DISCUSSION

4. DISCUSSION

Understanding higher temperature resistance of thermophilic and hyperthermophilic proteins, is essential for studies of protein folding and stability, and is critical for designing efficient enzymes that can work at high temperatures. The usability of high temperature stable enzymes from these bacterial sources in industrial processes found commercial use for the enzymes worldwide. However, difficulties in the procurement of patented thermostable enzymes, and natural bacterial sources from hot springs not available in India, combined with the need to culture such sources in larger amounts as a source of thermostable enzymes for industrial needs at considerable cost, directed our attention to seek and identify alternate natural sources of high temperature stable enzyme forms for their potential industrial and commercial use in the country.

Chan et al., (1989) and Amore et al., (1989) reported the expressions of *Escherichia coli* xylose isomerase in *Schizosaccharomyces pombe* and the *Bacillus* and *Actinoplanes* xylose isomerases in *Saccharomyces cerevisiae* for fermentation purposes. The expressed enzymes were however found to be partly insoluble and inactive. Since the gene constructs containing bacterial isomerase sequences in yeasts were found unsuitable for use in industrial fermentation processes, interest was directed to study the potential of the xylose isomerase gene from a eukaryotic source for its' function in the yeast. Within the ecology and environment of the Indian subcontinent, the existence of several xerophytic species of plant varieties growing in very arid and semi arid regions of this country attracted attention as a possible source of high temperature enzymes. Investigations were therefore initiated and directed to characterize the xylose isomerase enzyme activity that had an established industrial importance. Xerophytic plant species *Opuntia vulgaris* and *Cereus pterogonus*

were identified as the enzyme source for the purpose, as they grew abundantly in the coastal as well as in the arid neighborhoods of Puducherry, India.

Using the conventional protein isolation and characterization methods as reported in this thesis's studies employing the XI enzyme activity in the two xerophytic plant species (*Opuntia vulgaris* and *Cereus pterogonus*) has yielded material and data to establish the existence of high temperature stable xylose isomerase enzyme isoforms in these species, indicative of their significant role in isomerization process in these plant varieties.

By definition, high temperature stable enzymes, also called thermophilic enzymes, are those that exhibited their catalytic activities above 70°C. The existence of the T_{70}, T_{80} and T_{90} xylose isomerase isoforms in the xerophytic species employed in this study can therefore be categorized as thermophilic isoforms. The T_{40} and T_{60} isoform variety from both the species is therefore not thermophilic. The existence of the T_{60} and the T_{80} isoforms in *Cereus pterogonus* versus the existence of T_{40}, T_{70} and T_{90} isoforms in *Opuntia vulgaris* raised doubt if these isoforms were constitutively expressed in these plant species, or the thermophilic T_{70}, T_{80} and T_{90} isoforms were inducible enzymes, since the normal habitat temperature for these plant species were around 30°C. Additionally these enzymes were found to be stable at both acidic and alkali conditions in the pH range 5.0 - 8.0 during our studies, offering an added advantage for the potential use of these enzymes in industrial applications wherein both high temperature stability as well as acid stability were a matter of advantage for use of these enzymes.

Zoltowska (2001) reported the existence of two isoforms of amylase from the intestine and the muscle of *Ascaris Suum* (Nematoda). The intestinal amylase showed two optimum activities, at 40°C and 50°C. The muscle amylase also exhibited two optimum activities, at 30°C and 50°C. Nabi and Srikumar (2003) reported the existence of

temperature stable amylase isoforms from *Opuntia vulgaris*. The plant amylase showed two optimum activities, at 50°C and 90°C. The existence of two forms of temperature stable enzyme in these species was therefore considered as due to the different thermal sensitiveness.

Bhosale et al., (1996) reported the existence of a thermophilic xylose isomerase in microbes that had optimum temperature in the range from 60°C to 80°C that increased in the presence of Co^{2+}. The enzyme from *Streptomyces spp.*, *Bacillus spp.*, *Actinoplanes missouriensis*, and *Thermus thermosulfurogenes* was stable at high temperatures, but that from *Lactobacillus* and *Escherichia spp.* was less stable. The optimum pH range of XI is generally between pH 7.0 and 9.0. The enzyme from *Lactobacillus brevis* exhibiting a lower pH optimum (between 6 and 7), that is desirable for commercial applications of the XI. It however requires divalent cations such as Mg^{2+}, Co^{2+}, or Mn^{2+}, or a combination of these ions, for maximum activity. Co^{2+} was considered to be responsible for the stabilization of the enzyme through holding an ordered conformation.

Kristo et al., (1996) reported the presence of eukaryotic xylose isomerase in the barley *Hordeum vulgare*. The enzyme was fairly thermostable with an optimum temperature at 60°C. It showed maximum activity over a broad pH range (7.0 - 9.0). The enzyme required Mn^{2+} for its' activity.

Vieille and Zeikus (2001) reported that most industrially used XIs were isolated from mesophilic organisms (*Streptomyces spp.* and *Actinoplanes spp.*) because of by-product and color formation that occurred at high temperature and alkaline pH and because the isomerases themselves were not highly thermostable. Lee and Zeikus (1991) reported that thermostable XIs with neutral or slightly acidic pH optima have a potential for industrial applications. Performing isomerization at higher temperature and at neutral or

slightly acidic pH employing thermo-acid stable XIs therefore allowed for faster reaction rates, higher fructose concentration at equilibrium and decreased viscosity of substrate.

This report served an indirect support to the finding of thermophilic and neutrophilic or acidophilic xylose isomerases in the plant species *Cereus pterogonus* and *Opuntia vulgaris*. Observations made here are further supported by the findings of Lama et al., (2001) on the xylose isomerase from *B. thermoantarcticus* that was remarkably stable and resistant to high temperature. This enzyme, stable at acidic pH values, increased the efficiency of the process and reduced the possibilities of bye- product formation. This enzyme required the presence of divalent metal cations such as Mn^{2+}, Mg^{2+} or Co^{2+}, as essential cofactors for its' catalytic activity and was therefore a great potential source of thermophilic xylose isomerase for the isomerization process.

Two xerophytic plant tissue homogenate proteins were analyzed by electrophoresis and were found to have two forms of xylose isomerase by activity staining study. However, the electrophoretic analysis was based on differences on the net charge of the protein molecules. The presence of multiple forms of these enzymes based on differences in molecular mass could not be ruled out. Refourelet and Daugin (1999) suggested the existence of seven isoforms of the enzyme GDH with subunits presenting different isoelectric points. Dark and ammonium controlled forms of GDH were found to be more acidic and faster migrating ones in Native PAGE. Simpson et al., (1999) reported the existence of multiform of Fructan exohydrolase enzyme in *Lolium temulentum* L. Taylor et al., (1978) reported the presence of two glucoamylase in their strain of T. *lanuginosus* from shaken cultures on a complete medium that contained soya meal, molasses and corn steep liquor. Therefore, conclusions regarding the production of number of forms may be dependent upon the conditions of growth employed.

The purification strategy reported here for the isolation and purification of xylose isomerase from the two xerophyte species employed conventional approaches such as ammonium sulphate precipitation of protein, ion exchange chromatography, and gel permeation chromatography, to recognize and establish basic characteristics of the enzyme under these conditions. The cost factor involved in strategizing immuno-affinity approaches as well as the procedural protocols required to process expensive import supplies offered significant hurdles and precluded working out improved methodologies for the isolation and purification needs. Hence compromises had to be made for optimizing possible methods for our investigative purpose without having to miss out on the novelties of our observations.

Ammonium sulphate precipitation of protein in crude extracts has been used to concentrate and purify isomerase enzymes from a variety of sources, such as *Clostridium thermosulfurogenes* and *thermoanaerobacter* strain B6A (Lee and Zeikus, 1991), *Paenibacillus spp.* and *Alcaligenes ruhlandii* (Moneke et al., 1998), Thermophilic *Bacillus spp.* (Chauthaiwale and Rao, 1994), and *Streptomyces spp.* (Inyang 1995). However, Ammonium sulphate precipitation of isomerase activities during the fractionation resulted only in partial precipitation. On the other hand, a number of isomerases and other industrially important enzymes like cellulases have been concentrated by ammonium sulphate precipitation from thermophilic fungi, such as cellulases from *Thermoascur* a. *tiacua* (Khandke, 1986) and glucosidases from *S. thennophile* (Bhat and Maheshwari, 1987), xylanases from *Humicola lanuginosa* (Anand et al., 1990) and *Melanocarpus* (Prabhu, 1989).

Ion-exchange and gel-filtration chromatography techniques have been used to purify xylose isomerase enzymes (Nazmul Ahsan and Shugo Watabe, 2001). Two isoforms of Trypsin (aT - I and aT - II) have been separated from *Engraulis japanicus*. Multiple

forms of *A. oryzae* (Razzaque and Ueda, 1978) and *Penicillium oxalicurn* (Yamasaki et al., 1977) glucoamylase have been separated by anion-exchange chromatography. On the other hand, gel filtration was used to separate isoforms of *Rhizopus, spp.* glucoamylase (Takahashi et al., 1978). In the present study, ion-exchange chromatography on Dowex-1 (pH 7.0) was used successfully to clearly separate xylose isomerase isoforms with approximately 25 fold purification. Xylose isomerase isoform (T_{40} and T_{60}) activity eluted at lower salt concentration than that required to elute other forms. This suggested that isoform T_{40} and T_{60} is more electronegative than T_{70}, T_{80} & T_{90}. An effective step for the purification of xylose isomerase was found to be in the use of Sephadex G- 100 chromatography that separated xylose isomerase from major contaminants without significant loss in the enzyme activity. This step alone resulted in 46 and 58 fold purification of CPXI and OVXI, respectively. The enzyme obtained from this step however was associated with some contaminants. Information obtained through this purification procedure was that two forms of CPXI and three forms of OVXI existed. The recovery of xylose isomerase (34%) in the present study is comparable to that (40%) reported by Rao et al., (1981). Though the fold purification (46 and 58 fold respectively) and the yield of activity (34%) obtained following the purification strategy employed here matched well with earlier reports on the purification of xylose isomerase, strategic improvements in the purification process and the yield of activity required additional attempts to carry out more modern approaches. The lower recovery of activity in the purified protein can probably be due to the sensitivity of certain plant enzymes to extremely low abundant contaminants like heavy metals present in the chemicals employed for the isolation methods. Nevertheless, the purified enzyme protein from *Cereus pterogonus* and *Opuntia vulgaris* was found capable of generating 53-54% fructose from glucose in a single step during the 10 min assay at 90°C. Estimate for xylulose generation when xylose was used as substrate

indicated >55% efficiency. The ability to enrich fructose content in a single step in reaction assay points to the potential for use of this enzyme activity for the production of HFCS in the Indian context.

In most of the physicochemical properties studied, i.e., effect of temperature, pH optima and substrate specificity, the effect of metal ions on xylose isomerase enzyme activity from *Cereus pterogonus* and *Opuntia vulgaris* resembled that of other microbial xylose isomerase enzymes. The molecular mass of the protein could not be unequivocally established. However SDS-PAGE analysis identified the existence of four distinct polypeptides in the homogenate 10, 000 x g supernatant. These polypeptides also remained enriched in the T_{60}, T_{70}, T_{80} and T_{90} ion exchange fractions. All purified preparations of xylose isomerase on SDS-PAGE migrated as a single protein band ranging in MW between 68 kDa and 71 kDa. However, on gel-filtration at pH 7.0, the native enzyme eluted as a single protein peak of 66 kDa. The molecular mass observed here was close to that obtained by SDS-PAGE. The SDS-PAGE 68 kDa, polypeptide was generated following reduction of T_{60} & T_{80} isoforms of *Cereus pterogonus* as well as the T_{70} & T_{90} isoforms of *Opuntia vulgaris* with 2-mercaptoethanol. The *Opuntia vulgaris* T_{90} isoform however yielded only the 71 kDa molecular mass polypeptide under the conditions. In all the cases however, the 68-71 kDa polypeptide band yielded enzyme activity following the SDS-PAGE activity staining method, even though the proteins were treated with 2-mercaptoethanol, suggesting that these polypeptides singularly exhibited the enzyme activity and that each of the polypeptide bands existed as a holoenzyme unaffected by the

sulfahydryl reduction process. These observations suggested that the xerophytic xylose isomerases studied are monomeric proteins. Since migration of proteins on gel-filtration column depends on the Stokes radius rather than on the molecular mass of the protein, the molecular mass by gel-filtration will depend upon the globular nature of the proteins and tightly the protein remain packed during the permeation process. Further it became possible to detect this enzyme activity in native PAGE activity gels. The retention of the xylose isomerase enzyme activity in the activity stained gels was not surprising since studies carried out during the course employing DTT as a reductant of the inter or intra chain disulphide bonds had established that the xylose isomerase protein of *Cereus pterogonus* and *Opuntia vulgaris* continued to exhibit enzyme activity following such a treatment.

The molecular weight of XI varied from 52,000 to 191,000 (Chen, 1980). The subunit structure and amino acid composition of XI revealed that it is a tetramer or a dimer of similar or identical subunits associated with non covalent bonds and was devoid of inter chain disulfide bonds. The extracellular XI from *Bacillus spp.* is a trimer (Chauthaiwale and Rao, 1994). However Basuki et al., (1992) had reported the existence of isozymes of GI from *Streptomyces phaechromogenes*. These isoenzymes differed in their N-terminal amino acids and in the peptide patterns of the digests generated with trypsin, *Achromobacter* protease I, and cyanogen bromide. Each of the isoenzyme was a tetramer of non identical subunits.

Suekane (1978) reported on xylose isomerase from *Streptomyces olivochromogenes* and *Bacillus stearothermophilus* as having a molecular weight of 130 kDa containing one subunit and described as a monomer, while Lama et al., (1996) reported a molecular weight of 200 kDa for xylose isomerase isolated from the *Bacillus thermoantarcticus*. Lehmacher and Bisswanger (1990) reported on xylose isomerase

isolated from *Thermus aquaticus* having a molecular weight of 196 kDa containing four identical subunits, and described as a tetramer. Kawai et al., (1994) reported on xylose isomerase isolated from *Bifidobacterium adolescentis* having a molecular weight of 100 kDa containing two identical subunits, and described as a trimer. Kristo et al., (1996) reported on xylose isomerase isolated from eukaryotic *H. vulgare* having a molecular weight of 100 kDa containing two identical subunits, and described as a dimer.

It was interesting to note that the temperature related activity of the xylose isomerase isoforms was stabilizable by divalent Mn^{2+} and Co^{2+}, by employing critical concentration of these ions, whereas Ca^{2+} invariably contributed to a reduction in the enzyme activity, suggestive of xerophytic xylose isomerases being metalloenzymes, and the inhibition by specific ions as being due to a competition between the exogenously added cations and protein-associated cations, resulting in decreased metalloenzyme activity. This aspect can be appreciated based on the understanding that protein-metal interactions generally led to the formation of non covalent protein-metal complexes (Boel, 1990), yielding conformational alternatives exhibiting differences in activities. It is to be recognized that the formation of protein-metal aggregates at higher temperature acted as a protection mechanism for thermophilic enzymes in general, and contributed to the higher thermostability of these species. It may therefore be considered a mechanism for high temperature isomerization processivity in the xerophytes while growing at elevated ambient temperature in the environment. Experimental results obtained confirm the fact that the xylose isomerase isoforms were capable of functioning 10-12 minutes at 90°C, making them a potentially viable thermophilic alternative where required, to the thermophilic enzyme activity available from bacterial sources.

Marg and Clark (1990) reported that activity and metal content measurements have provided evidence for two distinct metal binding sites (Mn^{2+} and Co^{2+}) in glucose

isomerase from *Bacillus coagulans*. Callens et al., (1986, 1988) reported that XI requires a divalent cation such as Mg^{2+}, Co^{2+}, or Mn^{2+}, or a combination of these cations, for maximum activity. Although both Mg^{2+} and Co^{2+} are essential for activity, they play differential roles. While Mg^{2+} is superior to Co^{2+} as an activator, Co^{2+} is responsible for stabilization of the enzyme by holding the ordered conformation, especially the quaternary structure of the enzyme. Danno (1971) reported that direct metal ion-binding studies were carried out on GI from *Bacillus coagulans*. This fact was observed again during our current studies.

Kasumi et al., (1982) have reported the presence of four Co^{2+} ions per tetramer of XI from *Streptomyces griseofuscus*. The catalytic activity of XI was inhibited by metals such as Hg^{2+}, Cu^{2+}, Zn^{2+}, and Ni $^{2+}$ and to some extent by Ca^{2+}. This information is in direct support of our findings that Ca^{2+}, Hg^{2+}, Cu^{2+}, Zn^{2+}, and Ni^{2+} inhibited the *Cereus pterogonus* and *Opuntia vulgaris* xylose isomerase activities.

Ghatge et al., (1991) reported that xylose isomerase from *Streptomyces spp.* was inhibited by urea and that SDS reduced the xylose isomerase enzyme activity. Guanidine hydrochloride gave a mixture of active and inactive forms of the enzyme in assay tube. Again our findings are in good agreement with these reports.

The K_m and V_{max} values were obtained (Table 7) from the Lineweaver-Burk double reciprocal plot. 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 was subsequently being utilized in the pentose phosphate or Phosphoketolase pathway in other organisms (Lama et al., 2001; Brown et al., 1993).

The denaturation kinetic studies reported here were undertaken to establish the thermophilic nature of the xylose isomerase isoforms. Denaturation assays conducted for various intervals of time at selected higher temperature enabled to heat treat the enzyme species for a given length of time and allowed to measure the residual enzyme activity available in the sample to determine the enzyme stability at higher temperatures. Thermal denaturation assays of isoforms when repeated in the presence of specific divalent metal ions yielded denaturation profiles that indicated augmented thermal stability for the enzyme protein at higher temperatures. Inactivation was accompanied by heavy aggregation at the given enzyme concentration, and the difference in inactivation rate between the control and metal ions treated samples probably resulted from the trapping of some active soluble enzyme molecules in the aggregate. As the aggregate increased in size with inactivation time, more and more soluble enzyme may remain trapped in the insoluble pellet obtained following centrifugation. Hess & Kelly, (1999) suggested that the precipitated enzyme was completely inactive, and that the soluble fraction remained fully active. The apoenzyme was significantly less stable than the enzyme containing Co^{2+}, Mn^{2+}, or Mg^{2+}. Of the three metal cations, Mn^{2+} particularly stabilized enzyme isoforms. Co^{2+} was only slightly less stabilizing than Mn^{2+}. Mg^{2+} was significantly less efficient than the other two metals at stabilizing. This metal-specific Protection of XI enzyme against inactivation is very similar to the situation observed by Callens et al., (1988) with class I XIs: Mg^{2+} does not protect type I XIs from unfolding to the extent that Co^{2+} does. CPXI and OVXI isoforms have much higher thermal stabilities than their mesophilic counterparts, this is not evident from those non-metal mediated structural features previously proposed to enhance thermostability (Jaenicke et al., 1998; Vieille and Zeikus, 2001), such as hydrogen bonds (Tanner et al., 1996), ion pairs (Vetriani, et al., 1998), compactness (Zhu 1998), and hydrophobic surface burial (Maes et al., 1999). Generation of inactivation differences in

the decay profile of the denaturing enzymes at each selected temperature was noticed. The denaturation rate constant of each isoforms were determined by drawing tangents to the linear segments within each denaturation curve, from which the thermodynamic indicator for free energy (ΔG) was calculated. Arrhenius plots were then employed to determine the ΔH values. The activation enthalpy ΔH, is in agreement with previously measured activation energies for the unfolding of soluble proteins. Activation Gibbs free energies ΔG, which is measures of the spontaneity of the inactivation processes, is lower than the ΔH values. This is due to the positive entropy generated during the inactivation process. The activation entropy represents the difference in the extent of local disordering between the transition state and the ground state for the inactivation pathway. Thus, the positive ΔS are in agreement with increasing local disorder in the transition state when compared with the ground state. The increasing value of ΔS as a function of metal ion concentration is considered to be due to the degree of disorder in the structure required to reach the transition state for the unfolding process since the structural packing in the ground state probably remained firmly stabilized by the bivalent ion.

The melting point for control CPXI and OVXI were found to be 88°C and 92°C when determined by DSC. Whereas OVXI showed two melting transitions (60°C and 92°C) these transitions probably were indicative of either the existence of two phases during enzyme inactivation or the presence in solution of a heterogeneous population of enzymes containing Mn^{2+} and Co^{2+}. Nevertheless in presence of the metal ions, the melting point of the enzyme was found to be above 100°C. Indeed, the enzymes were however not EDTA treated prior to the experiments. It is probable that the enzymes may become denatured by simple unfolding or chemical modification as the temperature is raised above their threshold temperature as shown for example, in the DSC results presented in figures (46-49). In here, the unfolded proteins irreversibly aggregate preventing any possibility of

quantification of the relative contributions of stabilizing forces such as hydrogen bonding, hydrophobic interactions and salt bridging.

Ahren (1985) reported that enzymes are also subject to chemical modifications which may cause a loss of activity without necessarily resulting in the unfolding of the protein at high temperature. These include hydrolysis of peptide bonds, deamidation of labile amino acid residues, and destruction of disulphide bonds.

Fluorescence spectroscopic analysis enabled to recognize that the initial intrinsic tryptophan fluorescence of these proteins correlated well with an environment in which the tryptophan residues remained totally exposed, followed by decremental changes in the intrinsic fluorescence of these proteins with increasing temperature, reaching a minimum in the emission λ_{max} at 100°C, suggestive of significant burial of tryptophan residues within the protein structure or within the protein aggregates that formed at higher temperature, as evidenced employing Transmission Electron Microscopy of glutamine synthetases from thermophilic and mesophilic *Bacillus* species (Merkler, 1988). Fitter (2001) reported on a comparative analysis of the thermophilic and mesophilic amylases.

Laderman (1993) reported that the fluorescence emission (λ_{em}) of α-amylase from *P. furiosus* at 20°C exhibited maximum intensity at a (λ_{exc}) of 345nm. When the fluorescent spectrum was monitored over a range of temperatures, there was no shift observed in the emission λ_{max}, suggestive of that the tryptophan residues occupied a polar environment independent of temperature. A decrease in fluorescent intensity with increase in temperature is indicated as due to increased quenching caused by greater thermal motion (Galley and Edelman, 1964) in solution.

Results of the ICP-AES analysis carried out on the ammonium sulphate used for protein precipitation indicated the presence of chromium (21.43 ppm) in the reagent. Whether the level of chromium available to the enzyme during the precipitation process

affected the enzyme activity was not specifically determined. Since, the ICP-AES facility was only recently established, its' analytical sensitivity level of < 0.1 ppm for elemental analysis could not be employed during the purification work as a co-detection strategy for the determination of contaminant ions during this study. ICP-AES analysis of the tissue homogenate 10,000 x g supernatant indicated the presence of also Mn^{2+} (19-22 ppm) and Mg^{2+} (527-568 ppm). EPR studies did not however indicate the presence of manganese in the isoforms when investigated at room temperature, probably due to the relatively lower sensitivity of this detection technique compared with ICP-AES. However the presence of Mn^{2+} in the isoform samples was detected when EPR analysis was carried out at liquid nitrogen temperature of 77K. Surprisingly, the presence of Co^{2+} was also indicated in these samples analyzed by EPR at 77K where as Co^{2+} acted as diamagnetic at room temperature due to relaxation effects. EPR spectra can give information for determining the metal binding nature of the enzyme. For example T_{80} isoforms of CPXI and T_{90} isoforms of OVXI revealed one resonance line for Mn^{2+}.which exhibited a g value around 2.071, whereas the presence of Co^{2+}in these isoforms revealed eight resonance lines, having a g value of 2.304. Mg^{2+} levels were detected and were indicated to be greater by the ICP-AES analysis than Mn^{2+} levels in these same samples. Lack of adequate purified protein for analysis and the requirement of expensive argon gas for the ICP-AES analysis precluded wider studies on elemental analysis of the purified material employing the ICP-AES method. It was believed that the plant enzyme was sensitive to contaminants and/or cold temperature during the purification process and therefore contributed to a lower yield of the enzyme activity

The generation of polyclonal antibody serum against the T_{90} isoform of *Opuntia vulgaris* xylose isomerase aided to investigate cross reactivity between the antibody and the *Cereus pterogonus* and the *Opuntia vulgaris* xylose isomerase isoforms, which

indicated regions of commonality in the polypeptide sequences of these different isoforms. This was confirmed by the observation that the rabbit antiserum brought about inhibition of the xylose isomerase activities during in vitro test tube assays. Ouchterlony double immunodiffusion, dot-blot and western blot analysis of xylose isomerase isoforms showed that these isoforms activities were similar in their optimum pH, temperature and substrate specificity, and that they also probably had identical epitopes and a high level of sequence similarity between the two plant species' isoforms, despite their differing thermostabilities.

This study is supported by the findings of Brown et al., (1993), who reported on the immunological cross reactivity between *S. murinus* antisera and the *T. maritima* XI enzyme while investigating identical epitopes or sequence similarities between the two enzymes. Using a polyclonal antiserum against a purified recombinant enzyme (Lee et al., 2004) reported that recombinant arabinose isomerase was identical to the native arabinose isomerase present in an L-arabinose-induced culture of *T. maritima* later confirmed by Western blotting.

Bioinformatics tools aided to identify the conserved sequence regions in the 5' and the 3' -end of selected eukaryotic and prokaryotic xylose isomerase gene sequences. The multiple sequence alignment of *xylA* sequences from eleven sources cited earlier using ClustalW (1.83) established that the amino acids involved in the substrate and metal ion binding, as well as in the catalytic function were completely conserved. A *cis* peptide linkage between a glutamic acid and a proline was found to be responsible for the formation of the rigid structure at the active site that was also seen to be well conserved in all the XIs studied. Thus, the essential structure at the catalytic site XI appears to be analogous in all the enzyme species that were compared. The information on the conserved and homologous regions in the *xylA* sequences will be a valuable tool for isolating novel XIs with desirable catalytic properties (Bhosale et al., 1996).

For fermentation purposes *Escherichia coli* isomerase had been expressed in *Schizosaccharomyces pombe* (Chan et al., 1989) and *Bacillus* and *Actinoplanes* xylose isomerases had been expressed in *Saccharomyces cerevisiae* (Amore and Hollenberg, 1989). These enzymes however were reported to be partly insoluble and inactive. Since the expression constructs containing bacterial isomerase sequences in yeasts have not been suitable for use in industrial fermentation processes, we were interested in obtaining a xylose isomerase gene from a eukaryotic source and investigating its function in yeasts. So an attempt was made to amplify the xylose isomerase gene nucleotide sequence employing the use of *Cereus pterogonus* and *Opuntia vulgaris* genomic DNA templates for the PCR amplification process using the different pairs of sense - antisense primers synthesized for the purpose did not yield a full length sequence of *xylA* gene of 1.2 -1.4kb. The anticipation of a 1.2 -1.4kb PCR band was based on the reported average size of the eukaryotic xylose isomerase gene in the different mesophilic plant sources, and documented in the data base. The use of Poly A^+ RNA from these same species served as an alternate template and for the synthesis of full length cDNA for use in the PCR based sequence amplification process involving each of the three different pairs of sense-antisense primers designed for the purpose. The reverse transcriptase based PCR method also did not yield the 1.2-1.4kb xylose isomerase gene band on the agarose gels. Nonetheless, PCR amplification efforts employing the primer pair $BMBF_2/R_2$ resulted in the generation of an ethidium bromide stained specific PCR band, having a nucleotide length of 300bp. Similarly, using $BMBF_1/R_1$ as primers, PCR products of 600bp and 350bp for the *Opuntia vulgaris* and 250bp for *Cereus pterogonus* genomic templates were observed. $BMBF_1/R_1$ and $BMBF_2/R_2$ primers were thus unable to amplify the XI nucleotide sequence from the genomic DNA. Attention was therefore directed to the design and use of a set of degenerative primers. The degenerative primer nucleotide sequences were deduced to

complement the XI gene sequences of the three plant species *Arabidopsis thaliana, Oryza sativa and Hordeum vulgare*. PCR amplification efforts employing these primers resulted in the generation of an 800bp and 450bp of ethidium bromide stained bands for the *Cereus pterogonus* genomic DNA template and 600bp and 200bp bands for the *Opuntia vulgaris* genome DNA template, reproducibly. However, nucleotide sequencing data obtained for each of the PCR amplified product did not indicate homology with any of the known plant XI gene or cDNA sequences available in the database.

Work is therefore in progress to determine if the PCR bands obtained employing different primers could be used as probes to hybridize with the genomic template through the southern blot analysis method. Financial constrains and time limitations requiring the submission of this thesis precluded incorporation of additional work on the molecular biology study at this juncture.

The existence of a high degree of molecular divergence in the xylose isomerase gene sequences from various species makes this molecular biology investigation a very complex process that is being fraught with extreme difficulties. The generation of shorter length PCR amplified bands representing the xylose isomerase gene renders itself inconsistent to the expected nature and the size of its protein product.

Kristo et al., (1996) had reported that in order to obtain the full-length cDNA and genomic DNA by PCR, the first strand of cDNA was synthesized using an anchor-(dT)-oligomer as a primer. The cDNA obtained was used as a template in the PCR reaction. Later the anchor sequence and the sequence corresponding to nucleotides 620-644 within the cDNA sequence were used as primers. The PCR product so obtained was purified and cloned employing a TA cloning kit. Southern-blot hybridizations using the cDNA, confirmed that the isomerase gene was around 2.5 kilobases in length.

The primers designed for the PCR amplification process reported here targeted the conserved nucleotide sequence in the 5'- end and the 3'- end of the eukaryotic xylose isomerase gene in the selected plant varieties that reportedly exhibited a nucleotide length of 1.2-1.4kb. The BMB primers were 20-27 mers in length and exhibited a G+C content of 47-55%, in all the three pairs of sense-antisense combinations synthesized.

The existence of several intron-exon boundaries in the xylose isomerase gene potentially caused severe limitations to the polymerase enzyme during the amplification process. The genomic structure of xylose isomerase is very complex, with 20 introns. All intron sequences start with GT and end with AG dinucleotides, tacitly following the universal exon-intron rule. The exon sequences were shorter and ranged over 41 -130 nucleotides. The most striking feature of the structure is the presence of a large intron in the 5' untranslated region. The gene contained three large introns (528, 400 and 190 nucleotides) and the 400 nucleotide intron remained located in the 5' un-translated region (Kristo et al., 1996). Occurrence of an intron in the 5' noncoding region is not a common trait for plant genes and was found only in few plant gene families (Mittler and Zilinskas, 1992). The extreme molecular divergence exhibited by the xylose isomerase family of genes became apparent when a comparison of nucleotide sequence of the genes as available in the data base was carried out. Additionally, sequence homology between the 5' sense and the 3' antisense regions within each conserved domains was found to be much less than 50 % and made it difficult to use the available knowledge on eukaryotic xylose isomerase genes for the benefit of predicting probable numbers and nature of the intron-exon junctions within the thermophilic xylose isomerase genes. Extensive work therefore needs to be carried out further to achieve the goal.

Conclusion

Based on the results obtained in the present study, it is concluded that

- The xylose isomerase enzyme in *Cereus pterogonus* and *Opuntia vulgaris* existed as two and three isoforms respectively, one being mesophilic while the other one or two being thermophilic isoforms.
- The conversion of xylose to xylulose and glucose to fructose was seen to be proceeding at greater than 55% efficiency in the both thermophilic xylose isomerase isoforms.
- The existence of alternate forms of xylose isomerase may be the result of alternate splicing or due to proteolytic processing of a precursor protein.
- The T_{80} and T_{90} isoforms are independently active for different time periods, in the temperature range 30°C to 100°C, and upto a maximum of 10 min at 100°C in presence of specific metal ions as stabilizing factor.
- The addition of Co^{2+} and Mn^{2+} stabilized the half-life of each of the thermophilic isoforms in the higher temperatures.
- The molecular weights of these isoforms were determined to be 68 kDa for T_{60} and T_{80} (*Cereus pterogonus)* and 68 kDa for T_{70} and 71 kDa for T_{90} (*Opuntia vulgaris).*
- Km value for xylose is very low when compared to that for glucose as a substrate for this enzyme activity. Temperature dependent denaturation profiles yielded two or three independent phases for the denaturation process of the thermophilic isoforms.

- Differences in the rate constants and thermodynamic properties (Ea, ΔG, ΔH, ΔS) were noted for each isoform undergoing the denaturation process in the temperature range 40°C to 100°C.
- Immunological cross reactivity and sequence interrelationship existed between the isoforms.
- Fluorescence intensity changes detected during the denaturation process confirmed conformational alterations occurring to the enzyme species. The intrinsic tryptophan fluorescence decreased with increasing temperature suggestive of possible molecular aggregation leading to burial of tryptophan residues within the protein structure.
- Differential Scanning Calorimetry studies yielded information on the melting temperature (Tm) for the T_{80} and T_{90} XI species.
- Molecular Biology experiments yielded two PCR amplified nucleotide bands for each of the xerophytic species under study presumably representing the XI gene. Work carried out thus far has yielded inconclusive results to provide information on the nucleotide sequence data for the thermophilic xerophytic XI genes.
- Living systems are considered open systems that can exchange matter and energy with the environment. Plants remain especially sensitive to the environmental changes such as water availability, soil and air composition and light intensity. Changes in the behavior of catalytic activities observed in our studies due to variations in the divalent cations and their concentration, and the temperature changes are therefore indicative of the adaptability of these plant enzymes to different habitats for plant growth. Consequently, the thermodynamic parameters influenced the adaptability processes within the plant cell. The metal ion effects

observed is suggestive of a regulatory role for these cations within plant cells, for stabilizing enzyme activities when influenced by a biotic stress in their habitats.

- Temperature stable plant enzymes should find significant application in industrial processes.
- Xerophytic plant species are potentially an alternate source of thermostable enzymes.
- The XI gene can be employed as an established marker for the selection of transgenic plants.