth was 1 nm.

Principal Component Analysis and Redundancy Analysis

To classify the family type of XI from *A. kamchatkensis* G10, principal component analysis (PCA) was performed. The distances of sequence similarity between 14 *xylA* from each bacterium were used as the component in this study. The correlations between the *xylA* gene sequence component and GC content, optimum temperature, and K_m value were illustrated according to the redundancy analysis (RDA) function from the vegan library in R software [19].

Results

Expression of Recombinant XI

The D-xylose isomerase gene (*xylA*) from *A. kamchatkensis* G10 was cloned by PCR, as described in the Materials and Methods. The recombinant enzyme was expressed in *E. coli* and purified by Ni-NTA affinity chromatography. Although the recombinant XI was eluted from 100 mM imidazole, a relatively pure fraction from 150 mM imidazole was used for further study (Fig. 1A). After this one-step purification, the yield of the purified enzyme was 50.5%. In contrast, the specific activity was increased from 26.3 U/mg to 164.25 U/mg, which was a 6.5-fold increase in the purification rate (Table 1). In SDS-PAGE, the purified enzyme appeared as a single band located near the 50 kDa marker protein (Fig. 1A). This was a good match with the molecular mass, 49.61 kDa, calculated from the sequence. The oligomerization of recombinant XI was determined by gel filtration chromatography using a Superose 6 10/300 GL column. A single sharp peak was shown with a Stokes radius of 47.9 Å, which was similar to that of aldolase (158 kDa) and much bigger than ovalbumin (43 kDa). Therefore, the recombinant XI was estimated to be a tetramer (Fig. 1B).

Effects of Temperature and pH, and Kinetics of Recombinant XI

The optimum temperature of XI activity was measured after incubation of the enzyme at temperatures between 30°C and 100°C for 30 min. Recombinant XI from *A. kamchatkensis* G10 exhibited the maximum activity at 80°C (Fig. 2A). To evaluate the thermostability of the recombinant XI, the

Table 1. Purification yield of recombinant xylose isomerase from *A. kamchatkensis* G10.

	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude enzyme	296.4	7,789.4	26.3	100.0	1.0
Purified enzyme[a]	24.2	3,937.4	164.3	50.5	6.5

[a]The enzyme activity was measured after heat treatment and purification at 60°C for 30 min.

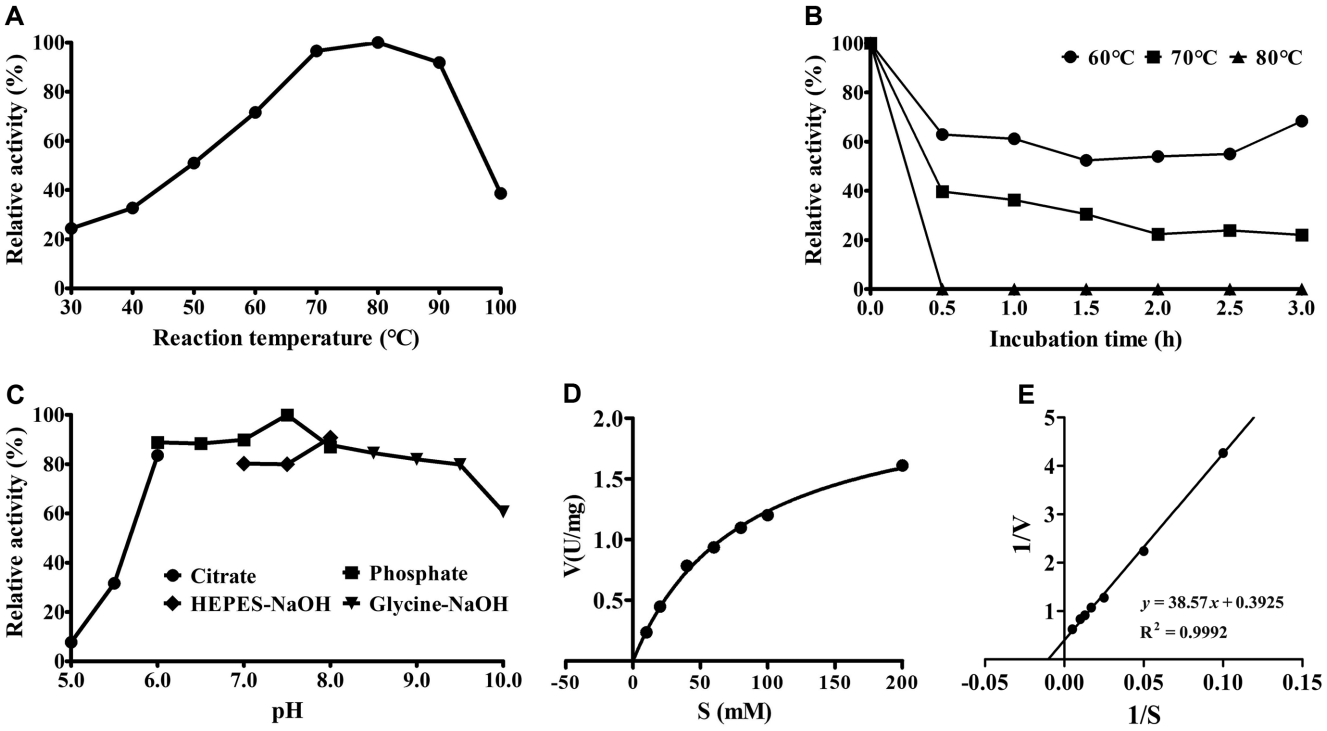

Fig. 2. Optimal conditions and kinetics of recombinant xylose isomerase.
(**A**) Optimum temperature; (**B**) thermostability; (**C**) optimum pH; (**D**) Michaelis-Menten kinetics; (**E**) Lineweaver-Burk plots. Optimal condition results represent the mean ± SE of three replicated experiments. The linear equation of Lineweaver-Burk plot and R-square value are shown.

enzyme was incubated at 60°C, 70°C, and 80°C for up to 2 h and the remaining activity was measured (Fig. 2B). Recombinant XI possessed 55% of remaining activity after 2 h of incubation at 60°C and an activity half-life of 30 min at 70°C, but no activity was observed after incubation for 30 min at 80°C. The optimal pH was 7.5, which was determined by testing pH conditions ranging from 3.0 to 10.0 (Fig. 2C). To determine the kinetic values of the recombinant XI, the reaction was performed in 50 mM sodium phosphate buffer (pH 7.5) at 60°C (Figs. 2D and 2E). The substrate concentrations in the reaction mixture were in the range from 10 to 200 mM. The kinetic parameters were calculated from the Lineweaver-Burk plot; the K_m and V_{max} values of the enzyme were calculated to be 81.44 mM and 2.237 μmol/min/mg, respectively.

Effects of Divalent Metal Ions and CD Spectra of the Recombinant XI

The relative activities with various divalent cations of the recombinant XI were measured (Table 2). The enzyme showed 12-fold activity with Mn^{2+} compared with the enzyme without the addition of cofactors. Increased activity was also seen with other cations; for example, Mg^{2+}, Co^{2+}, Zn^{2+}, and Ca^{2+}, increased the enzyme activity by approximately 10-, 7.4-, 3.9-, and 2.4-fold, respectively. Interestingly, divalent cations, such as Ni^{2+}, Cu^{2+}, and Al^{2+} resulted in enzyme inhibition, which was similar to the effect of a metal ion chelator, EDTA.

The two-dimensional structure of recombinant XI is illustrated in Fig. 3. In the experiment, the divalent cations were used at 20 mM. The CD spectrum of the apoenzyme

Table 2. Effects of divalent cations on xylose isomerase activity.

Ions / EDTA (20 mM)	None	$MnCl_2$	$MgCl_2$	$CoSO_4$	$ZnSO_4$	$CaCl_2$	$CuSO_4$	$AlNH_4(SO_4)$	$NiSO_4$	EDTA
Relative activity (%)	100.0	1202.0 ± 80.8	993.2 ± 109.2	743.3 ± 62.0	396.9 ± 36.1	239.9 ± 128.6	12.0 ± 7.6	11.8 ± 9.6	9.6 ± 3.7	9.4 ± 4.6

The activity was measured with divalent cations in 50 mM sodium phosphate buffer (pH 7.5) at 60°C.

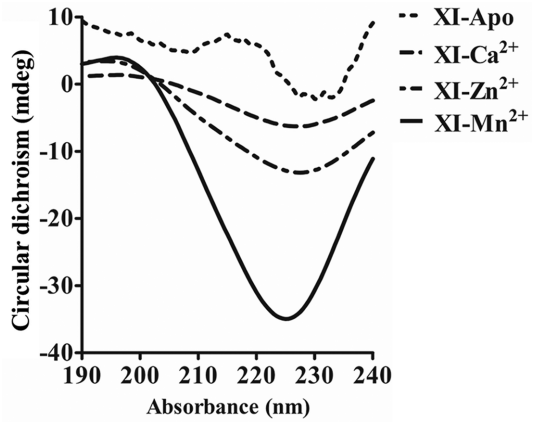

Fig. 3. CD spectrum of the xylose isomerase.
From top to bottom, the four graphs represent XI-Apo, XI-Ca^{2+}, XI-Zn^{2+}, and XI-Mn^{2+}, respectively. The depth of the trough between 220 and 230 nm indicates the enzyme active form with higher activity.

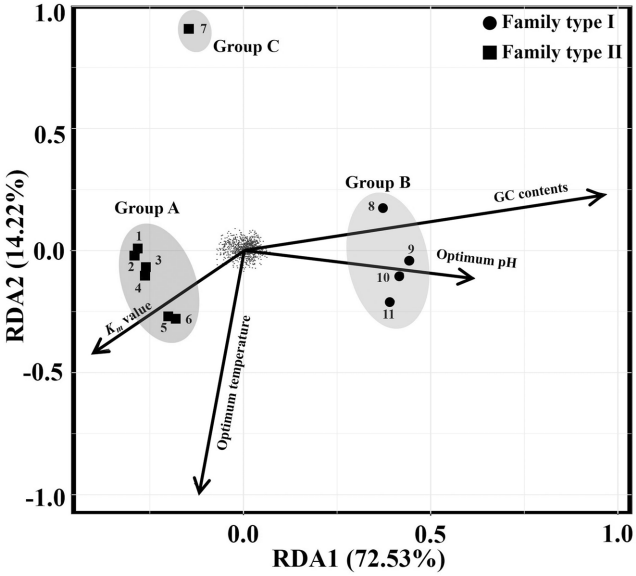

Fig. 4. Redundancy analysis (RDA) of xylose isomerase based on metadata.
The four arrows indicate the variables used in RDA. All numbers indicate that bacteria were used in the RDA plot, as shown in Table 3. 1, *Anoxybacillus kamchatkensis* G10; 2, *Anoxybacillus gonensis* $G2^T$; 3, *Geobacillus caldoxylosilyticus* TK4; 4, *Bacillus thermantarcticus* DSM 9572; 5, *Thermotoga maritima* MSB8; 6, *Thermotoga neapolitana* 5068; 7, *Fulvimarina pelagi* HTCC 2506; 8, *Arthrobacter* sp. B3728; 9, *Streptomyces chibaensis* J-59; 10, *Thermus aquaticus* HB8; 11, *Thermus oshimai* JL-2.

was not stable, but in the presence of other cations, such as Ca^{2+}, Zn^{2+}, and Mn^{2+}, more stable structures were formed. The XI-Mn^{2+} CD spectrum showed the most stable form and the highest activity. Combined with the other cations, Ni^{2+}, Cu^{2+}, and Al^{2+}, the CD spectrum of the enzyme was unstable (data not shown).

Classification and Correlation of Xylose Isomerase

In an RDA plot with the DNA sequence variations of each reference *xylA* gene, all XIs were classified into three major groups by variations of parameters such as GC content, optimum temperature, optimum pH, and K_m value (Fig. 4). In particular, *xylA* from *A. kamchatkensis* G10 was located in group A, with the genera *Geobacillus*, *Bacillus*, and *Thermotoga* (Fig. 4). All other bacteria were isolated from terrestrial samples, except for *Fulvimarina pelagi* HTCC 2506, which was derived from a marine sample [20]. Therefore, it is understandable that XI from *F. pelagi* was classified into a separate group (group C). The optimum temperature and K_m value were correlated to group A or type II, whereas higher GC content and higher optimum pH were correlated to group B or type I.

Discussion

The kinetic parameters of XI from other studies were collected and compared (Table 3). There are two types of XI families: compared with the type I family, the family of type II enzymes has approximately 40–50 more amino acid residues at the N-terminus [21]. The catalytic triad (His-101, Asp-104, and Asp-339), as well as almost all the other residues involved either in substrate or metal binding, is conserved among the two families of XI [22]. XI from *A. kamchatkensis* G10 was identified as a member of the type II family and correlated with the expected K_m values. Enzymes in the type I family were strongly correlated to GC content and optimum pH. The *A. kamchatkensis* G10 XI was most similar to other thermostable isomerases, with the exception of the enzyme from *Fulvimarina pelagi* HTCC 2506 in the XI of type II family. On the other hand, it was confirmed that *A. kamchatkensis* G10 XI and thermostable XI belonging to the type I family were not correlated.

There are two metal-binding sites in XI. For enzyme activity, Co^{2+}, Mg^{2+}, or Mn^{2+} metal cations are needed as cofactors [23]. The divalent cations play a key role in substrate binding, ring opening, and isomerization [24, 25]. In contrast, Ni^{2+}, Ca^{2+}, Cu^{2+}, Al^{2+}, and Zn^{2+} reduced the enzyme activity [26–28]. XI from *A. kamchatkensis* G10 also requires those metal ions to achieve higher activity than the enzyme without cations. The enzyme activity was inhibited by Ni^{2+}, Cu^{2+}, and Al^{2+}, similar to any other XI. However, Ca^{2+} and Zn^{2+} caused approximately 2.4-fold and 4-fold activation of the enzyme, respectively. If the enzyme used

Table 3. Metadata table of xylose isomerase from *A. kamchatkensis* G10 and other bacteria.

No.	Family types	Strains	Optimum temperature (°C)	Optimum pH	K_m value (mM)	GC content (%)	Accession No.	References
1	II	*Anoxybacillus kamchatkensis* G10	80.0	7.5	81.44	41.4	This study	This study
2	II	*Anoxybacillus gonensis* $G2^T$	85.0	6.5	25.00	41.7	JQ768452	[21]
3	II	*Geobacillus caldoxylosilyticus* TK4	80.0	6.5	20.58	44.0	AF170068	[29]
4	II	*Bacillus thermantarcticus* DSM 9572	90.0	7.0	33.00	43.7	X59466	[30]
5	II	*Thermotoga maritima* MSB8	105	7.5	74.00	46.2	L38994	[31]
6	II	*Thermotoga neapolitana* 5068	95.0	7.1	52.10	46.9	NZDS022272	[22]
7	II	*Fulvimarina pelagi* HTCC 2506	25.0	7.0	1.75	61.2	AE000512	[20]
8	I	*Arthrobacter* sp. B3728	60.0	8.0	3.30	64.4	HQ687480	[32]
9	I	*Streptomyces chibaensis* J-59	83.0	7.5	20.20	72.0	D90256	[33]
10	I	*Thermus aquaticus* HB8	70.0	7.0	15.00	69.5	FJ848379	[27]
11	I	*Thermus oshimai* JL-2	95.0	8.0	81.46	68.6	NC019386	[34]

in the process of converting glucose to fructose has resistance to Ca^{2+} inhibition, it is possible to reduce costs by avoiding a Ca^{2+} removal step in the process of saccharifying starch to glucose [26]. The detailed mechanism of this activation is unknown, but the stable CD spectra with those cations provided confirmation that this XI is unique.

Karaoglu *et al*. [21] reported a glucose isomerase from *Anoxybacillus gonensis* $G2^T$, which has exactly the same amino acid sequence as the XI from *A. kamchatkensis* G10. In the paper, they described that Ca^{2+} and Zn^{2+} ions suppress the enzyme, which was the opposite of our observation. Moreover, they claimed that there was about 80% of remaining activity after incubation at 85°C for 24 h, which is the highest thermal stablility among the bacteria. Conversely, our results have shown that this enzyme has lost its activity completely even after incubation at 85°C for 30 min. The difference between the two studies may be due to different substrates used in the experiments, or different research perspectives to the enzyme. In any case, we showed here the result of our best efforts.

As the property of resistance to divalent cations such as Ca^{2+} and Zn^{2+} is beneficial in the food industry, the metal ion properties of this XI from *A. kamchatkensis* G10 were observed. Simultaneously, because the enzyme has a relatively low optimal pH, high optimal temperature, and adequate thermal stability for industrial use, it may prove to be an alternative option for food processing techniques that use XI.

Acknowledgments

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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