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Purification and Characterization of a Novel Thermostable Xylose Isomerase from *Opuntia vulgaris* Mill

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Abstract Thermophilic xylose isomerase from the xerophytic eukaryote *Opuntia vulgaris* can serve as a good alternate source of enzyme for use in the production of high fructose corn syrup. The existence of two temperature stable isoforms having optimal activity at temperatures 70 °C (T_{70}) and 90 °C (T_{90}), respectively, is reported here. These isoforms were purified to homogeneity using column chromatography and SDS-polyacrylamide gel electrophoretic techniques. Only the T_{90} isoform was subjected to full biochemical characterization thereafter. The purified T_{90} isoform was capable of converting glucose to fructose with high efficiency under the assay conditions. The enzyme at pH 7.5 exhibited a preference to yield the forward isomerization reaction. The melting temperature of the native enzyme was determined to be 90 °C employing differential scanning colorimetery. Thermostability of the enzyme protein was established through temperature-related denaturation kinetic studies. It is suggested that the thermostability and the wide pH activity of this eukaryotic enzyme will make it an advantageous and dependable alternate source of catalytic activity for protected use in the high fructose corn syrup sweetener industry.

Keywords Xylose isomerase · Isoenzyme · O*puntia vulgaris* · Thermophilic · Purification

Introduction

D-xylose isomerase (XI) (D-xylose ketol-isomerase; EC 5.3.1.5) is an enzyme actively that reversibly catalyzed the isomerization between D-xylose and D-xylulose. This enzyme can also convert D-glucose to D-fructose and is referred to as glucose isomerase [1]. XI is

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widely used catalytic activity in the industrial production of high fructose corn syrup (HFCS) and ethanol from hemicelluloses [2–4]. HFCS is a sweetener used as an alternative for sucrose or invert sugar in the food and beverage industry [5]. The availability of novel thermostable XIs having neutral or slightly acidic pH optima has therefore great potential for industrial applications [6]. The ability to perform the isomerization process at a higher temperature and at neutral to acidic pH allowed for faster reaction rates [7, 8].

Bacterial thermophiles remained associated with natural hot springs and these XIs remain widely used in the industrial bioprocesses. While these bacteria (*Streptomyces* spp., and *Bacillus* spp.) were a good source of XI, few yeasts such as *Candida utilis* and *Candida boidinii*, and the fungus *Aspergillus oryzae*, also were shown to possess the XI activity [9, 10]. It is believed that environmental changes contributed to the evolution of thermostable enzymes in nature. The existence of earlier thermophilic enzyme in certain xerophytic plants was reported from the laboratory. The presence of high temperature stable xylose isomerase isoenzymes in *Cereus pterogonus* plant was also identified [11]. This article describes the purification and characterization of the thermostable xylose isomerase from the xerophytic plant *Opuntia vulgaris*. The observed physiochemical property of the enzyme makes it a potentially advantageous catalytic converter for use in the food and beverage industry.

Materials and Methods

Chemicals

Ammonium sulphate, phenylmethylsulphonyl fluoride (PMSF), Dowex-1, and Sephadex G-100 were obtained from Sigma Chemical, Co., St. Louis, MO, USA. SDS-PAGE chemicals and molecular weight markers were purchased from Bangalore Genei, India. All other chemicals used were of analytical grade obtained from Himedia laboratories, Mumbai, India.

Enzyme Extraction

The xerophytic plant *O. vulgaris* cladodes were weighed and chopped into small pieces after dethroning and removal of the hard cuticle layer using a sharp-edged razor blade and was homogenized in 50 mM Tris–HCl buffer, pH 7.5.0 containing 1 mM PMSF to prepare a 20% *w*/*v* tissue homogenate. The homogenate was filtered through cheese cloth to remove the debris, and the filtrate was centrifuged at 10,000×*g* for 20 min in a sorvall RC-5C refrigerated centrifuge to obtain a clear supernatant that was used as the enzyme source.

Enzyme Assay

Xylose isomerase enzyme assay was carried out in a 1-ml reaction volume containing 70 mM xylose, 10 mM $MnCl_2$, and 1 mM $CoCl_2$ and a fixed volume of the enzyme source in 50 mM Tris–HCl buffer (pH 7.5). For assay of glucose isomerase, the reaction mixture contained 0.8 M glucose, in the buffer instead of xylose. Samples were initially incubated for 30 min at 60 °C and were followed with the addition of 1 ml of 0.5 M $HClO_4$ to stop the reaction. The xylose-containing reaction mixture was then diluted 50-fold and the glucose-containing reaction mixture 10-fold, respectively, with double distilled water. The amount of xylose or fructose formed in the reaction was measured by the cysteine–carbazole–sulfuric acid method [12]. One unit (U) of isomerase activity was defined as the amount of

enzyme required to produce 1 μmol of product per minute under the assay conditions. Protein content of each sample was determined by the method of Bradford employing bovine serum albumin as a standard [13].

OVXI Purification

All operations were carried out at room temperature. *O. vulgaris* cladode homogenate (20% *w*/*v*) was prepared in 0.05 M Tris–HCl buffer pH 7.0 containing 0.1 mM PMSF. The extract was filtered through a cheese cloth and centrifuged at 10,000×*g* for 15 min in a refrigerated Sorvall RC5C centrifuge employing a GSA rotor. The supernatant was collected for used as the enzyme source. The supernatant obtained was then treated with solid ammonium sulphate to 80% saturation. Following mixing carried out for 16 h, the protein precipitate was collected by centrifugation at 10,000×*g*, and dissolved in 50 mM Tris–HCl buffer pH 7.5 containing 0.1 mM PMSF, 10 mM $MnCl_2$, and 1 mM $CoCl_2$, and dialyzed overnight against the same buffer with three changes of the dialysate at regular intervals. An aliquot of the dialyzed enzyme preparation was applied to a column (1.2×30 cm) of Dowex-1 that had been pre-equilibrated with the Tris–HCl buffer. The column was washed with the buffer (until OD<0.005), and the adsorbed proteins were eluted with a linear NaCl gradient (0–1 M) in the equilibration buffer. Fractions of 3 ml were collected and assayed for the isomerase activity and protein content. Active fractions were pooled and were then taken for gel filtration on a column (0.6×10 cm) of Sephadex G-100 pre-equilibrated with the Tris–HCl buffer at a flow rate of 40 ml/h. The column was calibrated using blue dextran and a set of protein molecular weight standards.

Enzyme Characterization

Molecular Weight Determination

Native *O. vulgaris* XI molecular weight was determined by size exclusion chromatography on Sephadex G-100 column and the subunit molecular weight by SDS-PAGE employing 10% acrylamide gels using a Mini-PROTEAN 3 electrophoresis cell (Bio-Rad), followed

Table 1 Summary of purification of *OVXI*

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Cladode homogenate	88	596.81	6.78	100	1.0
Ammonium sulfate	48	507.89	10.58	85	1.56
Dowex-1 anion exchange Chromatography					
T_{70}	1.2	106.4	88.67	18	13.07
T_{90}	1.65	143.87	87.19	24	12.85
Sephadex G 100 Chromatography					
T_{70}	0.63	83.92	133.21	14	19.64
T_{90}	0.45	119.89	266.43	20	39.2

Enzyme activity was assayed with xylose as a substrate. Protein concentrations were determined by the Bradford assay, using BSA as a standard. One unit (U) of isomerase activity was defined as the amount of enzyme required to produce 1 μmol of product per minute under assay condition

by quantization of the SDS-PAGE bands using a Syngene software. An amount of 10 μg of purified XI was applied per lane and the separated protein bands were stained and visualized using a solution of 0.25% Coomassie Brilliant Blue R-250 in 10% methanol/7% acetic acid followed by destaining the gels in the same solvent [14].

Optimum pH and Temperature

The pH optimum of *O. vulgaris* XI activity (T_{90} isoform) was determined using the assay protocol employing the appropriate buffer. For the pH range 4.0–6.0, 100 mM sodium acetate buffer, for pH range 6.0–8.0, 100 mM Tris–HCl buffer and for pH range 8.0–10.0, 100 mM glycine–NaOH buffer were used. Enzyme stability was assayed using the same buffers with addition of the divalent ions namely 10 mM $MnCl_2$ and 1 mM $CoCl_2$.

The temperature optima of the OVXI enzyme (T_{90} isoform) was determined by using the enzyme sample in 1 ml of a reaction volume containing 70 mM xylose, 10 mM $MnCl_2$, 1 mM $CoCl_2$, and 50 mM Tris–HCl buffer (pH 7.5) and incubating the reaction tubes at different temperatures for 30 min each. The reaction tubes were assayed as discussed earlier.

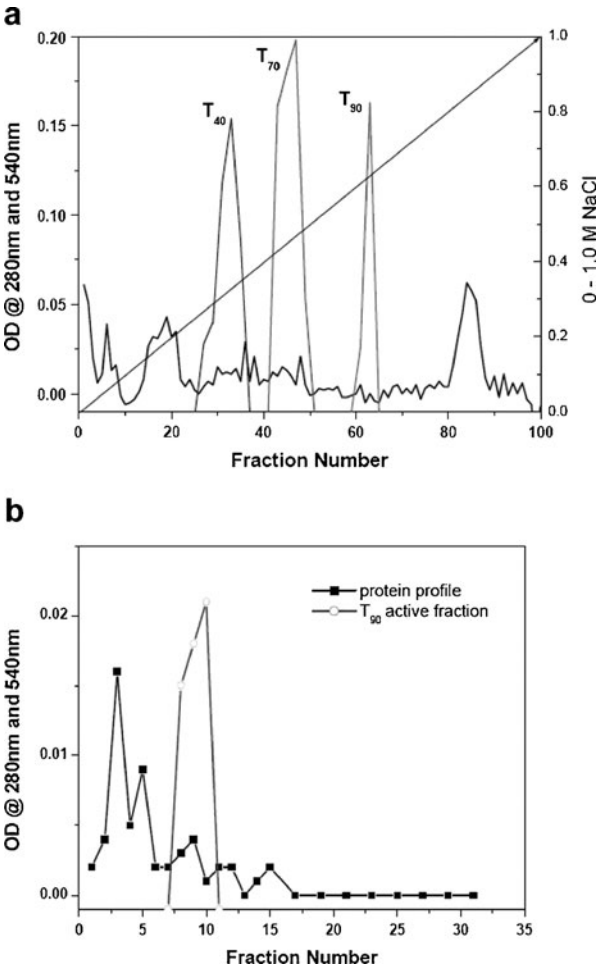

Fig. 1 Elution profile of *OVXI* on Dowex-1 ion exchange and Sephadex G-100 chromatography. Absorbance of fractions was estimated at 280 nm and XI assay was carried out using *Opuntia vulgaris* supernatant (10%, *w*/*v*). The bound enzymes were eluted with a linear gradient of NaCl in ion exchange chromatography

Effect of Cations

Endogenous metal ions in the enzyme sample was removed by dialyzing purified T_{90} isoform of OVXI at 4 °C for 8 h against 50 mM Tris–HCl (pH 7.5) containing 5 mM EDTA, followed by dialysis of the enzyme sample in the same buffer without EDTA. The enzyme activity was then determined in the presence of various concentrations of $CoCl_2$, $MnCl_2$, and $MgCl_2$ as per the assay protocol indicated earlier.

Kinetic Constants

The kinetic constants for the purified T_{90} isoform of OVXI were determined using a Lineweaver–Burk plot. The kinetic parameters K_m and V_{max} were calculated by measuring the initial rate of isomerization of xylose and glucose under varying substrate concentrations. Enzyme samples were incubated at 90 °C for 10 min with the respective substrates in 50 mM Tris–HCl, pH 7.5, containing 1 mM $CoCl_2$.

Thermostability Measurement

Enzyme samples (T_{90} isoform) were dialyzed overnight against 500 volumes of 50 mM Tris–HCl buffer pH 7.5 for establishing the baseline. The melting temperature (T_m) of the enzyme species was determined from the scan and was then scanned in the temperature range 30 °C to 105 °C with a Metler Toledo Differential Scanning Calorimeter (DSC) using a scan rate of 1 °C/min.

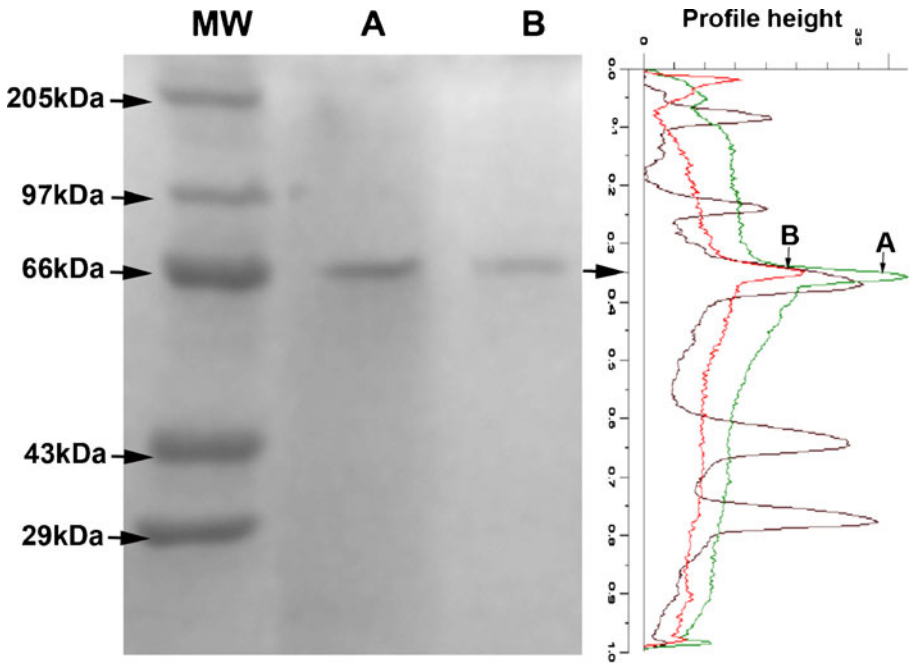

Fig. 2 SDS-PAGE of purified *OVXI* (*MW*—protein molecular weight standard; *lane A* Purified T_{70} isoform from OVXI; *lane B* Purified T_{90} isoform from OVXI)

The purified sample was taken for time dependant denaturation studies in presence and absence of metal ions (10 mM $MnCl_2$ and 1 mM $CoCl_2$). Aliquots of the purified enzyme sample were incubated at 90 °C for a period of 0–30 min and were assayed at intervals of 5 min.

Results and Discussion

Enzyme Purification

The xylose isomerase enzyme activity was stable at room temperature for several hours. Therefore, all steps of purification were performed at room temperature. The existence of temperature stable isoforms of xylose isomerase in the eukaryote *O. vulgaris* were identified

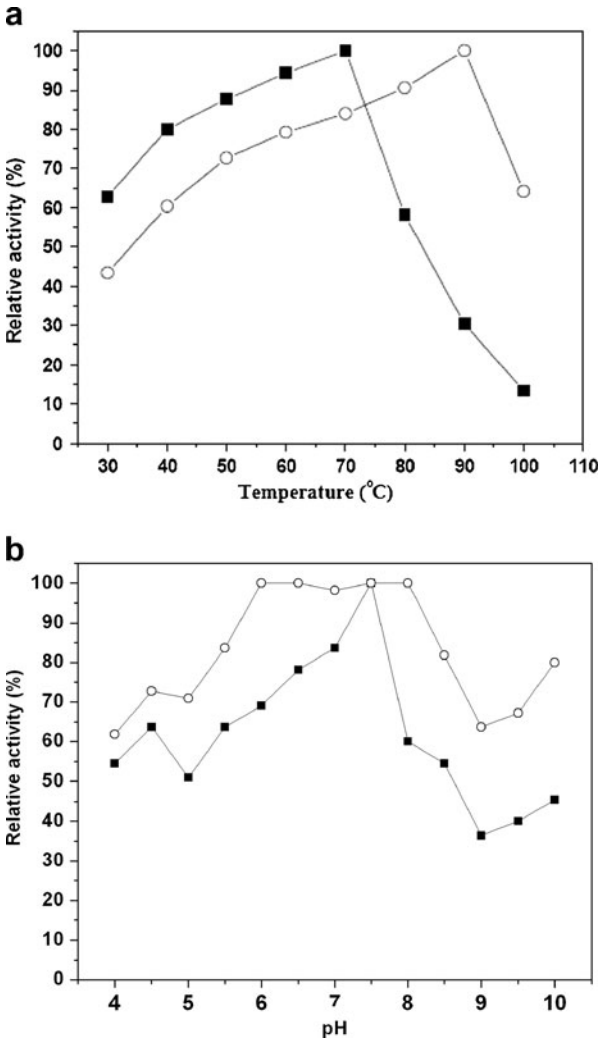

Fig. 3 **a** Temperature and **b** pH profile for purified *OVXI* isoenzyme; *filled square* indicates T_{70} isoenzyme and *circle* indicates T_{90} isoenzyme. Enzyme activities were determined as described in the "Materials and Methods" section. Values are the means of those from three independent assays

and separated by Dowex-1 anion exchange chromatography. The specific activity, fold purification, and yield of the enzyme activity are as shown in Table 1. As a result of Dowex-1 column chromatographic separation, three major peaks of XI activity were obtained having different temperature optimum and they were identified as T_{40}, T_{70}, and T_{90} enzyme isoforms. Fractions with activity for the T_{70} and T_{90} XI isoenzymes were independently pooled, dialyzed, and concentrated for further characterization. The overall recovery of T_{90} isoenzyme activity was 20%. Sephadex G-100 column chromatography of the T_{70} and T_{90} isoforms further purified these proteins to apparent homogeneity (Fig. 1). SDS-PAGE was used to visualize these isoenzymes as single polypeptide bands, following gel staining (Fig. 2). The purified xylose isomerase isoenzymes had a combined yield of 34%.

The existence of three isoenzyme forms made the purification process difficult for their isolation. This problem was settled using limiting ranges of linear salt gradient, while also limiting the fraction volume and controlling the flow rate, concentrating on the elution of the thermostable isoenzyme (T_{90}) during the purification process. It thus became important to select conditions under which the enzyme activity was extractable. We confined to the use of ammonium sulfate saturation of cladode homogenate 10,000×*g* supernatant to an optimum saturation of 80% (*w*/*v*). An estimated molecular mass of 66 kDa for the enzyme protein was determined by the gel filtration study. However, as shown in Fig. 2, SDS-PAGE yielded a single band with a molecular mass of 71 kDa for the T_{70} and 68 kDa for the T_{90} isoforms, suggestive of the monomeric nature of the enzyme protein.

Optimum Temperature and pH

The activity of D-xylose isomerase enzyme was measured at various temperatures. The temperature profile determination for each isoform was carried out independently. Isoform I (T_{70}) and II (T_{90}) showed temperature optimum at 70 °C and 90 °C, respectively (Fig. 3a). When the effect of pH on enzyme activity was measured in the wide pH range (4–10), the enzyme was found to be active in acidic as well as in basic pH. Although the pH optimum of the enzyme was found to be 7.5, the enzyme retained 60% of its activity even at pH 4.5. *OVXI* exhibited a broad range (5.5–8.5) of pH stability but denatured readily at pH values below 4 (Fig. 3b).

Table 2 The effect of metal ions on the activity of purified OVXI T_{90} isoform

Metal	Isomerase activity (% of maximum)
None	32.3
Mn^{2+} (1 mM)	54.8
Mn^{2+} (10 mM)	86.3
Mg^{2+} (1 mM)	49
Mg^{2+} (10 mM)	63.9
Co^{2+} (1 mM)	82.4
Co^{2+} (10 mM)	78
Mn^{2+} (10 mM)+Co^{2+} (1 mM)	100
Mg^{2+} (10 mM)+Co^{2+} (1 mM)	68

Enzyme was treated with EDTA, and the activities were determined as described in the "Materials and Methods" section. These data corresponds to an experiment representing a total of three independent experiments closely coincident

Table 3 Comparison of kinetic parameters for several xylose isomerase

Organism	K_m (mM)		V_{max} (μmol/min per mg)		
	Xylose	Glucose	Xylose	Glucose	Reference
G. stearothermophillus (60 °C)	100	220	44.5	6.0	[21]
T. aquaticus (70 °C)	15	93	15.9	9.1	[22]
S. olivochromgenes (60 °C)	33	250	21.8	5.3	[21]
O. vulgaris (90 °C)	46.4	259.6	9.88	9.1	This work

Enzyme activity was assayed with xylose and glucose as a substrate

Effect of Cations

The effect of cations on EDTA-treated XI from *O. vulgaris* is illustrated in Table 2. Mn^{2+} exhibited the strongest stimulatory effect, followed by Co^{2+}, while Mg^{2+} had no appreciable effect. This enzyme typically required the presence of Mn^{2+}, Co^{2+}, or Mg^{2+} for its activity [15, 16].

Kinetic Constants

The K_m and V_{max} values were obtained (Table 3) from the Lineweaver–Burk double reciprocal plot of specific activity versus substrate concentration. The kinetic features of the purified xylose isomerase employing two different substrates (xylose and glucose) were determined and compared. The enzyme displayed a lower K_m for xylose than for glucose, indicative of greater affinity for xylose. This reflected the presumed physiological function of the enzyme to produce xylulose, which subsequently was utilized in the pentose phosphate or phosphoketolase pathway [17, 18].

Thermostability Measurement

The thermostability of the purified *OVXI* enzyme activity in the absence of metal ions is illustrated by the DSC scan (Fig. 4). The melting temperature (T_m) for purified T_{90} isoform of

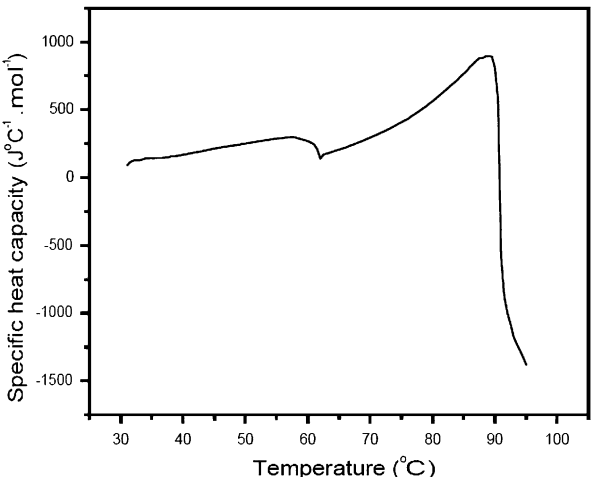

Fig. 4 Thermal denaturation of T_{90} isoforms of *OVXI*. DSC scans of OVXI isoenzymes are run in 50 mM Tris–HCl buffer (pH 7.0) with no metal (apo). Data are baseline corrected

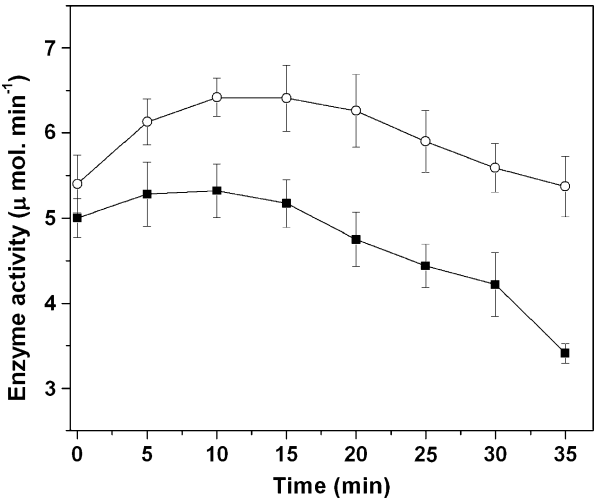

Fig. 5 Time-dependent denaturation study of T_{90} isoforms of *OVXI* enzyme activity; *filled square* indicates apo enzyme and *circle* indicates metal-treated enzyme (10 mM $MnCl_2$ and 1 mM $CoCl_2$). Values are the means of those from three independent assays

OVXI enzyme was found to be 90 °C. The enzyme denatured with rise in temperature through thermal unfolding, leading to destabilization of hydrogen bonds, hydrolysis of peptide bonds, deamidation of liable amino acid residues, hydrophobic interactions, and the salt bridges [19].

Time-dependent temperature study of purified enzyme in the presence and absence of metal ions for a period of 30 min indicated the temperature stability of both apo and metal-treated enzyme (Fig. 5). In the presence of metal T_{90} isoform of OVXI enzyme showed stable enzyme activity even after 30 min, whereas the apo enzyme activity decreased rapidly. This suggested the active involvement of the metal ion in the enhancement stabilization of the enzyme activity [20].

Comparison of Characteristics

The kinetic parameters calculated for the purified OVXI were compared with those available from literature. The enzyme preference for xylose as substrate over glucose is illustrated by a

Table 4 Comparison of specific properties of xylose isomerase from *Opuntia vulgaris* with other sources of the enzyme (based on literature data)

Xylose isomerase source	Operating temperature (°C)	Optimum pH	Metal ion requirement	Molecular weight	No. of subunits
E. coli [23]	45	6	NR	44,000	2
L. brevis [24]	40	7.1	Mn^{2+}, Co^{2+}	49,000	NR
S. violaceoniger [25]	>70	7.5	Mg^{2+}	43,000	4
B. coagulans [26]	75	7	Co^{2+}	49,000	3
A. missouriensis [27]	90	7.0-7.5	Mg^{2+}, Co^{2+}	40,000	2
Arthrobacter sp. [28]	-	5.5-9.5	Mn^{2+}	47,000	4
Thermus aquaticus [22]	70	5.5-8.5	Zn^{2+}	50,000	4
H. vulgare [29]	60	6	NR	50,000	2
O. vulgaris [This work]	90	7.5	Mn^{2+}, Co^{2+}	68,000	1

NR not reported

lower K_m and V_{max} toward this substrate. The V_{max} value toward glucose (the industrial substrate) compares favorably with the values for the enzymes from other sources listed.

Table 4 outlines a comparison of characteristics of the purified OVXI from our study with XI from mesophilic plant and thermophilic prokaryotes based on literature. The molecular weight and subunit nature of the purified XI were comparable to those from other sources and ranged from 40–130 kDa (by SDS-PAGE) comprising 1–4 subunits. The optimum pH of the purified XI from various sources ranged from pH 6–8. The temperature optimum of the purified XI in our study was comparable to that of *Actinoplanes missouriensis*, both exhibiting optimal activity at 90 °C. The optimum temperatures for XI from other sources were relatively lower than 80 °C. The purified XI required divalent cations Mn^{2+} or Mg^{2+} and Co^{2+} for its activity and stability.

Conclusion

In the current studies, an extremely thermophilic xylose isomerase from *O. vulgaris* plant species was purified to apparent homogeneity. The occurrences of temperature stable isoenzymes in plants were considered as a need for the adaptation of plants to desert type living conditions. The catalytic properties of the *O. vulgaris* isomerase generally resembled those of the thermostable prokaryotic xylose isomerase. However, the OVXI enzyme exhibited a wider pH activity range (pH 5.5–8.5) and was found temperature stable up to 90 °C. The catalytic efficiency of thermostable OVXI for the conversion of glucose to fructose at 90 °C was found to be remarkable for potential use in the food and beverage industry. The availability of thermophilic XI from this plant species within the Indian subcontinent makes *O. vulgaris* as a good source of thermophilic genes, for use in bioengineering strategies.

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