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Purification and characterization of thermostable xylose(glucose) isomerase from *Bacillus thermoantarcticus*

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Xylose isomerase produced by *Bacillus thermoantarcticus* was purified 73-fold to homogeneity and its biochemical properties were determined. It was a homotetramer with a native molecular mass of 200 kDa and a subunit molecular mass of 47 kDa, with an isoelectric point at 4.8. The enzyme had a K_m of 33 mM for xylose and also accepted D-glucose as substrate. Arrhenius plots of the enzyme activity of xylose isomerase were linear up to a temperature of 85°C. Its optimum pH was around 7.0, and it had 80% of its maximum activity at pH 6.0. This enzyme required divalent cations for its activity and thermal stability. Mn^{2+}, Co^{2+} or Mg^{2+} were of comparable efficiency for xylose isomerase reaction, while Mg^{2+} was necessary for glucose isomerase reaction. *Journal of Industrial Microbiology & Biotechnology* (2001) **27,** 234–240.

Keywords: *Bacillus*; thermophile; xylose isomerase

Introduction

Various microorganisms are able to grow on hemicelluloses, which amount to 40% of the plant biomass [34], as a sole carbon source. Exoenzymes degrade the polymer to D-xylose, which is transported into the cell, isomerized to D-xylulose and then phosphorylated to xylulose 5-phosphate, which enters either the pentose phosphate pathway [10,15,18,20,21] or the phosphoketolase pathway [15]. Isomerization of D-xylose is carried out by D-xylose isomerase (D-xylose keto-isomerase, EC 5.3.1.5). This enzyme has been used commercially because of its capacity to produce a high-fructose corn-enriched syrup (HFCS) by converting D-glucose into fructose [3,34]. D-xylose isomerase is also of industrial interest for the isomerization of D-xylose to D-xylulose, which can be ultimately fermented to ethanol by conventional yeasts [15]. Owing to the industrial significance of the enzyme, xylose isomerases from various microorganisms have been studied, and their catalytic and physicochemical properties have been reviewed [8,9]. Immobilization techniques and an isomerization process with the enzyme have been described [17].

Most commercially available xylose isomerases have been isolated from mesophilic microorganisms, including *Streptomyces*, *Actinoplanes*, *Flavobacterium* and *Bacillus* species [4,26]. These enzymes are generally thermostable, and are utilized in the immobilized form to enhance enzyme half-life [39]. These enzymes require metal ions for their activity and stability, and the pH optima for enzyme activity are in the range 7.5–9.0. The reaction temperature used in the current industrial process for sweetener production is limited to 60°C because of by-product and colour formation during reaction at high temperature and alkaline pH [7]. Reaction temperatures greater than 60°C have the advantage of faster reaction rates, higher equilibrium concentrations of products and decreased viscosity of the substrate and product stream. Therefore, thermostable xylose isomerases with neutral or slightly acidic pH optima have potential industrial application.

Thermophilic microorganisms produce intrinsically thermostable enzymes, which have evolved and adapted to the extreme environment of their natural habitats [2].

In the present work, D-xylose(glucose) isomerase of the thermophilic bacterium *Bacillus thermoantarcticus* (DSM 9572) [33] was purified and the physicochemical and enzymatic properties were studied and compared between the two types of isomerase reactions.

Materials and methods

Strain and growth conditions

B. thermoantarcticus (DSM 9572) was isolated from Antarctic geothermal soil near the crater of Mount Melbourne [33]. The strain was cultivated at 65°C on a medium containing 0.6% yeast extract (w/v) and 0.3% (w/v) NaCl at pH 6.0 (standard growth conditions). Production of the enzyme was investigated on media containing 0.1% (w/v) yeast extract, 0.3% NaCl and 0.5% of a different carbon source: xylose, xylan, arabinose, galactose, fructose, yeast, maltose or glucose. Growth was followed by measuring the absorbance at 540 nm. Cells were harvested, in late exponential growth phase, by centrifugation at 9000×*g* for 30 min.

Enzyme assays

D-xylose isomerase: The formation of D-xylulose from D-xylose was measured using the colorimetric assay of Dische and Borenfreund [12] in which 20 μl of the enzyme solution was added to 20 μl of a solution of 0.2 M D-xylose and 0.4 mM $MnSO_4$ in 20 mM Tris–HCl, pH 7.0, and incubated for 10 min at 80°C. One unit (U) of enzyme activity was equal to the formation of 1 μmol of D-xylulose per minutes at 80°C.

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D-glucose isomerase: The test for D-glucose isomerase activity was the same as that for D-xylose isomerase, with the exception that D-xylose and $MnSO_4$ were replaced by 0.4 M D-glucose, 10 mM $MgSO_4$ and 1 mM $CoCl_2$ in 20 mM Tris–HCl, pH 7.0.

Protein content

Protein content was determined by the method of Bradford [5] by using the Bio-Rad protein assay with bovine albumin as a standard.

HPAE-PAD

The products of reaction were identified and quantitated by HPAE-PAD Dionex, equipped with Carbo-Pac PA-1 column, using 100 mM NaOH as solvent system [31].

Enzyme purification

Preparation of the crude extract: Wet cells (5 g) were lysed by freezing and thawing, suspended in 20 mM Tris–HCl, pH 7.0 (25 ml) and, after ultrasonic treatment (Heat Systems Instrument) for 4 min, treated with 0.2 mg of DNase and 6 mg of lysozyme at 37°C for 30 min. The resulting suspension was centrifuged at 34,000×*g* for 30 min. The dialysed (20 mM Tris–HCl, pH 7.0, containing 0.4 mM $MgCl_2$ and 1 mM $CoCl_2$) supernatant was taken as crude extract.

Heat treatment: The crude extract of *B. thermoantarcticus* was heated for 15 min at 80°C, in the presence of 10 mM $MgSO_4$ and 1 mM $CoCl_2$, and cooled to 4°C. The soluble fraction was recovered after centrifugation at 15,000×*g* for 20 min.

Ion exchange chromatography: The above enzyme preparation was loaded on a column (2.5×40 cm) of Q-Sepharose Fast Flow pre-equilibrated with 20 mM Tris–HCl, pH 7.0, containing 0.5 mM $CoCl_2$ and 5 mM $MgSO_4$. The column was washed with 100 ml of the same buffer at flow rate of 2.5 ml/min and eluted with a 5-bed-vol linear NaCl salt gradient (0–0.6 M) in the same buffer. The active fractions were pooled and concentrated by ultrafiltration (YM 30 Amicon).

Gel filtration: Concentrated fractions were applied to a column of Sephacryl S-200 (1.5×80 cm). Equilibration and elution were carried out with 20 mM Tris–HCl, pH 7.0, containing 0.5 mM $CoCl_2$ and 5 mM $MgSO_4$ at flow rate of 0.4 ml/min. The active fractions were pooled and concentrated as above.

Hydrophobic interaction: A saturated ammonium sulphate solution was added to the enzyme solution to give a final concentration of 1.3 M. This solution was applied to a 1.5×16 cm Phenyl-Sepharose CL-4B column, previously equilibrated with 20 mM Tris–HCl, pH 7.0, containing 1.3 M $(NH_4)_2SO_4$. The column was washed with 2 bed vol of the same buffer, and eluted with a 10-bed-vol linear gradient of 1.3–0 M $(NH_4)_2SO_4$ at a flow rate of 0.5 ml/min. Fractions containing enzyme activity were pooled, concentrated as above and dialysed overnight against 20 mM Tris–HCl, pH 7.0.

Fast protein liquid chromatography: The enzyme was further purified by FPLC on Mono Q HR/5/5 column in 20 mM Tris–HCl, pH 7.0. The column was washed with 10 ml of the same buffer and the enzyme was eluted with a linear gradient 0–0.6 M NaCl (30 ml) at a flow rate of 0.5 ml/min.

Electrophoresis and M_r determination

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli [19]. Native PAGE was performed without SDS, and Tris–HCl buffer (pH 8.6) was used during polyacrylamide gel preparation. Staining of protein bands was done with Coomassie brilliant blue R-250 (Bio-Rad). M_r values of purified xylose isomerase were determined by gel filtration on a Sephacryl S-200 column (2.5×49 cm); Blue Dextran (M_r 2,000,000) and Pharmacia Gel Filtration Calibration Kit (high and low molecular weight) were used as M_r standards.

Subunit M_r values were estimated by SDS-PAGE using the Pharmacia high- and low-molecular-weight electrophoretic standards.

The isoelectric point of the enzyme was determined by using the Mini Protean II (Bio-Rad) system with IEF Ready Gel (range pH 3–10). Protein bands were made visible by Coomassie brillant Blue G-250 staining.

Metal ion effects on enzyme activity and stability

To prepare a metal-ion-free enzyme solution, xylose isomerase purified from *B. thermoantarcticus* was dialyzed at 4°C for 24 h against 20 mM Tris–HCl, pH 7.0, containing 10 mM EDTA, followed by dialysis against buffer without EDTA (two changes). Enzyme activity was determined in the presence of various concentrations of $MgCl_2$, $MnCl_2$ and $CoCl_2$ using the standard assay.

Thermostability was determined by measuring residual activity under optimum assay conditions after preincubation of the enzyme at different temperatures in the presence of various metal ions.

Effect of temperatures and borate on isomerization ratio

The isomerization reaction was carried out at different temperatures at pH 7.0. This reaction was also conducted at 70°C in the presence of different quantities of sodium tetraborate (0–0.5 M). All reaction products were quantified by HPAE-PAD.

Table 1 Induction of synthesis of D-xylose (XI) and glucose (GI) isomerase from *B. thermoantarcticus*

Carbon source (0.5% w/v)	Specific activity (U/mg)	
	XI	GI
Xylose	0.4	0.18
Xylan	0.136	0.009
Arabinose	0.0085	0
Galactose	0.048	0
Fructose	0.041	0
Yeast	0.018	0.007
Maltose	0.007	0.003
Glucose	0	0.005

Values are the means of those from three independent assays.

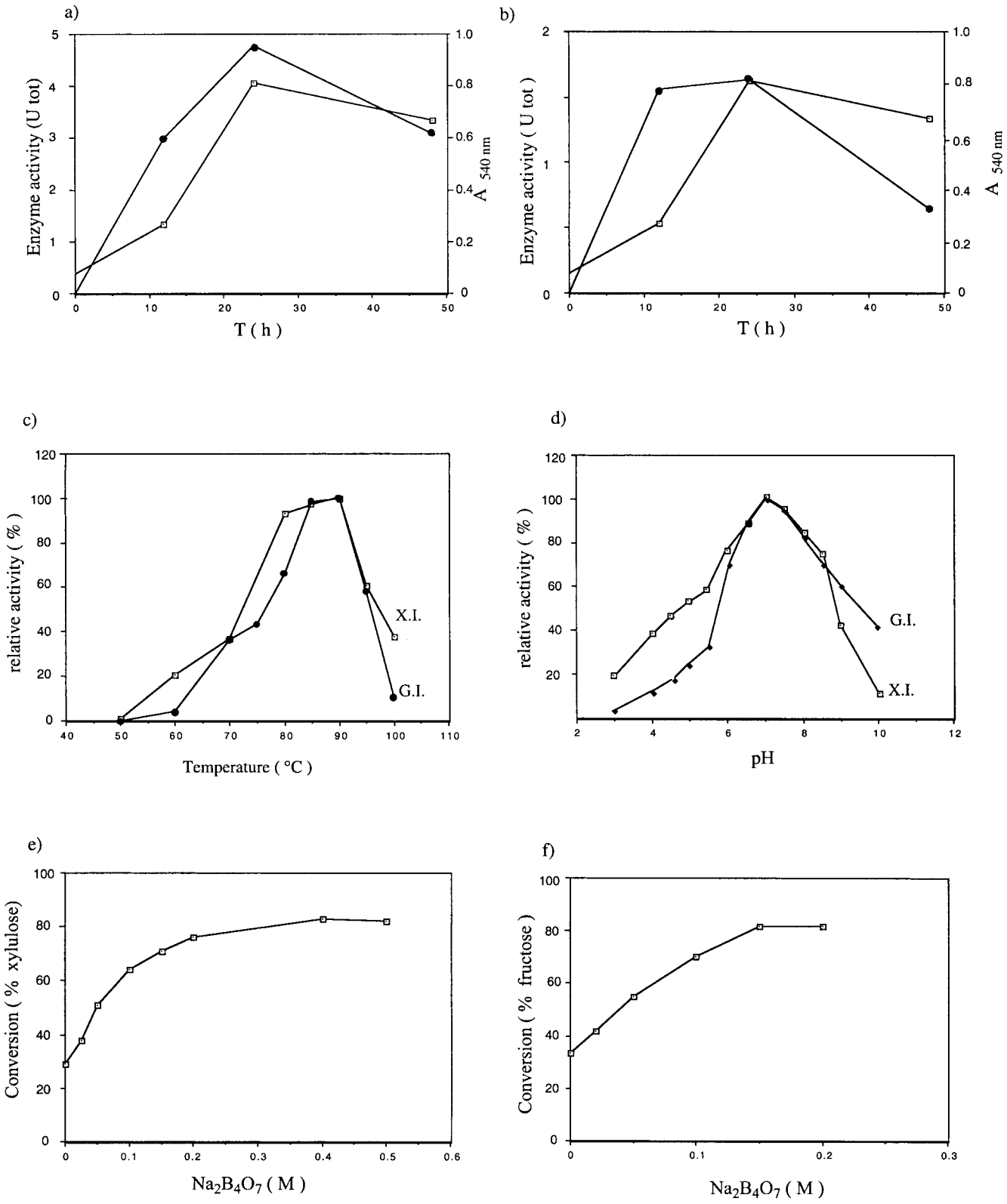

Figure 1 (a) Production of D-xylose isomerase from *B. thermoantarcticus*. Cell density of culture (open symbols); enzyme activity (closed symbols). (b) Glucose isomerase from *B. thermoantarcticus*. Cell density of culture (open symbols); enzyme activity (closed symbols). (c) Effect of temperature on D-xylose (XI) and D-glucose (GI) isomerase activity. (d) Effect of pH on purified D-xylose (XI) and D-glucose (GI) isomerase activity at 80°C. Enzyme activities were assayed under standard conditions in 50 mM glycine buffer, pH 3.0, 4.5, 7.5, 8.5, 9.0, 10.0; 50 mM sodium acetate buffer, pH 4.0, 5.0, 5.5; 50 mM phosphate buffer, pH 6.0, 6.5; Tris–HCl buffer, pH 7.0, 7.5, 8.0, 8.5. (e,f) Effect of borate on equilibrium displacement of isomerization. Isomerization was carried out at 70°C with 1 M D-xylose (e) or D-glucose (f) and sodium tetraborate (0–0.5 M).

Table 2 Purification of D-xylose/glucose isomerase from *B. thermoantarcticus*

Purification step	Total protein (mg)		Total activity (U)		Specific activity (U/mg)		Yield (%)		Purification (fold)	
	GI		XI	GI	XI	GI	XI	GI	XI	GI
Crude extract	135		49.6	12	0.37	0.09	100	100	1	1
Heat treatment (80°C×15 min)	49.5		51.3	12.7	1.0	0.26	103	106	2.7	2.9
Q-Sepharose FF	12.8		47.2	11.7	3.7	0.91	95	97.5	10	10.1
Sephacryl S-200	8.5		47	11.6	5.5	1.36	94.8	96.7	14.9	15.1
Phenyl Sepharose CL-4B	2.3		29.5	7.4	12.8	3.22	59.5	61.7	34.6	35.8
Mono Q/FPLC	0.4		10.8	2.8	27	7	21.8	23.3	73	78

Results and discussion

Strain and induction of the D-xylose isomerase activity

The antartic thermophilic microorganism *B. thermoantarcticus* classified as a novel species of *Bacillus* [33] on the basis on the phenotypic, taxonomic and genetic studies possesses an endocellular D-xylose(glucose) isomerase that can be induced by growth under certain conditions.

In many organisms, D-xylose isomerase was induced by its substrate D-xylose [24,30,41]. Induction of the enzyme by a range of compounds was tested in *B. thermoantarcticus*. The relative activity obtained is shown in Table 1. D-xylose was the most potent inducer of both D-xylose isomerase and D-glucose isomerase.

The cost of enzyme production is an important factor in the evaluation of its suitability for industrial application. Intensive efforts have been made to optimise the fermentation parameters for the production of xylose isomerase with a view to developing an economically feasible technology; one aspect of this is the optimization of the fermentation medium. Xylose is very expensive and hence impractical for use on a commercial scale.

B. thermoantarcticus grew on xylan, a cheaper medium whose presence in the growth medium enhanced the expression of xylose isomerase about eightfold with respect to standard growth medium. The organism possesses extracellular xylanase and β-xylosidase [21] that are able to degrade xylan in xylose. Maximum activity of the xylose isomerase was expressed in a late exponential growth phase (Figure 1a and b).

Purification of D-xylose/glucose isomerase

The enzyme activity was stable at room temperature for several hours; therefore, all steps of purification were performed at that temperature. Also, the enzyme activity was stable to freezing and thawing with no loss of enzyme activity.

The effects of various techniques on the specific activity, fold purification and yield are shown in Table 2. The last purification step was significant for both activities; in fact, purification was about twofold higher than after the previous step. D-glucose isomerase activity co-purified exactly with D-xylose isomerase activity. The purified enzyme was homogeneous by the detection of a single protein band on SDS-PAGE and native PAGE. An approximate molecular mass of 200 kDa was estimated from the gel filtration step. SDS-PAGE analysis showed a single band with a molecular mass of about 47 kDa (Figure 2, lane 2), suggesting that the enzyme was a homomeric tetramer, which is the most common oligomer form for xylose isomerases [22].

Isoelectric focusing of the purified D-xylose isomerase gave a single protein band corresponding to an isoelectric point of 4.8.

Physicochemical properties

The enzymic activity of D-xylose isomerase was measured at various temperatures utilizing D-xylose and D-glucose as substrates. For both substrates, the optimum temperature was 90°C (Figure 1c) and Arrhenius plots yielded straight lines up to a temperature of 85°C and declined thereafter.

The optimum pH with both substrates was around 7.0 and showed 80% of its maximum activity at pH 6.0 (Figure 1d). Stability of the xylose isomerase as a function of pH was tested by preincubation of the enzyme at 25°C at different pH values for 2 h. After incubation, the activities of xylose isomerase and glucose isomerase were measured at pH 7.0 — both activities were stable in the broad pH range 5.5–10.0 — and readily denatured at pH values lower than 5.0.

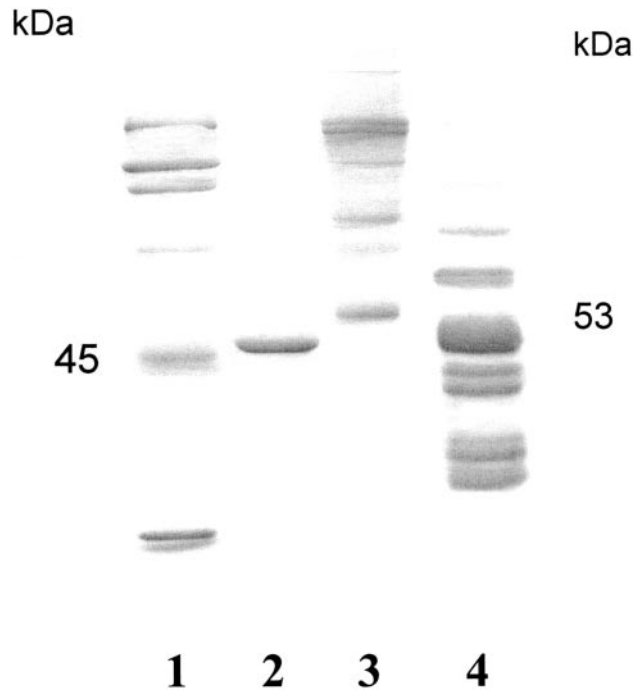

Figure 2 SDS-PAGE of purified D-xylose (glucose) isomerase from *B. themoantarcticus*. Lanes 1 and 3 contain M_r marker proteins: myosin (212,000), α_2-macroglobulin (170,000), β-galactosidase (116,000), phosphorylase b (97,000), transferrin (76,000), serum albumin (66,200), glutamic dehydrogenase (53,000), ovoalbumin (45,000), carbonic anhydrase (31,000). Lane 2 contains purified D-xylose (glucose) isomerase (5 μg). Lane 4 contains crude extract.

Table 3 The effect of metal ions on thermal stability of EDTA-treated xylose (glucose) isomerase from *B. thermoantarcticus*

Preincubation of enzyme	Thermal stability (% residual) with											
	No metal added		Co^{2+} (1 mM)		Mg^{2+} (5 mM)		Mg^{2+} (5 mM) Co^{2+} (1 mM)		Mn^{2+} (5 mM)		Mn^{2+} (5 mM) Co^{2+} (1 mM)	
	XI	GI	XI	GI	XI	GI	XI	GI	XI	GI	XI	GI
70°C×24 h	38	15	100	141	102	111	104	130	103	0	104	28
80°C×1 h	35	15	88	84	98	124	104	106	100	0	101	53
90°C×30 min	0	0	50	44	13	20	74	102	70	0	80	39

D-xylose(glucose) isomerase from *B. thermoantarcticus* was remarkably stable and resistant to high temperatures. Therefore, this enzyme, stable at acidic pH values, may increase the efficiency of the process and may reduce the possibilities of by-product formation [16].

In addition, the combination of saccharification and isomerization is an ideal development in the progress of HFCS production, and it is likely to be put into operation once an acid-stable enzyme was discovered.

Because of the high thermostability and the slightly acidic pH optimum for the enzyme, xylose isomerase from *B. thermoantarcticus* could be a better candidate for the production of high-fructose syrup and ethanol [23].

The effect of various metals on thermal stability of EDTA-treated xylose/glucose isomerase from *B. thermoantarcticus* is shown in Table 3. The xylose isomerase requires Mg^{2+}, Mn^{2+} or Co^{2+} for high thermal stability; glucose isomerase needed Mg^{2+} or Co^{2+}, while Mn^{2+} had no effect. The combination of Mn^{2+} and Co^{2+} showed an inhibitory effect for glucose isomerase.

Catalytic properties

The K_m and V_{max} values were obtained from Lineweaver–Burk plots of specific activities at various substrate concentrations. For xylose isomerase, K_m and V_{max} were 33 mM and 57 U/mg, respectively, while for glucose isomerase, they were 167 mM and 6.3 U/mg. The enzyme preference for xylose as substrate over glucose was illustrated by a lower K_m and higher V_{max} toward this substrate. This reflected the presumed physiological function of the enzyme, which acted in the organism to produce xylulose, which was subsequently metabolized *via* either the pentose phosphate pathway or phosphoketolase pathway.

Xylose isomerases typically require the presence of divalent metal cations such as Mn^{2+}, Mg^{2+} or Co^{2+} as essential cofactors for their catalytic activity [1,6,25]. The effect of various metal ions on the activity of EDTA-treated enzymes from *B. thermoantarcticus* is illustrated in Table 4. Treatment of purified enzyme with EDTA resulted in an almost complete loss of enzyme activity. However, the activity could be restored by the addition of metal ions. In particular, increasing amounts of Mn^{2+}, Mg^{2+} or Co^{2+} (each up to 10 mM) were able to restore only 60–80% of the original xylose isomerase activity. For glucose isomerase, 10 mM Mg^{2+} was required to restore 80% of the original activity, whereas Mn^{2+} did not enhance enzyme activity. As is common with other isomerases, 10 mM Mn^{2+}, in combination with 1 mM Co^{2+}, had the ability to restore full xylose isomerase activity, while 10 mM Mg^{2+} plus 1 mM Co^{2+} restored total glucose isomerase activity.

The results of equilibrium conversion of D-xylose and D-glucose at different temperatures are shown in Table 5. Temperature had a profound influence on the equilibrium concentration of products.

Xylose isomerase from *B. thermoantarcticus* isomerized xylose to xylulose up to an 80:20 equilibrium ratio, while glucose to fructose equilibrated up to 50:50, as also reported by Chen [9].

It is well known that equilibrium of the enzyme-catalyzed isomerization between aldose and ketose was shifted to the ketose side when borate was added to the reaction mixture [32].

Isomerization of D-xylose and D-glucose with various amounts of sodium tetraborate was shown in Figure 1e and f. The shift in equilibrium in both cases was almost linearly

Table 4 The effect of various metal cations on the activity of EDTA-treated xylose (XI)/glucose (GI) isomerase purified from *B. thermoantarcticus*

Metal	Isomerase activity (% of maximum)	
	XI	GI
None	8	2
Mg^{2+} (1 mM)	40	45
Mg^{2+} (5 mM)	70	65
Mg^{2+} (10 mM)	80	82
Mn^{2+} (1 mM)	55	0
Mn^{2+} (5 mM)	59	0
Mn^{2+} (10 mM)	62	0
Co^{2+} (1 mM)	35	0
Co^{2+} (5 mM)	48	0
Co^{2+} (10 mM)	60	0
Mg^{2+} (10 mM) Co^{2+} (1 mM)	90	105
Mn^{2+} (10 mM) Co^{2+} (1 mM)	110	0

Table 5 Effect of temperature on equilibrium of isomerization of D-xylose isomerase and D-glucose isomerase

Temperature (°C)	Conversion (%)	
	X1	GI
60	17	15
70	21	33
80	27	48

Values are the means of three independent assays.

Table 6 Physico-chemical properties of xylose/glucose isomerases from thermophilic microorganisms

Microorganisms	Molecular weight (kDa)	Optimum temperature (°C)	Optimum pH	Thermostability
B. thermoantarcticus	200	90	7.0	90°C×30 min
B. stearothermophilus	130	80	7.5–8.0	75°C×10 min
Thermot. maritima	200	105–110	6.5–7.5	100°C×10 min
Thermot. neapolitana	200	95	7.1	half-life 95°C×24 min
Thermus thermophilus	200	95	7.0	85°C×8 h
Thermus aquaticus HB8	196	85	7.5	70°C×1 month
Thermoanaerobacterium saccharolyticum B6A-R1	200	80	7.0–7.5	85°C×1 h
Thermoanaerobacterium JW/SL-YS 489	200	80	6.8	80°C×1 h
Clostridium thermosulfurogenes	200	80	7.0–7.5	85°C×1 h
Streptomyces sp. (PLC)	183	80	7.0	53°C×10 days
Streptomyces sp. SK	185	95	6.0	80°C×5 h

affected by the quantity of tetraborate; the introduction of additional tetraborate did not increase the equilibrium concentration of products when more than 80% of substrates had been converted. This saturation phenomenon was also observed by Takasaki [38].

Concluding remarks

Xylose isomerase produced from *B. thermoantarcticus* was purified 73-fold and was a homotetramer with an isoelectric point at 4.8. This enzyme was able to recognise both xylose (K_m 33 mM) and glucose (K_m 167 mM) as substrates. In both reactions, the enzyme required divalent cations for thermal stability and catalytic activity, although their requirements were different.

Several thermophilic xylose isomerases were also described [6,11,25,37]. The molecular weight of about 200,000 for the native D-xylose isomerase from *B. thermoantarcticus* and of 47,000 for its subunits suggests that the enzyme was similar to those enzymes formed by *Thermotoga maritima*, *Thermot. neapolitana*, *Thermus aquaticus* and *Thermus thermophilus* [6,11,13,25], while the D-xylose isomerase from *B. stearothermophilus* was described as a monomer with a M_r of 127,000 [37] (Table 6).

B. thermoantarcticus enzyme showed a highly thermostability and an optimum temperature of 90°C, similar to the isomerase from *Thermot. maritima* that has an optimum temperature at 100°C (Table 6) [6].

The optimum pH was 7.5–8.0 for the *B. stearothermophilus*, 6.5–7.5 for the *Thermot. maritima*, 6.8 for the *Thermoanaerobacterium*, 6.0 for *Streptomyces* and 7.0 for the *B. thermoantarcticus* (Table 6).

Therefore, kinetic characteristics for xylose or glucose isomerization were similar to xylose isomerases from distantly related bacteria [14,27–29,36] such as the once from *Thermotoga* and *Thermus* (Table 6) [6,13,25,40].

The high stability of the xylose isomerase from *Bacillus* is further illustrated by the fact that the enzyme could be immobilized by different techniques without severe losses of enzyme activity (data not reported) [35].

In fact, preliminary studies on immobilized cells of *B. thermoantarcticus* in sodium alginate, using xylose as substrate, showed the capability to produce xylulose, reaching the same conversion of the free enzyme within 24 h.

Further studies, such as large-scale process, other immobilization techniques and cloning of genes in homologous hosts, are necessary to assess biotechnological use of this enzyme.

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