MATERIALS AND METHODS

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2.1 MATERIALS

2.1.1 Chemicals

Molecular weight markers Myosin (MW 200 kDa), Alcohol Dehydrogenase (MW 150 kDa), BSA (MW 66 kDa), Ovalbumin (MW 45 kDa), Carbonic Anhydrase (MW 29 kDa), Dowex-1 (chloride form), Sephadex G-100, Phenyl methyl sulphonyl fluoride, Ammonium Sulphate, Acrylamide, Bis-acrylamide, Sodium dodecyl sulphate, TEMED, Tris, β-mercapto ethanol, Triton X-100, Blue dextran, and Urea were obtained from Sigma Chemicals, St.Louis, Mo, USA. Freunds complete and incomplete adjuvants were purchased from Bangalore Genei, India. All other chemicals and biochemical's were of analytical grade purchased in India. Glass distilled water was used for the preparation of all reagents.

2.1.2 Equipments

The equipments used for the study included Systronics 119 UV-Visible single beam and Shimadzu UV - 1700 double beam UV-Visible Spectrophotometers, Sorvall RC -5C refrigerated centrifuge, Hettich EBA 12 Eppendorf microfuge, JEOL (JES-TE 100 with X-Band microwave) Electron Paramagnetic Resonance Spectrometer, Hitachi-F-4500 Fluorescent Spectrophotometer, Metler Toledo Differential Scanning Calorimeter, Imported GLF water bath, DIGIQUAL digital (±0.01°C) thermometer, Inductively Coupled Plasma - Atomic Emission Spectrophotometer (JOBIN YVON Ultima-2), Bio-Rad electrophoresis unit and Mini Trans Blot Electrophoretic Transfer cell with power packs, Eppendorf Master Cycler gradient PCR machine and Syngene Gel Documentation Unit.

2.2 METHODS

2.2.1 Biochemical Studies

2.2.1.1 Collection of cladode

Young to mid grown cladodes of *Cereus pterogonus* and *Opuntia vulgaris* were collected throughout the year and the xylose isomerase enzyme activity was assayed according to the method of Lee & Zeikus (1991).

2.2.1.2 Preparation of Cladode homogenate as enzyme source

Plant cladode tissue was weighed and chopped after dethroning and removal of hard cuticular layer into small pieces using a sharp edged razor and homogenized in 0.1M Tris-Hcl, pH 7.0, containing 1mM PMSF using a waning blendor (4 min), to prepare a (20% w/v) cladode homogenate. The homogenate was filtered through muslin cloth, to remove the cell debris. The filtrate was then centrifuged at 10,000 x g for 20 min in a refrigerated centrifuge RC-5C to obtain a clear supernatant that was used as the enzyme source.

2.2.1.3 Assay for xylose (glucose) isomerase enzyme activity

The xylose isomerase enzyme activity in the cladode homogenate 10,000 x g supernatant was assayed by the method of Lee & Zeikus (1991), as described below

Xylose (glucose) isomerase activity was assayed by measuring the amount of xylulose formed during the isomerization reaction employing xylose as substrate. Xylose isomerase activity was measured by incubating a 1 ml reaction mixture that contained 70mM xylose, 10mM $MnCl_2$, 1mM $CoCl_2$ and the enzyme (100 µl of an appropriate diluted purified or crude extract) preparation in 100mM Tris-HCl buffer, pH 7.0, The reaction mixture was incubated for 10 min at 60°C and the reaction was stopped by the addition of 0.5 M- $HClO_4$. The mixture was further diluted to 50 and 10-fold with double

distilled water and assayed for the amount of xylulose generated by the addition of cysteine – carbazole - sulphuric acid (Dische & Borenfreund, 1951). When the glucose isomerase enzyme activity was monitored, 0.8 M glucose instead of xylose was used as the substrate in the same reaction, and the amount of fructose formed was measured instead of xylulose. To the diluted reaction mixture, 0.2 ml of a 1.5% solution of cysteine hydrochloride was added. This was followed by the addition of 6 ml of a mixture of 70% concentrated sulfuric acid and 0.2ml of 0.12 % alcoholic solution of carbazole. The mixture was then shaken and left standing at room temperature. The intensity of the purple colour developed in the sample due to xylulose formation was measured at 540nm, employing a Shimadzu UV - 1700 double beam UV-Visible Spectrophotometer. Quantitation of fructose generated was however done at 560nm. One Unit (U) of isomerase activity is defined as the amount of enzyme needed to produce 1 μmol of either product formed per minute under the assay conditions.

2.2.1.4 Estimation of total protein content in the enzyme preparation.

The protein content of each enzyme preparation was estimated by Bradford's method (1976).

Protein sample, calibration standard, and buffer (each 0.2 ml) was added to 5 ml of the coomassie dye reagent, mixed and incubated for 10 min at room temperature. The protein reacts with coomassie dye (Brilliant blue G250) to give a blue coloured complex in acid pH. During the reaction the triphenylmethane group of the dye binds to non polar structures in proteins, and the anion sulfonate groups interact with protein cationic side chains of arginine and lysine. The intensity of the colour developed was a measure of the amount of protein present in a sample and was read at 595nm in the spectrophotometer.

2.2.1.5 Physico - Chemical Studies

2.2.1.5.1 Effect of temperature on enzyme activity

The xylose isomerase activity in the enzyme source was determined in the temperature range 30°C – 100°C at intervals of 10°C, and by incubating the reaction mixture for 10 min at each temperature before assaying the enzyme activity as described earlier (Lee & Zeikus, 1991).

2.2.1.5.2 Effect of pH on enzyme activity

The xylose isomerase activity in the enzyme source was measured by incubating a fixed volume (200µl) of the enzyme sample in the reaction mixture at selected pH values and was assayed as described earlier (Lee & Zeikus, 1991). Measurement of pH stability was carried out in the pH range 4.5 - 9.5, followed by incubating the enzyme sample at 37°C for 30 min employing different buffers (0.1M sodium acetate buffer, pH 4.0-6.0, 0.1M Tris-HCl buffer, pH 6.0 to 8.0 and 0.1M glycine - NaOH, pH 8.0-10.0).

2.2.1.5.3 Effect of metal ions on enzyme activity

Studies related to the effect of metal ions on the xylose isomerase enzyme activity in the enzyme source was carried out employing divalent cations in their chloride form using 0.1M Tris-HCl buffer, pH 7.0, and incubating a fixed amount of the enzyme sample each time for 10 min. The metal ions were used at 1, 5 and 10mM concentration in independent reactions. The activity of xylose isomerase determined in the absence of metal ion served as the control.

2.2.1.5.4 Analytical polyacrylamide gel electrophoresis of enzyme source.

Protein in the cladode homogenate 10,000 x g supernatant of *Cereus pterogonus* and *Opuntia vulgaris* were analyzed by electrophoresis on a 7.5% polyacrylamide gel according to the method of Davis (1964). The protein samples were mixed with 0.25

volumes glycerol (20% v/v) containing 0.1% bromophenol blue as tracking dye and applied directly to the top of each gel. The electrophoresis was carried out at room temperature for 5-8 hrs at 100 volts using 50mM Tris-glycine (pH 8.3) as electrophoresis buffer that contained 0.1% SDS.

Proteins were stained with 0.25% CBBR-250 in methanol: acetic acid: water (50:12:38 v/v/v) for 2-3 hrs at room temperature. Gels were then destained in methanol: acetic acid: water (50:12:38 v/v/v) and stored in 5% acetic acid.

2.2.1.5.5 Activity staining of xylose isomerase

For visualization of xylose isomerase activity in polyacrylamide gel, following electrophoresis the gels were incubated with 50mM xylose in the presence of 10mM $MnCl_2$ and 1mM $CoCl_2$ in 20mM Tris-HCl buffer pH 7.0 for 15 min at 50°C. The gels were washed with distilled water for 0.5 min at 4°C, and dipped in a solution of 0.1% 2, 3, 5 triphenyl tetrazolium that was freshly prepared in 1N NaOH. The reaction was stopped by incubating the gels in 2N HCl for 15 min at 20°C. Gels were finally washed with water. A clear zone around a protein band indicated the presence of the active enzyme.

2.2.1.6 Isolation, Purification and Characterization Studies

2.2.1.6.1 Ammonium sulphate precipitation of proteins in cladode homogenate

Cladode homogenate (20% w/v), 10,000 x g supernatant was taken for solid ammonium sulphate addition to a final concentration of 80%, at room temperature, with constant stirring and without any froth formation. The salt was allowed to react for 15-20 min using a magnetic stirrer. The ammonium sulphate treated supernatant was allowed to stand for an additional 2 hrs at room temperature without stirring. The ammonium sulphate precipitated proteins were collected by centrifugation of the sample at

10,000 x g for 45 min using the Sorvall RC-5C refrigerated centrifuge. The precipitate obtained was re-dissolved in 0.1M Tris-HCl pH 7.0 and taken for dialysis.

2.2.1.6.2 Dialysis

The resolubilised ammonium sulphate precipitate was dialyzed for 18 hrs against 0.1M Tris-HCl buffer, pH 7.0, at room temperature, with three changes of the dialysate. The dialyzed protein sample was then taken for ion exchange chromatography.

2.2.1.6.3 Ion exchange column chromatography

DOWEX-1 anion exchanger was taken in 0.05M HCl and the suspension was allowed to stand for 1 hr. Following this, the supernatant was discarded and 0.1 M Tris-HCl buffers (pH 7.0) was added to the resin and allowed to stand for an additional 30 min. The material was then packed into a column (28 cm x 0.83 cm) without air bubbles. The packed gel matrix was equilibrated with 0.1 M Tris-HCl pH 7.4 for 3-5 bed volumes.

2.2.1.6.4 Enzyme fractionation

Aliquots (6 ml) of the dialyzed protein sample obtained following ammonium sulphate precipitation and dialysis of the cladode homogenate 10,000 x g supernatant were applied to the equilibrated ion exchange resin column. The protein sample volume was adjusted to be 10 % of the column bed volume, and was loaded using a pasteur pipette without disturbing the bed surface. Ion exchange chromatography of the proteins was carried out at 27°C, fractions of 3 ml each were collected manually. At the end of sample application, the column was washed with the equilibrating buffer, until the absorbance at A_{280} of the wash exhibited < 0.05. The adsorbed proteins were then eluted using a linear gradient of NaCl (0-1.0 M) in the wash buffer. Protein content of each fraction was monitored at A_{280} employing a UV-Visible double beam spectrophotometer. Xylose isomerase activity in the fractions was determined using the assay described earlier.

2.2.1.6.5 Enzyme Kinetics

Reaction tubes contained increasing concentration of xylose (7–140mM) and glucose (0.02 - 1.1M) and a fixed amount of the xylose isomerase activity from the *Cereus pterogonus* and *Opuntia vulgaris* enzyme sources and the assays were carried out at 60°C as described earlier (Lee & Zeikus, 1991). Kinetics of T_{60}, T_{70}, T_{80} and T_{90} xylose isomerase isoforms were also carried out by the same method.

2.2.1.6.6 Determination of molecular weight by gel permeation column chromatography.

Sephadex G-100 was allowed to swell in 0.1 M Tris-HCl buffer, pH 7.0 for 72 hrs at room temperature. The slurry was then slowly packed without any air bubbles into a glass column (18 cm x 0.5 cm) filled one third with the elution buffer that was allowed to flow at a rate 0.5 ml/ min. The slurry was applied using a Pasteur pipette with gentle stirring of the gel-buffer interface in the column. After the desired bed volume was obtained, the column was equilibrated with several column volumes of the elution buffer. A sample of Blue dextran (5 mg/ml) was then applied to the column without disturbing the bed surface to determine the void volume (Vo) of the column. Fractions of 1 ml were collected and the absorbance in each fraction was measured at 650nm. Following the blue dextran run, the column was calibrated employing known standard proteins in separate column runs, to determine the elution volume (Ve) of each standard protein. The enzyme sample was finally applied to the column. The enzyme fractions were collected and the absorbance in each was measured at 280nm for the presence of proteins and at 540nm to detect the presence of enzyme activity following the specific assay. The molecular weight was determined from a standard plot of Ve vs Vo obtained from the column run of standard proteins and Blue dextran, and the Ve of the enzyme fractions.

2.2.1.6.7 Determination of molecular weight by SDS PAGE

SDS-PAGE of protein samples was carried out in 10% polyacrylamide gel by the method of Laemmli (1970). The marker proteins and enzyme samples were dissolved in sample buffer (0.0625 M Tris-glycine, pH 6.8) containing 2% SDS, 0.01% bromophenol blue and 10% glycerol. Marker proteins were denatured by keeping the protein solution in a boiling water bath for 5 min. Enzyme samples were mixed with sample buffer but not subjected to heat treatment. The electrophoresis was carried out at room temperature for 2 hrs at 100 volts using 50mM Tris-glycine (pH 8.3) as electrophoresis buffer containing 0.1% SDS.

Proteins were stained with 0.25% Coomassie brilliant blue R 250 in methanol: acetic acid: water (50:12:38 v/v/v) for 2-3 h at room temperature. Gels were destained in methanol: acetic acid: water (50:12:38 v/v/v) and stored in 5% acetic acid.

2.2.1.6.8 Activity staining of xylose isomerase

For visualization of xylose isomerase isoenzyme (T_{70}, T_{80} and T_{90}) activity in polyacrylamide gels after electrophoresis, the gels were incubated in 20mM Tris-HCl pH 7.0 containing 50mM xylose, 10mM $MnCl_2$ and 1mM $CoCl_2$ for 15 min at 50°C. The gels were washed with distilled water for 0.5 min at 4°C, and dipped in a solution of 0.1 % 2, 3, 5 triphenyl tetrazolium freshly prepared in 1N NaOH. The reaction was stopped by incubating the gels in 2N HCl for 15 min at 20°C. Finally the gels were washed with water. Results showing a clear zone around a protein band indicated the presence of active enzyme.

2.2.1.6.9 Effect of denaturants on purified enzyme activity

The influence of protein denaturants urea, sodium dodecyl sulphate and guanidine hydrochloride on the xylose isomerase enzyme activity was carried out at 80°C for the *Cereus pterogonus* enzyme and at 90°C for the *Opuntia vulgaris* enzyme. The

concentrations of the denaturants used were 0.5M - 4.0M Urea, 20mM - 100mM SDS and 1mM -10mM DTT. The enzyme activity was assayed as described earlier (Lee & Zeikus, 1991).

2.2.2 Biophysical Methods

2.2.2.1 Denaturation Kinetics

Denaturation kinetics of the xylose isomerase enzyme activity was carried out in the temperature range 30°C – 100°C using an imported GLF waterbath and a temperature sensitive probe capable of sensing ± 0.01°C to assess both the stability of the enzyme activity as well as the rate changes in the denaturation process due to the temperature effect. Aliquots of the T_{70}, T_{80} and T_{90} ion exchange fractions in Tris-HCl buffer were taken for incubation at different temperatures in a thermostated water bath and the protein samples were maintained in the water bath for a maximum of 30 min. The reaction temperature was monitored with a sensitive (± 0.01°C) DIGIQUAL digital thermometer with its probe placed into the reaction tube. A fixed volume (0.2ml) of the incubated enzyme protein was withdrawn from the reaction using a positive displacement pipette, at regular intervals of 5 min and was taken for the xylose isomerase enzyme assay employing a set of reaction tubes as described earlier. The temperature dependent denaturation process was repeated in independent denaturation experiments, using fixed concentration of different divalent cations (10mM Mn^{2+}, 1mM Co^{2+} and 10mM Mg^{2+}), followed by assay of the residual xylose isomerase activity in the reaction tube. Cation concentration was selected based on the higher activity of the enzyme in presence of the indicated cation level. The first order rate constant k, for thermoinactivation was obtained by linear regression in semilog coordinates. Enzyme half life was calculated from the equation: $t_{1/2}=\ln 2/k$.

2.2.2.2 Differential Scanning Calorimetry

Differential Scanning Calorimetry (DSC) is a technique in which the sample and the reference materials are subjected to a precisely programmed temperature change for measuring directly both the temperature and the enthalpy of transition or the heat of a reaction. It is often used for differential thermal analysis. Temperature expansion per 0.2°C/cm when combined with a high calorimetric sensitivity of 0.01mw/cm, allows to record thermal occurrences in a reaction system that produced even very small amounts of heat. For maximum accuracy, a base line thermogram is first obtained and is then subtracted from the sample thermogram to determine the heat capacity. Any energy difference detected between a sample and the reference at a given programmed temperature is recorded by the instrument. Thus thermal events within a sample appear as deviations from the DSC base line, indicating an endothermic or an exothermic reaction.

DSC experiments were performed on a Metler Toledo Differential Scanning Calorimeter. Samples were scanned from 30°C to 100°C for both CPXI (T_{80}) and OVXI (T_{90}) with a scan rate of 1°C/min. The apoenzymes (1 mg/ml) were scanned against 10 mM MOPS (pH 7.0) at room temperature and similarly the enzyme samples (1 mg/ml) were scanned against 10mM MOPS buffer (pH 7.0) containing 1mM Co^{2+} and 5mM Mn^{2+} and at room temperature. Buffer having the sample composition but without the enzyme was in the reference cell. A buffer baseline was stored and subtracted from the displayed data to obtain the normalized excess-heat-capacity function (NEF) curve.

2.2.2.3 Fluorescence Spectrophotometric analysis of thermophilic xylose isomerase isoforms.

The temperature effects on fluorescence intensity and fluorescence emission spectra of the xylose isomerase isoforms were studied using a Hitachi-F-4500 spectrofluorimeter. The emission wavelength was 280nm fixed in the range to 430nm. The excitation

wavelength was fixed at 280nm. The concentration of enzyme taken for the study was 90mM in 0.1 M Tris-HCl buffer, pH 7.0. The temperature range selected was 40-100°C. The protein was heated for 15 min at each temperature, cooled at room temperature, and the fluorescence spectrum was taken for each of the isoforms and recorded.

2.2.2.4 Determination of rate constant, free energy (ΔG), enthalpy (ΔH) and entropy (ΔS)

The experimental rate constant is determined from a slope of the reaction obtained by plotting enzyme activity vs time over a period.

For first order kinetics, the rate of a reaction is independent of the substrate concentration. The differential rate law is therefore given as

$\mathbf{d(A)/dt = k}$ Where, d(A) = change in substrate concentration

dt = change in time

k = reaction rate constant

The integrated form of the rate equation is

$$\mathbf{[A] = -kt + [A]_0}$$

The unit of rate constant is expressed in **mol.** $\mathbf{L^{-1}.s^{-1}}$. Rate constant for most of the reactions closely follow an equation of the form

$$\mathbf{k = Ae^{-Ea/RT}}$$

which expresses the dependency of the rate constant on temperature, and is called the Arrhenius equation.

Where, e = base of natural logarithms

Ea = activation energy

R = gas constant 8.31 J/ (K. mol)

T = absolute temperature

A = called the frequency factor which is assumed to be a constant

2.2.2.4.1 Calculating Free energy (ΔG)

Free energy of denaturation of the T_{70}, T_{80} and T_{90} xylose isomerase isoforms at each temperature was determined employing the reaction rate constants (k) from a plot of percent enzyme activity retained vs time, in the equation

$$\Delta G = -RT \ln k$$

where R = gas constant (1.987 Cal/ mole)
T = absolute temperature

The unit of free energy (ΔG) is expressed in **kJ/mol**

2.2.2.4.2 Calculating Energy of activation (Ea)

Energy of activation required to establish during the denaturation process, was determined employing the slope of an Arrhenius plot generated by the relationship

log k versus 1/T where k = reaction rate constant
T = absolute temperature

The units of energy of activation (Ea) is expressed in **J/mol**

2.2.2.4.3 Calculating enthalpy (ΔH)

Enthalpy is defined as an extensive property of a substance that is used to obtain the heat absorbed or evolved in a chemical reaction at constant pressure.

The enthalpy of activation can be calculated from the formula given below

$$\Delta H = -R\,[d \ln k / d\,(1/T)] - RT$$

(or)

$$\Delta H = Ea - RT$$ where $Ea = -R\,[d \ln k / d\,(1/T)]$
Ea = activation energy
R = gas constant 8.31 J/ (K. mol)
T = absolute temperature

The unit of enthalpy (ΔH) is expressed in **kJ/mol**

2.2.2.4.4 Calculating entropy (ΔS)

Entropy is defined as a thermodynamic quantity that is a measure of the randomness or disorder in a system and is determined by the relationship

$\Delta S = (\Delta H - \Delta G)/T$ where ΔH = enthalpy of a reaction
ΔG = free energy change
T = absolute temperature

The unit of entropy (ΔS) is expressed in $\mathbf{kJ.mol^{-1}k^{-1}}$

2.2.2.5 EPR analysis

The presence of endogenous manganese and cobalt ions in the enzyme preparation was investigated employing the Electron paramagnetic resonance (EPR) technique. The T_{60}, T_{70}, T_{80} and T_{90} ion exchange column fractions were taken for EPR studies in an EPR sample tube and analyzed using a JEOL-JES-TE100 EPR spectrometer operating at X-band frequency having 100 kHz field modulation to obtain first derivative EPR spectrum The EPR signal due to Mn^{2+} and Co^{2+} ions was obtained at room temperature and at liquid nitrogen temperatures. A standard sample of Mn^{2+} (1mM) and Co^{2+} (1mM) were used as controls to monitor changes during the course of the reaction.

2.2.2.6 Elemental analysis of soil, water, cladode homogenate and ammonium sulphate

Detection for the presence of low abundance elements was carried out using the Inductively Coupled Plasma – Atomic Emission Spectroscopy (ICP-AES) method as discussed below.

Principle of ICP-AES method

The Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) is a conventional type spectroscopic analytical technique employed for the quantitative determination of a spectrum of elements. This technique uses inductively coupled argon

plasma, yielding a linear instrumental response to rates of analyte feed typically over five orders of magnitude. This is a spectrum based technique and detection limits for this technique falls within the range of 1-100µg/L. The recommended sample volume requirement is generally less than 500µl. The analyte is applied through a pneumatic or ultrasonic nebuliser to a central tunnel of argon flow directed to the flat base of argon plasma produced by inductive heating employing very high frequency alternating current. The analyte is therefore raised instantly to a temperature range of 7,500 k leading to atomization of the analyte followed by excitation and ionization. The atomic emission spectrum of the analyte thus generated is observed close to a region above the bright plasma and is measured spectroscopically (using polychromator or scanning monochromator type) against a low back ground. Light emission from the excited atoms or ions in the analyte is converted to electrical signals employing a photomultiplier with in the spectrophotometer and the intensity of the electrical signal produced is compared to the measured intensity of signals generated using elemental standards. The typical geometry of the plasma torch endows the ICP-AES technique with many unique spectroscopic properties (Nicholas Walsh, 1989).

Investigation for the presence of metal ions in the distilled water, ammonium sulphate solution, soil from around the xerophyte habitat, and the 10,000 x g homogenate supernatant of *Cereus pterogonus* and *Opuntia vulgaris* cladode were carried out specifically for Copper, Iron, Magnesium, Manganese, Cadmium, Cobalt, Zinc, Chromium, Nickel, Lead, Phosphorus and Calcium, employing the Inductively Coupled Plasma-Atomic Emission Spectrophotometric technique using a JOBIN YVON Ultima - 2 ICP Spectrophotometer. While the distilled water, ammonium sulphate solution and the, *Cereus pterogonus* and *Opuntia vulgaris* samples were analyzed directly, the soil sample required digestion as a preparative method for the ICP-AES analysis.

2.2.2.6.1 Soil preparation for ICP analysis

Soil sample (150 gm) was coned and quartered four times to prepare a homogenous sample containing similar amount of coarse and fine particles. Approximately 20 gm of this sample was powdered to a size of 200 meshes using an agate mortar. The powdered sample (0.5 gm) was taken in a covered Teflon crucible to which 10 ml of HF and 5 ml HCl were added and maintained over a hot plate at 80°C for digestion. The acid was evaporated in 4 hrs and the dried sample, was treated with 5 ml of HF, 10 ml HNO_3 and 1 ml HCl and evaporated further. The sample was then treated with 5 ml of HNO_3, and the process was repeated three times and dried to remove even traces of HF in the sample. Finally, the acid dried sample fraction was dissolved in 10 ml of 2N HNO_3 and made up to volume in a 100 ml volumetric flask, and stored for use in ICP analysis.

2.2.3 Immunological Methods

2.2.3.1 Generation of polyclonal anti xylose isomerase rabbit antibodies

Polyclonal rabbit antibodies to partially purified xylose isomerase from *Opuntia vulgaris* was generated using the enzyme protein 2 U/ mg specific activity (1mg/ml) as the immunogen mixed with an equal of Freunds complete adjuvant. The protein adjuvant emulsion (1 ml) was injected into the dorsal skin of a male rabbit prepared for the enzyme in several spots. The rabbit was maintained on normal diet for 14 days thereafter. On the 14^{th} day, the animal was given a booster dose (1mg/ml) of xylose isomerase in equal volume of Freunds incomplete adjuvant. A second booster dose was administered on day 21, and a third booster was given exactly on 28^{th} day. All booster doses were prepared employing Freunds incomplete adjuvant. On day 29, blood was collected from the ear lobe of the animal clotted and serum was separated by centrifugation at 700 x g for 3 min. The serum as a source of the polyclonal rabbit anti xylose isomerase antibody was collected and stored for further use at 4°C.

2.2.3.2 Immunodiffusion studies

Ouchterlony double immunodiffusion is a method of allowing antigen and antibody to interact on agarose matrix where immunoprecipitation occurred that stayed visible to the naked eye as a white precipitin line. In certain instance, the immunoprecipitation can be stained with coomassie blue dye.

A 1% agarose solution was prepared in 0.85% NaCl, and the agarose was placed on glass microscope slides to form the gel matrix. Wells were then cut at equidistance in the agarose matrix using a gel cutter. 10 µl of the rabbit antiserum (300 µg) was applied in the central well. This was followed by the application of 10 µl of purified xylose isomerase protein of *Cereus pterogonus* and *Opuntia vulgaris* isoforms (>200 µg) into the antigen wells. The immunodiffusion plates were then allowed to develop at 6°C for 48 hrs.

2.2.3.3 Inhibition studies

The polyclonal rabbit antiserum was also used in test tube enzyme assays for evaluating the potency of these antibodies to block the xylose isomerase activity when assayed in vitro. *Opuntia vulgaris* xylose isomerase antiserum (200µg) in each reaction tube were incubated at 4°C overnight with fixed volumes of purified protein isoenzymes (T_{60},T_{70}, T_{80}, T_{90}) in 0.1M Tris HCl buffer (pH 7.0). The residual xylose isomerase activity was measured in the supernatant after centrifugation (3000 x g) of the antibody–antigen complexes and was compared with control enzyme active samples.

2.2.3.4 Dot blot analysis

Xylose isomerase of *Cereus pterogonus* and *Opuntia vulgaris* (2 µg) in 25mM Tris-HCl buffer, pH 7.5 was spotted specifically onto a nitrocellulose membrane and dried. After incubating for 1 hr at room temperature in Tris buffered saline (TBS) containing 0.1% Tween-20 supplemented with 5% skim milk powder to block non specific binding

sites, membranes were incubated at 4°C for 12 hrs in the same buffer (with 1% skim milk powder) containing 1:250 dilution of *Opuntia vulgaris* xylose isomerase antiserum. The membrane was washed three times (10min/wash) and incubated for 1 hr at room temperature in fresh blocking buffer containing a 1:1000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG (Bangalore Genei, Bangalore, India). The enzyme protein specific circular spots were detected using TMB/H_2O_2 as substrate, in the dark.

2.2.3.5 Western blot analysis

Western blot analysis was carried out as described by Towbin et al (1979). Equal quantities (50 μg) of protein \ lane were separated on a sodium dodcyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) run at 100 V for 3 hrs and then electrophoretically transferring the separated proteins onto a nitrocellulose membrane at a constant current of 50 V in a buffer containing 25 mM Tris-HCl, 19 mM glycine and 20% methanol for 6 hrs. After incubating for 1 hr at room temperature in Tris buffered saline (TBS) containing 0.1 % Tween-20 supplemented with 5% skim milk powder, to block non specific binding sites, membranes were incubated for 12 hrs in the same buffer (with 1% skim milk powder) containing 1:250 dilution of *Opuntia vulgaris* xylose isomerase antiserum at 4°C, washed three times (10 min each wash) and incubated (1 hr at room temperature) in fresh blocking buffer containing a 1:1000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG (Bangalore Genei, Bangalore, India) and the specific proteins were detected using TMB/H_2O_2 as a substrate in the dark.

2.2.4 Molecular Biology

Attempts to characterize the thermophilic xylose isomerase genes of *Cereus pterogonus* and *Opuntia vulgaris* were carried out by isolating their genomic DNA and RNA from the cladodic cells of these species devoid of the cell wall, followed by

amplification of the XI gene sequence and the message (mRNA) employing the PCR technique, with a view to sequence the PCR amplified product to establish the nucleotide sequence of these xerophytic xylose isomerase genes. The oligonucleotide primer sequence that was required for the PCR amplification process was designed and synthesized based on information retrieved from gene database on thermophilic microbial xylose isomerase (9 species) and mesophilic plant xylose isomerase (3 species) gene sequence, since there is no existing data on thermophilic plant xylose isomerase genes. The methodology adopted for the bioinformatics exercise to arrive at primer designs was carried out as given in the chapter on **Bioinformatics**.

2.2.4.1 Isolation of DNA

The cellular genomic DNA was isolated from cladodic cells employing the Cetyl tri methyl ammonium bromide method of Tel-Zur et al., (1999).

Fresh cladode (1gm) was weighed and ground to a fine powder in liquid nitrogen using a mortar and pestle. The frozen powder was transferred to a 50 ml capped centrifuge tube and 20 ml extraction buffer was added to this and kept on ice for few minutes. The tubes were then centrifuged at 10,000 x g for 10 min at 4°C. The pellet was resuspended in 20 ml extraction buffer, and the procedure was repeated twice more. The pellet was finally resuspended in 5 ml extraction buffer, to which 3.5 ml of high salt CTAB buffer was added and incubated for 60-90 min at 55°C. Equal volume of chloroform and isoamyl alcohol was then added, centrifuged at 10,000 x g for 10 min. The supernatant was transferred to a fresh tube to which 2/3 volume of cold absolute iso-propanol and with 1/10 volume of sodium acetate solution was added and mixed. The sample was centrifuged at 10,000 x g for an additional 10 min at 4°C. The pellet obtained was washed with 75% cold ethanol, air dried and redissolved in 200 µl of TE buffer. A 10 µl volume of RNase stock solution was added to the sample and incubated further for 40 min at 37°C. The solution was then transferred

to a 1.5 ml tube and extracted with equal volume of Tris saturated phenol: chloroform (1:1). The extract was centrifuged at high speed for 10 min using a micro centrifuge at room temperature. The aqueous phase containing DNA was carefully pipetted out into a fresh tube and the DNA was precipitated by addition of two volumes of absolute cold ethanol followed by the addition of 1/10 volume of sodium acetate solution and placed at -20°C for 30 min. When required the sample was centrifuged at high speed for 15 min and the pellet was rinsed with cold 75% ethanol and air dried. The pellet was then redissolved with 50-100µl of TE buffer and used for PCR work.

The concentration of DNA was estimated by measuring the optical density (O. D) of the sample at 260 nm in a spectrophotometer and using the formula of Sambrook et al., (1989).

$$\text{DNA concentration } (\mu g / \mu l) = \frac{\text{O. D at 260} \times 50}{1000}$$

2.2.4.2 ISOLATION OF RNA

RNA isolation was performed by the extraction of total RNA and was based on guanidine thiocyanate phenol: chloroform method of Chomoczynsky and Sacchi, (1987).

Fresh cladode (1gm) was weighed and ground to a fine powder under liquid nitrogen using a mortar and pestle. The frozen powder was transferred to a 1ml of denaturing solution and to this 1ml of water saturated phenol and 200µl of chloroform-isoamyl alcohol mix (49:1) were added to homogenate. The contents were mixed thoroughly and incubated on ice for 15 min and centrifuged at 10,000 rpm for 20min at 4°C. The upper aqueous phase was transferred carefully to another tube. 1 ml of 100% isopropanol was added to it and incubated at -20°C for 30 min to precipitate RNA. This mixture was centrifuged at 10,000 rpm for 20 min at 4°C and the supernatant was

discarded. The RNA pellet was resuspended in 75% ethanol and incubated at room temperature for 10-15 min to dissolve residual amounts of guanidine thiocyanate and centrifuged at 10,000 rpm for 20 min at 4°C and the supernatant was discarded. The pellet was dissolved in 40-50 µl of diethyl pyrocabonate (DEPC) treated water and incubated for 10-15 min at 55°C to ensure complete solubilisation of RNA. Total RNA was stored at -70°C.

The concentration of RNA was estimated by measuring the optical density (O. D) at 260 nm in a spectrophotometer and using the formula of Sambrook et al., 1989.

$$\text{RNA concentration } (\mu g/\mu l) = \frac{\text{O. D at 260 X 40}}{1000}$$

2.2.4.3 cDNA Synthesis

The cDNA was synthesized using 10 µg poly (A^+) RNA isolated from the plant species *Cereus pterogonus* and *Opuntia vulgaris* and used as a template, employing the cDNA synthesis kit obtained from Bangalore genei (India) as per suggested protocol. The procedure employed the use of AMV-Reverse transcriptase for the synthesis of the cDNA stand.

The reaction mixture contained 10µg of total RNA sample was taken into a sterile RNase free vial and the volume was made upto 9µl with sterile water. To this 1µl of Oligo dT primer was added. The samples were kept in a thermal cycler at 65°C for 10 min followed by the addition of Nuclease free water (1µl), RNase inhibitor (1µl), 0.1M DTT (1µl), 5X RT buffer (4µl), 30mM dNTP mix (2µl) and Reverse transcriptase (0.5µl) at room temperature. The content of the vials were mixed gently and was kept in the thermal

cycler at 42°C for one hr. At the end denaturation of the sample at 95°C was done for 2 min to separate RNA - cDNA hybrids and the samples were stored at 4°C.

2.2.4.4 Polymerase Chain Reaction (PCR)

PCR was performed employing the synthesized cDNA and genomic DNA as templates. Oligonucleotides primers for the purpose were obtained from IDT, USA as per sequence determined and submitted to the firm based on standard bioinformatics approach to primer designing. Forward primer and reverse primer nucleotide sequences were as follows ($BMBF_1$) 5' CCG TAG CGT TTT GGC ATA CGT TC 3', (BMBR1) 5' TCG TAC TGG TGT TTT GTG GGT TC 3', (BMBF2) 5' TGC TTC CAT GAC AGG GAT ATT GC 3', (BMBR2) 5' CCT TCT CTT CCA CCC CAG AAT AC 3' and (BMBF3) 5' GGG GAT CCA TGA WRR VGB DRA GYT YYT K-3', (BMBR3) 5'GGA AGC TTY TAY AKG CRG MTT GAS AKM ATC 3'. These primers were used to amplify *Cereus pterogonus* and *Opuntia vulgaris* xylose isomerase gene nucleotide sequence from genomic DNA and cDNA. The PCR reaction (50 µl) contained 50 ng of primer, 100 ng of template DNA, 1x *Taq* DNA polymerase buffer, 0.025 units of *Taq* DNA polymerase, 0.2 M deoxyribonucleotide triphosphate, and 1.5 mM $MgCl_2$. Amplification was performed in the DNA thermal cycler (eppendorff Master Cycler gradient PCR machine) employing a gradient program for an initial denaturation (94°C for 2 min) followed by 30 cycles of 1 min denaturation at 94°C, 1 min annealing at varying temperatures and 1 min extension at 72°C with an final extension at 72°C for 10 min based on observation of the PCR generated product on agarose gel following electrophoresis, the annealing temperature specific for use of primers F_1/R_1 and F_2/R_2 as well as for the degenerate primers were established for rerun of the PCR amplification process.

2.2.4.5 Agarose Gel Electrophoresis

Agarose gel electrophoresis was carried out as a routine for evaluating the quality and nature of the DNA and RNA preparations as well as to identify the nature of the PCR products as described by Southern et al., (1979). The required amount of agarose was weighed and dissolved in 1x TAE buffer to obtain a final agarose concentration of 0.7%, and melted in a microwave oven for 10 min. The solution was allowed to cool to 50°C, and a volume of ethidium bromide (10 mg/ml) was added. The agarose solution was poured on to a gel casting plate. The gel was allowed to set with sample comb in place, for 30 min. The comb was then removed and the gel was placed on a horizontal gel tank filled with sufficient 1x TAE buffer to submerge the agarose gel. The PCR-DNA and PCR-cDNA samples were premixed with the sample loading buffer and were loaded into each well of the agarose gel. The electrophoresis was carried out at 50 V until the tracking dye indicated the end of the gel run. The gel was then removed from the electrophoresis unit and examined under a UV-transilluminator short wavelength UV lamp for the presence of ethidium bromide stained DNA and PCR amplified bands.

2.2.5 Bioinformatics

Comparative amino acid sequence analysis of selected xylose isomerase enzyme proteins was initially carried out using information available in the database. The softwares used for the analysis were, **(1) SWISS – PROT**, a protein sequence database that strives to provide a high level of annotation (such as the description of the function of a protein, its domain structure, post translational modifications, variants etc), a minimal level of redundancy, and a high level of integration with other data bases (Bairoch, 2000). **(2) TREMBL**, A complete annotated supplement of SWISS-PROT that contained all the translations of EMBL nucleotide sequence entries that are not already integrated in to the

Swiss prot (Bairoch and Apweiler 2000) and **(3) ClustalW,** a general purpose multiple sequence alignment program for DNA or protein sequences (Thompson et al., 1994). This program is recognized to produce biologically meaningful multiple sequence alignments of divergent sequences. This program also calculates the best match for the selected sequences, and lines them up for identity, (similarities and differences). Evolutionary relationship can also be determined by viewing the cladogram or the phylogram.

Protein sequences were randomly selected from mesophilic plant xylose isomerase and thermophilic bacterial xylose isomerase (having accession numbers **Q8LFA4, Q8H3Q7, Q40082, Q9X1Z5, P45687, Q2WMA6, P22842, Q9KGU2, P30435, P77995, P19148)** employing the **Swiss prot** (see Table). The retrieved sequences were aligned with **ClustalW** to determine the **conserved regions** in the amino acid sequences between each species. Following determination of the amino and carboxy terminal conserved regions in each xylose isomerase enzyme protein sequence, specific conserved amino acid sequence were selected to design the required oligonucleotide primers.

ORF finder searches for open reading frames in the DNA sequences were made for sequence matches. This program identifies the length of each ORF along with its amino acid translation code. **ORF finder** was used to determine the coding sequence for each conserved amino acid to establish the corresponding conserved nucleotide sequences.

The oligonucleotide primers designed on the basis described above, were ordered for and purchased from Integrated DNA Technologies, USA. The primer sequences were carefully analyzed for potential primer dimerization possibilities, self complimentarity and for their net GC content. Primers were obtained at 0.2 μM purity. Following is a table of reference for the Mesophilic plant and thermophilic bacteria (xylose isomerase) data base accession numbers.

Species	Accession number
Mesophilic plants	
Arabidopsis thaliana	Q8LFA4
Oryza sativa	Q8H3Q7
Hordeum vulgare	Q40082
Thermophilic Bacteria	
Thermotoga maritima	Q9X1Z5
Thermotoga neapolitana	P45687
Clostridium beijerincki NCIMB 8052	Q2WMA6
Thermoanaerobacter ethanolicus	P22842
Thermoanaerobacter yonseii	Q9KGU2
Thermoanaerobacter saccharolyticum	P30435
Thermoanaerobacterium spp.	P77995
Thermoanaerobacter thermosulfurogenes	P19148

Primer design for amino and carboxy terminal domain amino acids based on anticipated conserved amino acid sequence in these domains of the thermophilic *Cereus pterogonus* and *Opuntia vulgaris* xylose isomerases as deduced from the conserved sequences in the XI domains of the above mentioned species was as follows -

Amino terminal	Carboxy terminal
FSVAFWHTF	**EPTKHQYD**
CFHDRDIAP	**YVFWGGRE**

Though these amino acid sequences are contiguous, their localization within the amino terminal and carboxy terminal regions varies within the mesophilic plant and the thermophilic bacterial species.

Following is the converted nucleotide sequence corresponding to the amino and carboxy terminal domain conserved amino acid sequences deduced for the primers

	Amino terminal	Carboxy terminal
I	**F1 5' CCG TAG CGT TTT GGC ATA CGT TC 3'**	**R1 5' TCG TAC TGG TGT TTT GTG GGT TC 3'**
II	**F2 5' TGC TTC CAT GAC AGG GAT ATT GC 3'**	**R2 5' CCT TCT CTT CCA CCC CAG AAT AC 3'**

Since there are no reports on the nucleotide sequence or the amino acid sequence of xylose isomerase from thermophilic plant sources, it became necessary to employ the conserved nucleotide sequence available thus for our searches, to function as a potential reference for the primer design. Based on this background, the following primer sequences were considered two of which ($BMBF_1R_1$ and $BMBF_2R_2$) were designed based on conserved sequences and the third ($BMBF_3R_3$) was a degenerate sequence to function as Forward (sense) and Reverse (antisense) primers for the PCR amplification process reported in this work.

	Forward primer (sense)	Reverse primer (antisense)
1	5' CCG TAG CGT TTT GGC ATA CGT TC 3'	5' TCG TAC TGG TGT TTT GTG GGT TC 3'
2	5' TGC TTC CAT GAC AGG GAT ATT GC 3'	5' CCT TCT CTT CCA CCC CAG AAT AC 3'
3	5' GGG GAT CCA TGA WRR VGB DRA GYT YYT K-3'	5'GGA AGC TTY TAY AKG CRG MTT GAS AKM ATC 3'

Melting temperature and G+C content of BMB primers

Particulars	Melting Temperature (Tm)	GC Content (%)
BMBF1	58.7	52.1
BMBR1	57.4	47.8
BMBF2	57.4	47.8
BMBR2	56.8	52.1
BMBF3	61.0	55.5
BMBR3	59.4	50.0

2.2.6 Statistical Methods

All data in the present study is expressed as mean of three independent determinations ± SE. The analysis was carried out using the software SPSS 7.0.