INTRODUCTION

1. INTRODUCTION

Thermophily is the phenomenon of exhibiting stability to high temperature environments. Organisms that have been recognized as having adapted to growth at high temperature are the group of thermophilic bacteria and a certain group of plant species classified as the xerophytes. While the former is representative of the prokaryotes found growing in terrestrial habitats and in the hot springs around the world, the latter remain distinctly eukaryotic generally found growing in arid habitats. The biology of these living species has adapted to synthesize thermostable enzymes that facilitated their normal course of metabolism while growing in extreme environments. By definition thermophilic enzymes are those that exhibited catalytic activity at temperature above 70°C. The high degree of stability of thermostable class of enzymes to high temperature as also chemical denaturants made them extremely useful biocatalysts in biotechnological applications. Heat stable enzymes therefore remain as important research material for understanding the basic principles governing the thermostability of proteins and their adaptation to high temperatures.

Microorganisms that were adapted to grow optimally at high temperatures (60-108°C) have been isolated from high temperature terrestrial and marine habitats. In more recent years, many heterotrophic extreme thermophilic (60-80°C) and hyperthermophilic (80-110°C) microorganisms have been discovered that utilized natural polymeric substrates as their carbon and energy source. Microorganisms capable of growing optimally at temperatures between 50°C and 60°C are designated as moderate thermophiles. Most of these microorganisms belong to many different taxonomic groups of the prokaryotes such as protozoa, fungi, algae,

streptomycetes and cyanobacteria, which comprise the mesophilic species. Moderate thermophiles, that are closely related phylogenetically to the mesophiles have secondarily adapted to life in the hot environments. Extreme thermophiles, in contrast grow optimally between 60°C and 80°C, and are widely distributed amongst the *Bacillus*, *Clostridium*, *Thermoanaerobacter*, *Thermus*, *Fervidobacterium*, *Thermotoga* and *Aquifex*. Hyper thermophiles, on the other hand, grow optimally between 80°C and 110°C (Stetter, 1998), which includes Archaea and Bacteria. They are found to facilitate the enzymatic degradation of polymeric substrates such as starch, cellulose, xylan, pectin and chitin (Stetter, 1996; Kristjansson and Hreggvidsson, 1995; Bertoldo and Antranikian, 2001). The occurrence of starch-hydrolyzing enzymes abundantly in extremophiles suggested that they had an important role in the bacterial metabolism. Enzymes from the use of thermophilic microorganisms are therefore being increasingly studied from a basic and an applied stand point. In this context, recognizing the existence of the xylose isomerase, in the eukaryote xerophytic plant species in alternate sources especially that represents a useful choice for a study of this enzyme physical, chemical and biological property. The exploitation of natural sugars glucose / fructose such in the syrup industry, the synthesis of non-fermentable carbohydrates and anti-salting agents in baking industry are all commercially important end use in the food industry.

Following is a list of thermophilic enzymes, their source and industrial applications for a comparative reference.

Thermophilic organisms, thermophilic enzymes and their applications

Enzyme	Origin	Application	References
Endo-1,4-β-glucanase	*T. maritime*	Cellulose degradation	Liebl (1996)
Endoxylanase	*Thermotoga spp. Strain FjSS3-B.1*	Paper pulp bleaching	Saul (1995)

β –Xylosidase	*Thermotoga spp. Strain FjSS3-B.1*	Paper pulp bleaching	Ruttersmith and Daniel (1993)
β –Mannanase	*Rhodothermus marinus*	Softwood pulp bleaching, coffee bean treatment and coffee extraction	Gomes and steinver (1998)
α – Glucosidase	*Thermoanaerobacter ethanolicus*	Starch processing	Saha and Zeikus (1991)
β- Glucosidase	*P. furiosus*	Regio-and stereoselective gluco conjugate synthesis by trans glycosylation	Kengen et al., (1993)
Trehalose synthase	*Thermus caldophilus*	α ,α-Trehalose production; used in food, cosmetics, medicine, and organ preservation	Koh et al., (1998)
Hydantoinase	*Bacillus stearothermophilus*	Synthesis of D-amino acids as intermediates in the production of semi-synthetic antibiotics, peptide hormones, pyrethroids, and pesticides	Lee et al., (1995)
Esterase	*P. furiosus*	Transesterification and ester synthesis	Ikeda and Clark (1998)
Aldolase	*S. solfataricus*	Synthetic chemistry; C-C bond synthesis	Buchanan et al., (1999)
Pectin methylesterase	*T. thermosulfurogenes*	Fruit juice clarification, wine making	Schink and Zeikus (1983)
Pectate lyase	*Thermoanaerobacter italicus*	Fruit juice clarification, wine making, fruit and vegetable maceration	Kozianowski et al., (1997)
Taq polymerase	*T. aquaticus*	PCR technologies	Bergquist and Morgan (1992)
Carboxypeptidase	*S. solfataricus*	C-terminal sequencing	Colombo et al., (1992)
α – Amylase	*Desulfurococcus mucosus*	Starch processing	Canganella et al., (1994)
β- Amylase	*Thermotoga maritime*	Starch processing	Schumann et al., (1991)
Pullulanase	*Bacillus flavocaldarius*	Starch processing	Kashiwabara et al., (1999)
Glucoamylase	*Clostridium thermosaccharolyticum*	Starch processing	Specka et al., (1991)
Xylose isomerase	*Thermoanaerobacterium thermosulfurogenes*	Starch processing	Lee and Zeikus (1991)
Polygalacturonate hydrolase	*T. thermosulfurogenes*	Fruit juice clarification, wine making	Schink (1983)
β –Fructosidase	*T. maritime*	Confectionery industry; production of invert sugar; hydrolysis of inulin to produce HFCS	Liebl et al., (1998)
Keratinase	*Fervidobacterium pennavorans*	Degradation of poultry feathers and production of rare amino acids (i.e., serine and proline)	Friedrich and Antranikia (1996)

α -Galactosidase	*T. maritime*	Sugar beet processing removal of raffinosefromsucrosesyrups; oligosaccharide synthesis through glycosyl transfer reactions	Liebl et al., (1998)
β -Galactosidase	*T. maritime*	Production of lactose-free dietary milk products	Gabelsberger (1993)
Alkaline phosphatase	*T. neapolitana*	Diagnosis enzyme labeling applications where high stability is required	Dong and Zeikus (1997)

Hither to investigations had not been carried out on the native of enzymes in the eukaryotic plant species characterized on xerophytes. Their growth in arid habitat and their ability to withstand extreme of terrestrial temperatures led to believe that some or all of their members were probably a rich source of eukaryotic thermophilic enzymes. Investigations were therefore initiated and directed at isolating and characterizing a few industrially important enzymes that during the course of these investigations were realized to be thermophilic in native. Xerophytic species have thus been identified and established as a primary source of eukaryotic thermophilic enzymes. Xerophytes are group of desert plants that includes the *Cereus pterogonus* and *Opuntia vulgaris* species. They have so far not been exploited by man for their catalytic, structural, flowering or fruiting capabilities. The present study aimed at a Physicochemical and biological characterization of xylose / glucose isomerase catalytic activity obtained from the cladodes of *Cereus pterogonus* and *Opuntia vulgaris*.

1.1 Cactus Evolution

Cenozoic cooling occurred around 50 million years ago and resulted in the formation of distinctly arid geographical regions, and contributed to the evolution of the group of plants called the xerophytes represented by the Cacti type of plants (Fensome and Williams, 2001). Cacti developed their physiological traits as a response to changing climatic conditions that

occurred several million years ago (Jacobsen, 1954c). Cacti were later defined by seven characteristics that were recognized in a special type jungle thorn bush (Chidamian, 1958). The thorn bush thus came to be established as the ancestor of all cacti. Although Cacti originated in the West Indies, they spread too many parts of the World, populating deserts in the Southwest America with hundreds of varieties (Schultz and Floyd, 1979) like the Beavertail Cactus (Boissenvain and Davidson, 1940; Proctor and Meyer, 1954) and the Jumping Cholla (Armstrong, 1982; Armstrong, 1992). A popular Lemon Vine, a hardy succulent, tropical, leafy and woody shrub produced the delicious "Barbados gooseberry" and resembled the ancestor cactus very closely.

Cacti that are capable of storing large quantities of water in their leaves, stems, or roots, giving them a fleshy (succulent) character are called succulent xerophytes. Most of the plants grow in drought-prone, nearly arid climates or physiologically dry soil (e.g. frequently frozen or periodically salty soils). This allowed them to adapt with minimal utilization of available water, and survive long periods without rainfall. In botanical ecology, plants exhibiting these qualities were called xerophytes.

Desert plants have adapted to the extremes of heat and aridity similar to microbial adaptations to extreme habitats using both physical and behavioral mechanisms. The Xerophytes have adapted by developing altered physical structure. They exhibit the presence of specialized tissues capable of storing and conserving water. Often they have few or no leaves and therefore exhibited reduced transpiration.

The plant surface generally appears tough and leathery, with the presence of ribs and spines and sometimes even fur. These are considered very smart adaptations, that helped the plant survive the heat, and perhaps even used for defense. Small, thick leaves with limiting

surface area, and the presence of thick cuticle restricted water loss from plants. The tough leathery skin being impermeable to water reduced evaporation from the surface of the plant (Benson, 1982, Cullmann et al., 1986).

Cacti also keep their stomata closed during day time especially when very hot preventing further loss of water through evaporation. Succulents have adapted such that their stomata remain closed during the day and are opened at night, allowing them to store carbon dioxide in their tissues as crassulacean acid that is converted to carbon dioxide during the day time. The plants absorbed carbon dioxide during the night, when the ambient temperature was lower, and reduced water vapour loss (Russell and Felker, 1987; Nobel, 1989). They converted carbon dioxide into organic acids and used them in photosynthesis during the day. This process called crassulacean acid metabolism or CAM is a very smart way of respiring in the desert conditions (Cullmann et al., 1986). Cacti however, depend upon chlorophyll in the outer tissue of their skin and stems to conduct photosynthesis for the manufacture of food. Some xerophytes lost their leaves in the dry months, and others even had their leaves rolled up to prevent water loss (with stomata in pits and surrounded by hairs to prevent water loss).

The juicy, slimy tissues in xerophytes contained significant amount of moisture for use during the dry seasons. Water and food is stored in the region between the pith and the palisade parenchyma occupied 85% of the plant's volume. This is a major adaptation found in desert plants. The plants remained completely alive during the dry season and continued to grow to larger sizes. The stored supplies facilitated cacti to energize flowering during spring, and grow much faster (Schwantes, 1957).

Root behavior is another adaptation that cacti have made for survival; Xerophytes however have an extensive and deep root system to extract maximum amount of water from

the soil. In addition extensive shallow radial root systems, allowed for quick acquisition of large quantities of water during rains. Since they stored water in the core of their stems and roots, cacti are well-suited to dry climates and they would survive years of drought using water collected from a single rainfall (Benson, 1982; Cullmann et al., 1986).

Some desert plants used behavioral adaptations, to develop lifestyles in conformance with the seasons of greatest moisture and/or coolest temperatures. Some of them also exhibited short life cycles that coordinated with the rain season. These types of plants were usually (probably inaccurately) referred to as perennials. They lived for several years, while annuals lived only for a single season. Desert trees and shrubs adapt by eliminating their leaves, replacing them with thorns (Riha and subik, 1981). They bear greatly reduced leaf size to eliminate transpiration (loss of water to the air). During stress, plants will often bend over as the 'hygroscopic' pressure (turgor) inside the plant diminished. The hygroscopic pressure held the plant erect, and as it reduced the plant experienced reduced ability to stand erect (Barthlott, 1977). When the plant bends over, it's exposure to the sunlight gets reduced, and much of the plant remained shaded by the overhead portion, thereby reducing moisture loss.

1.2 Protein Thermostability

The types, nature and properties of cacti enzymes though have not been studied in detail earlier; Cacti are expected to contain all various types and class of enzymes generally encountered in other eukaryotic systems. It was therefore exciting to venture and characterize individual catalytic activities available in the different cacti species, especially if they exhibited unique **Physicochemical** and biological properties that would feed commercial or industrial applications. Cacti as a class being xerophytic, targeting them as a source for the characterization of thermophilic catalytic activities was presumably well founded. Enzymes

that were stable at high temperature exhibited distinct Physicochemical properties that conferred thermostability on these biomolecules. The efficiency of many industrial processes had been improved by the use of thermo stable enzymes. Thermo stable proteins in general provided insight into the general mechanisms of protein folding and stabilization (Vogt and Argos, 1997). Stabilizing forces such as hydrogen bonding, hydrophobic interactions, ionic bonding, disulphide bridges and metallation are generally considered as responsible for the thermostability of an enzyme. There is also a delicate balance between these stabilizing interactions and destabilization due to the loss of conformational entropy of the folded protein (Matthews, 1987).

Amongst the stabilizing factors, hydrophobic interaction is considered a dominant force for structural stability of the macromolecules (Matthews, 1993). It is believed to provide the energy required for proteins to fold in aqueous solutions. As protein cores are typically hydrophobic, increased packing efficiency is often correlated with increased hydrophobicity (Sandberg and Terwillige, 1989). Site-directed (Ishikawa et al., 1993) and random (Turner et al., 1992) mutagenesis studies have demonstrated that the potential to increase thermostability lay in filling hydrophobic cavities in a folded structure.

Salt bridges represented another type of common interaction that stabilized proteins. Studies on the molecular mechanisms of irreversible thermal denaturation of α-amylase enzymes from *Bacillus licheniformis*, *Bacillus amyloliquefaciens* and *Bacillus stearothermophilus* by (Tomazic and Klibanov, 1988) suggested that the higher degree of thermostability of the enzyme from *B. licheniformis* was due to the presence of salt bridges involving a few specific lysine residues. These stabilizing electrostatic interactions reduced the extent of unfolding of the enzyme molecule at high temperatures making it less prone to

forming incorrect structures and thereby decreasing the overall rate of irreversible thermal denaturation. (Karshikoff and Ladenstein, 1998) suggested that electrostatic interactions are a common factor regulating the thermal tolerance of proteins from thermostable organisms. Based on the studies of the hexameric glutamate dehydrogenases from hyperthermophiles *Thermococcus litoralis* and *Pyrococcus furiosus*, (Vetriani et al., 1998) suggested that the formation of an extensive ion-pair network may provide a general strategy for manipulating enzyme thermostability for multi-subunit enzymes. (Vogt and Argos, 1997) noticed, from their studies on 16 protein families, a consistent increase in the number of hydrogen bonds and in the polar surface area fraction that increased with thermostability.

1.3 Establishment of High Fructose Corn Syrup (HFCS)

Earlier reports on xylose isomerase from *Lactobacillus pentosus* and *Pseudomonas hydrophila* showed that the enzyme converted D-xylose to D-xylulose, suggestive of an isomerization process (Mitsuhashi and Lampen, 1953; Hochster and Watson, 1954). The first purified and crystallized enzyme extracted by Slein from *Lactobacillus brevis* in 1955 exhibited ability for the inter-conversion of D-glucose and D-fructose and less efficiently for the conversion between D-ribose and D-ribulose. Later in 1957, Marshall and Kooi confirmed that D-xylose isomerase enzyme converted D-glucose to D-fructose (Sudfelt et al., 1990).

A partially immobilized xylose isomerase developed in Japan (1967) by Takasaki and co workers was later used in the commercial production of a fructose corn syrup (FCS) by USA, This product later on in 1970 became available as a 55% enriched fructose corn syrup (EFCS) on an industrial scale. The EFCS that was generated was used as a substitute sweetener for sucrose in the soft drinks industry and lead to production of High Fructose Syrup (HFCS+EFCS) (Pedersen, 1993).

1.4 Sources of Xylose isomerase

The existence of XI in barley malt (Bartfay, 1960) and wheat germ (Pubols et al., 1963) had been reported. The organisms that are commercially important as XI producers are *Actinoplanes missousriensis, Bacillus coagulans, Streptomyces rubiginosus, Streptomyces phaeochromogenes, Arthrobacter spp.*, and *Streptomyces olivaceus.* The highest yield of enzyme produced by *Lactobacillus brevis* was found to be active at low pH but was found unstabilized at higher temperature and resulting not suitable for economic exploitation. Yeasts such as *Candida utilis* (Wang et al., 1980) and *Candida boidinii* (Vongsuvanlert and Tani, 1988) have also been found to produce xylose isomerase. *Aspergillus oryzae* is the only fungus that is reported to possess XI activity. Xylose isomerase produced by the bacteria and actinomycetes was found to be active in the absence of arsenate. Extracellular XI in *Streptomyces glaucescens* (Weber, 1976) and *S. flavogriseus* (Chen and Anderson, 1979) was a consequence of a change in their cell wall permeability and a partial lysis of these cells. Purified extracellular XI had been obtained from *Chainia spp.* (Srinivasan et al., 1983; Vartak, 1984) and an alkalothermophilic *Bacillus spp* (Chauthaiwale and Rao, 1994) as well as from *Streptomyces spp.*, and several other *Bacillus spp.*

1.5 Chemistry and Biochemistry of xylose isomerase

Xylose isomerases have been found in at least 65 different organisms. Its enzymatic and Physicochemical properties have been extensively studied. Xylose isomerase belongs to the ketol isomerase group. Usually these enzymes are active on glucose and xylose. Some are able to convert other aldoses such as D-ribose, D-allose, L-arabinose, and L-rhamnose into the corresponding 2-ketoses (Sanchez and Smiley, 1975; de Raadt et al., 1994). These enzymes

generally occur intracellularly (Pawar et al., 1988). A D-xylose specific extracellular isomerase had been isolated from *Chainia spp.*, (Khire et al., 1990) but had no activity on D-glucose or D-ribose.

When the kinetic constants of xylose isomerases from different organisms were compared employing D-glucose or D-xylose as substrates, the general picture that emerged showed that V_{max} was of the same order of magnitude, but the K_m was one to two orders of magnitude greater for glucose than for xylose. The recommended name for the enzyme therefore became xylose isomerase.

The subunit structure and amino acid composition of xylose isomerase from different microbial sources have been examined. The molecular masses of xylose isomerase of different organisms determined by different methods showed that the enzyme may exist as tetramer, trimer, dimer or monomer, the monomer i.e. subunits remaining associated by noncovalent bonds. It has no interchain disulfide bonds. Nevertheless most xylose isomerases are found to be tetramers. The molecular weight of xylose isomerase also varied remarkably from 52 kDa to 191 kDa depending upon the source of the enzyme. Tetrameric forms of the enzyme existed in *Arthrobacter* spp. (Rangarajan and Hartley, 1992), *Streptomyces flavogriseus* (Chen and Anderson, 1979), *Thermotoga neapolitana* (Hess et al., 1998), *Thermus aquaticus* (Lehmbacher and Bisswanger 1990), *Lactobacillus xylosus* (Yamanaka and Takahara, 1977). *Bacillus coagulans* (Danno, 1971) and *Bifidobacterium adolescentis* (Kawai et al., 1994) were found to produce trimeric forms of xylose isomerase. Xylose isomerase reported by existed in dimeric forms in *Escherichia coli* (Schellenberg et al., 1984), *Hordeum vulgare* (Kristo et al., 1996) and *Streptomyces olivochromogenes* (Suekane et al., 1978). Monomeric forms of the enzyme were found in *Geobacillus stearothermophilus* (Suekane et al., 1978, Pawar et al., 1988).

1.5.1 Primary structure

XIs have been divided into two groups based on their sequence differences, class I and class II (Vangrysperre et al., 1990). Class I XIs contained 390 amino acids, while class II XIs typically contained around 440 amino acids, more in the N-terminal region by 30-40 amino acid (Bhosale et al., 1996). The functional role of the N-terminal insert in the class II enzymes remains unknown. Xylose isomerase from *S. violaceoniger* contains 388 amino acids and the molecular weight of the monomer is 43 kDa (Drocourt et al., 1988). Xylose isomerase from *Clostridium thermosulfurogenes* is a polypeptide of 439 amino acids with a molecular weight of 50 kDa. The amino acid sequence of this XI displays greater sequence identity with xylose isomerase from *B. subtilis* (70%) and *E. coli* (50%) than with isomerases from *Ampullariella* (22%), *Arthrobacter* (23%) and *S. violaceoniger* (24%) (Lee et al., 1990). Xylose isomerase from *S. rubiginosus* reportedly has 400 amino acids and two metal ions per monomer unit (Wong et al., 1991) and a typical tetrameric structure. Eventhough the enzyme required divalent cations (Co^{2+}, Mg^{2+}, and Mn^{2+}) for its stability and activity (Bogumil et al., 2000; Callens et al., 1988; Marg and Clark, 1990; Van Bastelaere et al., 1992), their relative importance differed for class I and class II XIs (Hartley et al., 2000). The primary structure of xylose isomerase from many microbial sources is known (Wong et al., 1991). For instance xylose isomerase from *A. missouriensis* contains 394 amino acids and its molecular mass per monomer is 43.5 kDa. The gene showed 74-92% homology with xylose isomerase genes from *Streptomyces*. The homology with *Bacillus* and *E. coli* isomerase genes however is found to below (Amore and Hollenberg, 1989). A number of three-dimensional structures have been solved for class I XIs (Carrell et al., 1989; Jenkins et al., 1992; Henrick et al., 1989 and Lavie et al., 1994) and have been shown to be essentially identical, and explains the similar

biochemical and thermostability properties of these enzymes. Comparisons between the structures of the most thermostable class I XIs from *Thermus caldophilus* and *Thermus thermophilus* with those from less thermophilic class I XIs from *Arthrobacter B3728* and *Actinoplanes missouriensis* revealed common thermostabilizing features such as increased ion pairing, lower surface to volume exposure, fewer exposed labile amino acids, and shortened loops (Chang et al., 1999). Similar comparisons among class II XIs have not been reported. Class II XIs have not been studied to the same extent as class I enzymes, probably because none are currently used commercially.

1.5.2 Secondary and tertiary structures

X-ray crystallographic structures have been determined for the group I isomerases from a number of bacteria. Structures differed only in detail. The crystallographic asymmetric unit is a dimer which in the crystals formed tetramers characterized by a 2x2x2 symmetry relating to four identical subunits. The monomers comprise two domains: a larger N-terminal domain (300-340 residues) forming a parallel eight stranded $\alpha\backslash\beta$-barrel, and a smaller C-terminal domain (60-70 residues) which forms a loop of helical segments stretching away from the barrel (Rey et al., 1988) The barrel of xylose isomerase is 3nm long and 4nm in diameter (Farber et al., 1987, Farber et al., 1988, Dauter et al., 1989; Mrabet, 1992). Tightly coupled dimers are formed by the loops of each monomer embracing and making extensive contacts with the barrel structure of a second monomeric unit. Pairs of these dimers associated weakly to form the tetramer. In solutions, xylose isomerase from *S. olivochromogenes* exists in a dimeric form, while xylose isomerase from *S. violaceoniger* existed as a tetramer (Glasfeld et al., 1988, Dauter et al., 1990).

Dissociation of the tetramer and monomer unfolding has been correlated with activity, and four distinct species have been detected (Callens et al., 1988; Rangarajan et al., 1992). They are (1) the active native tetramer (2) active dimers formed reversibly by 0.1% SDS treatment (3) an inactive dimer from guanidine-HCl or urea treatment and (4) an inactive monomer after heat treatment at 100°C for 5 min.

The tetramer was changed into dimers, whereas dimers do not refold readily into the tetramer with the same avidity. In case of *S. olivochromogenes* metal ions of xylose isomerase were bridged by the carboxylate of glutamate (Glu-216). Additional co-ordination existed to the side-chains of six other conserved amino acid residues, located C-terminus of β-strands to the β-strands of the α/β-barrel. Mg (1), although has four carboxylate ligands (Glu-180, Glu-216, Asp-244 and Asp-286) and an average Mg - O distance equal to 2.4 A°, the inter-atomic angles do not contribute to any ideal geometry. The second metal, Mg^{2+} secures to exhibit a near octahedral geometry by coordination to oxygens of Glu-216, Asp-256, imidazole ε-nitrogen of His-219, the two carboxylate oxygens of Asp-254, and a non-protein ligand, modeled as a hydroxide because of its short Mg – O distance (190 pm). However with Mn^{2+} at this site, the involvement of two additional water molecules is modeled to contribute to a recognizable octahedral coordination (Carrell et al., 1989; Whitlow et al., 1991). These ligating residues are grouped to make a highly charged region, Nevertheless. With in 800 pm exists a set of conserved hydrophobic residues (Phe-93, Trp-15, Trp-136 and Phe-25 from a second monomer) of the pocket, yielding a high hydrophobic contrast. Further, Lys-182, His-53 and Glu-185 also exists as conserved residues in this region.

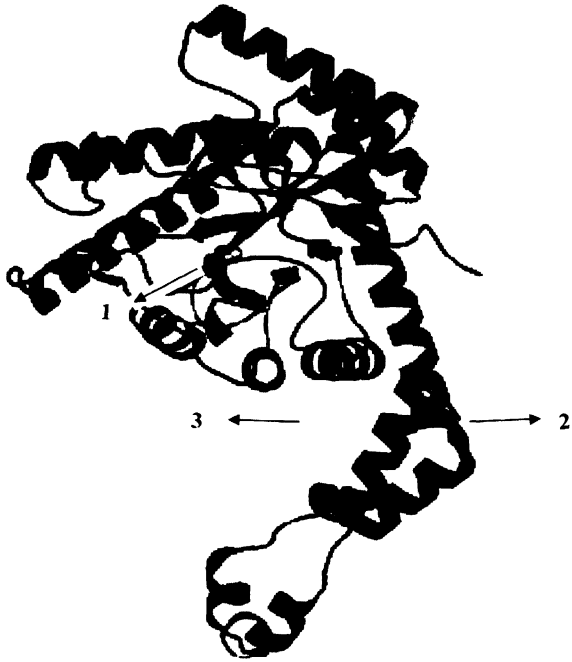


Fig. I Schematic view of folding structure of Glucose\xylose isomerase.
Figure by Z. Kovári (Budapest) using MOLSCRIPT.

1. The metal binding site; 2. Substrate binding site; 3. Catalytic domain.

Site directed mutagenesis of xylose isomerase in *S. olivochromogenes* was designed to remove the tight tetra co-ordinated Mg^{2+} binding site (site 1, Mg-1). Glu-180 was replaced with Lys-180. The stoichiometry of metal binding to the *S. olivochromogenes* mutant E180K was 1 mole of magnesium per mole enzyme. The enzyme ably opened the substrate ring but did not catalyze the isomerization of glucose to fructose (Allen et al., 1994b). Metal 2 was also involved in stabilization of the open chain intermediate and was able to coordinate both O1 and O2 during the catalysis. These observations indicated that both metal cations were necessary for catalysis (Lambier et al., 1992). The pH optimum changed from 7.2 to 6.2. Mutations also caused changes in substrate binding strength and substrate specificity. Thus, altered metal binding ability was generated by mutation of amino acid residues involved in the metal ion binding process (Allen et al., 1994a).The Schematic view of folding structure of Glucose \ xylose isomerase was represented in Fig. I.

1.5.3 Active site

Catalytically active amino acids are investigated employing chemical modifications for their residual activities. Mutations are targeted to alter specific amino acids (Batt et al., 1990). The activity, stability and other properties were then compared with the wild type enzyme. By such methods it has been shown that xylose isomerase contained histidine residues in its active site. Selective replacement of histidines 101 and 271 in *E. coli* xylose isomerase by site directed mutagenesis confirmed their importance as active site residues. However no significant change in the conformation of the mutant enzymes was observed by such mutations. The active site of *S. rubiginosus* xylose isomerase also contained two histidine residues, His-54 and His-217 corresponding to His-101 and His-271 in *E. coli* isomerase (Batt et al., 1990). Modification of arginyl, tryptophyl and tyrosyl side chains caused only minor

changes in the enzyme activity, suggesting that these groups had evidently little or no functional role in the enzyme function (Gaikwad et al., 1989).

In the case of xylose isomerase from *A. missouriensis* it has been shown that mutation in both His-220 and His-54 in the active site reduced the catalytic rate constant, suggesting that both histidines were important but not essential for the catalysis. Further, the observation suggested that His-54 affected the anomeric specificity (Lambier et al., 1992). The E186D and E186Q mutant enzymes are both active and their metal specificity is different from that of the wild type. The E186Q enzyme is most active with Mn^{2+} and exhibits a different pH optimum. With Mn^{2+}, both mutants perform like the wild type on xylose at neutral pH. At acidic pH the E186Q enzyme is significantly better than the wild type, its pH optimum being down shifted by more than one pH unit. Glu-186 is a conserved residue located near the active site but not in contact with the substrate and not with a metal ligand. In the presence of Mg^{2+}, the carboxylate group of the Asp-255 residue turns toward Gln-186 and hydrogen bonds to its side-chain amide. Mg^{2+} is not bound at metal site 2. Movement of Asp-255 occurred in the wild-type enzyme (Van Tilbeurgh et al., 1992).

(Matthews et al., 1987) proposed that stability of a protein can be increased by selective amino acid substitutions that decreased the configurational entropy of unfolding. Glycine, lacking a ß-carbon, has more backbone flexibility than alanine, with a β-carbon. More energy is therefore required to restrict the conformation of glycine compared with other residues, during the change from an unfolded to a folded state. Replacements with proline residues reduced the conformational degree of freedom in the main polypeptide chain and increased protein stabilization (Watanabe and Suzuki, 1998). In some thermostable enzymes α-helix stabilization has been observed (Menéndez-Arias and Argos, 1989). Comparison of 3-phosphoglycerate kinase enzymes of *B.stearothermophilus* and yeast have shown that

several α-helix lysine residues that are present in the yeast enzyme are replaced by glutamine, being a better helix stabilizer (Davies et al., 1993). In some cases disulphide cross-links have been found to have an important role in protein stability. Most of the early efforts to increase thermostability of proteins by site-directed mutagenesis focused on the introduction of non-native disulphide cross-links (Matthews, 1987).

1.5.4 Reaction mechanisms

Enzyme catalysis involves molecular recognition and rate acceleration. Molecular recognition, involves in bringing of the substrate and the enzyme into close proximity. In rate acceleration, the substrate-enzyme complex undergoes changes that resemble intramolecular reactions. Intramolecular reactions proceed at much faster rates than analogous intermolecular reactions. Isomerizations are reactions in which the chemical changes occur within a single molecule.

1.5.5 Recognition of substrate anomers

It has been shown that xylose isomerase is specific for the α-anomeric form of D-xylose instead of the β-anomer. Protons of carbons 1, 2, and 5 of the enzyme-bound α-D-xylose are equidistant from the bound Mn^{2+}. In the isomerization reaction of D-glucose the reactive species is the α-pyranose form (Young et al., 1975). The α-pyranose form of D-glucose is the primary product of isomerization of D-fructose. D-fructofuranose is the primary product in the reverse reaction, but due to the fast mutarotation of fructose, it has not been possible to resolve with certainty whether the α- or β- anomers of D-fructofuranose are formed (Pedersen, 1993; Carrell et al., 1994). The steric restrictions in the active site of the enzyme prevent a β-pyranose from binding in the same way. For the ketose isomers, the reactive species are α-furanose forms. Ketose forms are more rapidly interconverted between

α- and β- furanose forms (Feather et al., 1970; van Bastelaere et al., 1991; Collyer et al., 1992). Xylose isomerases of *Streptomyces spp.* however do not catalyze mutarotation of their substrates (Schray and Rose, 1971).

1.5.6 Substrate ring opening

Some investigations showed that the histidine at the active site is involved in substrate ring opening. This was supported by the observation that site directed mutants wherein histidine (101 in *E. coli*, 54 in *S. rubiginosus*) was replaced with another amino acid yielded catalytically inactive enzyme forms (Jamieson and Batt, 1992).

Trp-49 and 188 (*E. coli*) play an important role in efficient catalysis presumably in stabilizing the transition state during ring opening. Substitution of Trp-49 inactivates the enzyme while residual activity remains for substitutions of Trp-188. A consequence of this orientation is that the residue corresponding to His-101 might catalyze the intramolecular transfer of a proton from the anomeric hydroxyl to the ring oxygen of the substrate during the ring opening reaction (Henrick et al., 1989)

The initial enzyme-cyclic sugar complex is not the lowest point on the reaction free-energy profile. The enzymatic reaction is faster than spontaneous ring opening of pyranose sugars, so the enzyme must catalyse ring opening. Only a little is known about how the ring opening actually happens. Crystal structures of substrate-isomerase complexes show that the substrate is bound in an open and extended form (Whitlow et al., 1991; Carrell et al., 1989).

In the presence of open-chain ligands, metal site 1 is apparently identical in *A. missouriensis, Arthrobacter,* and *S. rubiginosus* xylose isomerases, with octahedral coordination to four carboxylate oxygens of equivalent residues and to O2 and O4 of the ligand. In the crystal structure of the *S. rubiginosus* enzyme in complex with Mn^{2+} and xylose,

Mn^{2+} has two alternative positions 1.76A° apart. Only the one with lower occupancy binds to the substrate (Whitlow et al., 1991). Metal ion binding at both sites is required for enzymatic activity. All available structural data on all xylose isomerases show that metal 1 binds to the substrate in cyclic form through O3 and O4 and in open form through O2 and O4. Shorter metal-oxygen bonds and a more favorable octahedral geometry in the second case may be the driving force for ring opening (Jenkins et al., 1992).

Metal 2 is not essential for substrate binding and ring opening. The *S. rubiginosus* enzyme bound substrate in the absence of metal, largely a cyclic species (Whitlow et al., 1991). *A. missouriensis* xylose isomerase mutants D255A and D257K also bound the substrate and aided ring opening in a similar way ring open. Both of these mutants had their metal binding site 2 destroyed. However, metal 2 was found essential for isomerization. This involved a hydride transfer between C2 and C1 and proton transfer between O2 and O1 (Jenkins et al., 1992).

Structures of enzyme complexes containing analogues in cyclic forms have been resolved. 5-Thio-α-D-glucose bound to Mn^{2+} isomerase from *Arthrobacter* was the first model of a complex studied (Blow et al., 1992). The sugars are in their chair conformations, with O3 and O4 of the pyranose ring bound to Mn (1). There are no direct interactions with Mn (2), but the imidazole of His-53 is placed close to sulphur of 5-thio-α-D-glucose, and within hydrogen bonding distance of the anomeric oxygen in 5-thio-α-D-glucose. This probably was the way that the aldose substrate initially bound the enzyme. The imidazole of His-53, which is polarized by the carboxylate of Asp-56, catalyzed opening of the ring by providing a correctly positioned base to transfer a proton from O1 to O5 of a pyranose sugar. The ring opening is initiated by proton transfer to histidine from the anomeric oxygen. This is followed by rate

limiting hydride shift assisted by the metal cation. The cation provided electrostatic stabilization of an anionic transition state, (Whitlow et al., 1991). However, in the x-ray crystal structure of the isomerase from *S. rubiginosus* complexed with 5-thio-α-D-glucose, His-54 is not positioned to deprotonate the anomeric oxygen (Whitaker, 1995). Involvement of this histidine as a catalytic base in ring opening has been challenged (Carrell et al., 1989).

1.5.7 Hydride shift or enediol intermediate

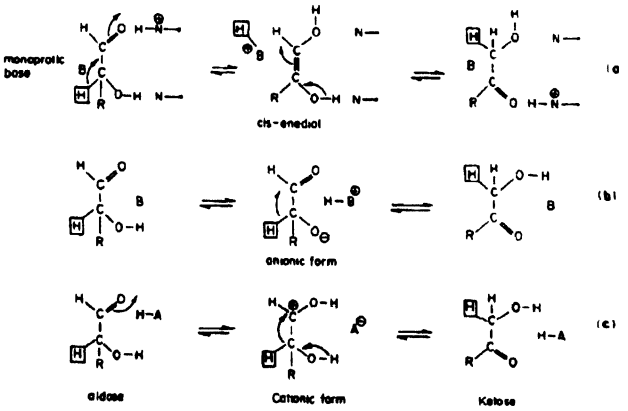
There are two mechanistic hypotheses of how the isomerization reaction with xylose isomerase proceeds; the first suggests an enediol intermediate and the latter suggests a hydride shift mechanism. The 1, 2-enediol intermediate was suggested to be analogous to the mechanism of triose phosphate isomerase and glucose-6-phosphate isomerase (Rose et al., 1969; Rose, 1981). Later observations pointed out that the expected proton exchange with the medium could not be detected. Experimental data showed that the position of bound substrate with respect to the active-site residues was not suitable for base catalyzed proton abstraction because no base was observed in a reasonable location. This was strong evidence to support the metal ion mediated hydride shift mechanism. This mechanism was consistent with the presence of two defined metal ions close to the catalytic site. Crystallographic studies strongly supported the hydride shift pathway (de Raadt et al., 1994; Allen et al., 1994a; Collyer and Blow, 1990). The hydride shift mechanism is presented in scheme Ia (Whitlow et al., 1991).

Exchange of a solvent proton with the migrating hydrogen has not been observed (Rose et al., 1969). Reaction of C2-tritiated glucose, over a range of reaction conditions designed to reveal hidden proton transfers, showed a loss of less than one proton in every billion turnovers. This observation is strong evidence against an enediol intermediate as well as the absence of fluoride release from the competitive inhibitors 3-deoxy-3-fluoroglucose and

3-deoxy-3-fluoroallose. The rearrangement is stereo selective, with isomerization of D2-deuterioglucose yielding (R)-D1-deuteriofructose as the only detectable product and D1-deuterioglucose yielding (S)-D1-deuterofructose (Bock et al., 1983; Farber et al., 1989; Smart et al., 1992)

1.5.8 Temperature dependency and pH optimum

Microbial xylose isomerases are active and stable at high temperatures (even at 80°C). The temperature stability of the enzyme obtained from *S. phaeochromogenes* increased by 10°C in the presence of Co^{2+} (Table 1). The enhanced thermostability (90°C) of xylose isomerase from *Thermophilus spp.* is proposed to be dependent on the number of intra- and inter-subunit ion pairs (Chang et al., 1999; Quax et al., 1991).



Scheme Ia. Mechanism of action of Glucose \ xylose Isomerase. (a) *cis*-Enediol. (b) Proton transfer. (c) Hydride shift. Boxes indicate the hydrogen atoms that are transferred stereospecifically.

Generally, xylose isomerases function well in the pH range 7.0–9.0. The enzyme tolerates even higher pH values and can be preserved at pH 10-11 at 30°C for several days. Xylose isomerase is stable in the pH range of 6.5 to 8.5 at 30 °C for at least 2 h in the absence of metal ions. Below pH 6.0 the enzyme is rapidly inactivated. Co^{2+} gave 15% protection down to pH 5.0, below which the enzyme was not protected by either Co^{2+} or Mg^{2+} (Mrabet et al., 1992).

The pH optimum of the enzyme from *Streptomyces spp* except that from *S. flavogriseus* is higher than that from other bacteria. The activity decreased rapidly at lower pH values. An exception to this is the enzyme from *T. aquaticus,* which is active at pH 3.5 and has full activity at pH 5.5. The reported values of the isoelectric point are between 4 and 5. Properties of xylose isomerases from different bacterial sources are summarized in table I.

1.5.9 Inhibitors

The catalytic activity of xylose isomerase is inhibited by heavy metal ions such as Ag^{+}, Hg^{2+}, Cu^{2+}, Zn^{2+}, Ni^{2+} and to some extent by Ca^{2+} (Callens et al., 1986). Other inhibitors are generally substrate analogs xylitol, arabitol, sorbitol, mannitol and lyxose (Takasaki et al., 1969; Schray and Mildvan, 1972; Lehmacher and Bisswanger, 1990). Cyclic α-xylopyranose analogues such as 5-thio-α-D-xylopyranose, α-D-xylopyranosylfluoride, glucal, and 1,5-anhydroxylitol are weak competitive inhibitors. The K_i values are one to two orders higher than the K_m for the substrates they were designed to model (Allen et al., 1994a; 1995). The metal chelating agent EDTA removes Mg^{2+} easily and yields an inactive enzyme. In contrast, elimination of Co^{2+} takes more time suggesting tighter binding of Co^{2+} than Mg^{2+}. The activity

of EDTA treated enzyme can be restored by adding activating metal ions into the medium (Callens et al., 1988;; Danno, 1971; Yamanaka, 1968).

Table I. Some properties of xylose isomerases from different microbial sources

Microorganism	Operating temp. °C	Optimum pH	Metal ion requirement	Molecular weight	No. of Subunits
E. coli	45	6	-	96 000	2
B. subtlis	50	7.5	-	-	-
L. brevis	40	7.1	Mn^{2+},Co^{2+}	195 000	-
Arthrobacter B3728	80	8	Mg^{2+}	180 000	4
Ampullariella sp 3876	75	-	-	170 000	4
S. violaceoniger	>70	7.5	Mg^{2+}	172 000	4
Streptomyces YT-5	80	8-8.5	Mg^{2+},Co^{2+}	157 000	4
S. olivochromogenes	80	10.8	Mg^{2+},Co^{2+}	172 000	2
S. flavogriseus	70	7.5	Mg^{2+},Co^{2+}	171 000	4
S. griseofuscus	85	8.5	Mg^{2+},Co^{2+}	180 000	4
S. violaceruber	80	7.5-9.5	Mg^{2+},Co^{2+}	-	-
S. rubiginosus	80	7.5-8.5	Mg^{2+},Co^{2+}	172 420	4
B. coagulans	75	7	Co^{2+}	160 000	4
B. stearothermopillus	80	7.5-8.0	Co^{2+}	130 000	-
T. aquaticus HB8	70	7	Mn^{2+},Mg^{2+}	-	-
A. missouriensis	90	7.0-7.5	Mg^{2+},Co^{2+}	80 000	2

1.5.10 Inactivation of xylose isomerase

Several physical or chemical conditions can cause inactivation of xylose isomerase, which seems to be an amazingly stable enzyme form. Most potent inactivators in industrial conditions are temperature, inactivating metal ions such as calcium, and microbial or chemical contaminants. Glucose is reportedly an allosteric modifier of this enzyme activity when xylose was used as the substrate. In addition, various denaturing agents can cause irreversible dissociation of the tetrameric structure to its inactive monomers.

1.5.10.1 SDS

Addition of 0.1% SDS to the enzyme solution caused the dissociation of the tetrameric enzyme into active dimers with 70% of the initial activity remaining. Traces of tetrameric and monomeric enzyme are also detected. Removal of SDS resulted in reassociation to the tetrameric species. Neither Co^{2+} nor Mg^{2+} affected the dissociation into dimers. Treatment with 3% SDS at 70 °C (1 hr) led to formation of either inactive monomers or inactive aggregates (Callens et al., 1988; Danno, 1971).

1.5.10.2 Urea and Guanidine-HCl

Urea (8M) at 35°C and pH 8.0 only partially destroyed the structure of xylose isomerase. Both Co^{2+} and Mg^{2+} protected the active enzyme conformation. The original activity could not be recovered by dilution of the urea solution to 0.4M. The inactivation being due to the dissociation of the active structure into monomers. Urea did not remove Co^{2+} from the enzyme. The effect of guanidine-HCl was more destructive. A concentration of 2M guanidine-HCl caused an immediate and total loss of activity. Formation of the monomers and inactive aggregate was obvious after 1 hr treatment of apoenzyme and Co^{2+}- enzyme with 5M

guanidine-HCl (Callens et al., 1988). The same kind of behaviour was found with xylose isomerase from *Arthrobacter* NRRL B3728. Almost identical inactivation was observed when xylose isomerase from *Streptomyces spp.* NCIM 2730 was tested with urea and guanidine-HCl (Rangarajan et al., 1992).

1.5.11 Thermal inactivation

Incubation of the apoenzyme at 80°C led to a rapid inactivation in contrast to the increase in specific activity observed for metal loaded enzyme samples. Addition of 1 eq Co^{2+} per monomer is sufficient to yield the thermostable isomerase. No further stabilization occurred with further addition of Co^{2+} (Callens et al., 1988).

The inactivation of xylose isomerase of *S. olivochromogenes* has been studied by Volkin and Klibanov, (1989) who concluded that deamidation is responsible for the inactivation at high temperatures. They further found that immobilized glucose isomerase gradually lost its activity at 80°C, pH 8.0. This process closely adhered to first order kinetics. Thermal inactivation was irreversible. Immobilization did not stabilize this enzyme. The free enzyme aggregated upon heating but the immobilized one did not and yet the two had comparable thermostabilities. Aggregation was probably an accompanying phenomenon rather than a cause of irreversible thermoinactivation (Chang et al., 1999).

The thermostability of *Thermoanaerobacterium thermosulfurogenes* xylose isomerase was enhanced by site-directed substitutions of aromatic amino acids in the active site leading to reduction of the area of water-accessible hydrophobic surface (Meng et al., 1993). Mutants with one or more disulfide bridges or additional salt bridges between subunits A and A* showed no change in enzyme activity or stability compared with the wild-type enzyme,

suggesting that subunit dissociation does not lie on the pathway of thermal deactivation (Lin, 1986).

1.5.12 Reaction kinetics and thermodynamics

Xylose isomerases generally follow Michaelis-Menten kinetics, and some values for K_m and k_{cat} were determined at 35°C (van Bastelaere et al., 1992) Pentoses are better substrates than hexoses. The isomerization of glucose to fructose is a reversible reaction. The equilibrium conversion of glucose to fructose is, under industrial process conditions, about 50% and increases to 55% at 90°C and to 60% at 115°C. The reaction is slightly endothermic, with an enthalpy of 5 kJ/mol (Pedersen, 1993; Tewari and Goldberg, 1985). From the effect of temperature on the equilibrium constant, it was found that the heat of the reaction, ΔH, of the formation of D-fructose from D-glucose was +2.22 kcal/mol. The endothermic heat of reaction in a packed bed reactor calculated from the slope of the Arrhenius plot is +5.42 kcal/mol. The difference in the heat of reaction could be due to diffusional limitations (Converti and Del Borghi, 1997, 1998).

1.6 Molecular biology of the Xylose isomerase gene

Proteins in general have evolutionary signatures engraved in their sequences. To extricate those unravelled valuable data extensive bioinformatic tools have been used nowadays. To elucidate the structure-function relationship of the D-xylose isomerases (XIs), the *xylA* gene sequences from various organisms have been compared. The nucleotide sequences of a number of XI genes have been determined, and their deduced amino acid sequences are available. Many such studies on xylose isomerases indicate that the amino acid composition differentiates them into two main groups, The D-xylose isomerases of

Escherichia coli and *Bacillus subtilis* constitute one group, and the other group includes the enzymes from *Actinoplanes*, *Ampullariella*, and *Streptomyces spp.* Meaden et al., (1994) have compared the deduced amino acid sequences of the 19 bacterial XIs obtained from the databases (Swiss-PROT version 23) and those described by Scheler et al., (1991) and Rygus et al., (1991). Considering the degree of similarity of the proteins and their statistical significance, it was inferred that all the proteins were homologous and made up a single family. On the basis of the G+C content of the DNA and the physicochemical properties of XI, the proteins were divided into two distinct clusters, such as the proteins from organisms with high G+C DNA content, and those from organisms with low G+C DNA content. The first cluster represents XI from four *Streptomyces spp.*, *Thermus thermophilus*, *Actinoplanes missouriensis*, *Ampullariella spp.*, and *Arthrobacter spp.* The second cluster includes XI from three *Bacillus* sp, *Staphylococcus xylosus*, two *Lactobacillus spp.*, *Escherichia coli*, and *Klebsiella pneumoniae*. The distribution of the proteins between the two clusters relates to the phylogenetic relationships among the organisms. Analysis of the aligned sequences revealed two signature sequences, namely, VXW(GP)GREG (YSTA)E and (LIVM)EPKPX(EQ)P, which recognized all XI and no other proteins in Swiss-PROT. In spite of the low homology between the *Streptomyces* and *Escherichia coli* or *Bacillus* enzymes, the amino acids involved in the substrate and metal ion binding, as well as in catalysis, are completely conserved. The *cis* peptide linkage between the adjacent glutamic acid and the proline which is responsible for the formation of the rigid structure at the active site is also well conserved in all the XIs studied. Thus, the essential structure at the catalytic center of XI appears to be analogous in all the enzymes that were compared. The information on the conserved and homologous regions in the *xylA* sequences will therefore be a valuable tool for isolating novel XIs with desirable catalytic properties.

1.6.1 Xylose isomerase gene cluster

Genetic studies on *Salmonella typhimurium* provided evidence for the existence of four clustered genes (*xyl* operon) that were responsible for xylose catabolism (xyl operon), they are *xylT*, the gene regulating xylose transport across membranes, *xylA*, the glucose/xylose isomerase gene; *xylB*, the xylulokinase gene; and *xylR*, a regulatory element essential for transcription of *xyl* genes (Shamana and Sanderson, 1979a, 1979b). In the case of *Escherichia coli* an analogous genetic organization and a similar xylose utilization pathway was identified (Maleszka et al., 1982). Feldmann et al., (1992) have suggested the presence of a regulatory gene in the 59 nucleotides upstream of the *xylA* gene of *Klebsiella pneumoniae*, responsible for the *xyl*-negative phenotype in recombinant *E. coli* mutants.

1.6.2 Divergent Promoters

In silico analysis has revealed that the *xylA* gene of *Streptomyces violaceoniger*, *xylA* and *xylB* promote transcription in opposite directions (Bhosale et al., 1996). The existence of divergent promoters in *Streptomyces spp.* and other prokaryotes was reported previously (Beck and Warren, 1988). Sequence analysis has indicated the presence of a third reading frame, which encodes a regulatory protein. The two genes are separated by a short region (195bp), which revealed the presence of an element with palindromic symmetry typical of bacterial operators. It is suggested that a regulatory molecule may act within the divergent transcription unit to control the expression of opposite genes and also regulate its' own synthesis.

Wong et al., (1991) have conducted a detailed analysis of the genetic organization and regulation of the xylose degradation genes in *Streptomyces rubiginosus*. His study reveals that the genetic organization of *xylA* and *xylB* genes in this organism differs considerably from that in the other bacteria. The two genes are transcribed divergently from within a 114bp sequence

separating the two coding regions, in contrast to the earlier observation that *xylA* and *xylB* genes are part of an operon. The transcription initiation sites are 40 and 20bp upstream of the translation initiation sites of *xylA* and *xylB*, respectively. The promoters of the genes share a 33bp overlapping sequence in the untranscribed region between the two genes. The transcription of *xyl* genes in *S. rubiginosus* is induced by xylose and repressed by glucose. It was believed that the 114bp nucleotide intergenic region provides the binding site(s) for the regulatory proteins.

All these studies explain how the relationship of structure and function of the xylose isomerase operate coordinately, offering the organization of *xylA* and *xylB* to be highly conserved in all bacteria. These two genes are always adjacent to each other, but a closer inspection revealed marked divergence in their organization. In *Bacillus subtilis*, the *xylR* gene has a polarity opposite to that of the *xylA* gene, unlike in *Staphylococcus xylosus* and *Lactobacillus spp.* In *Streptomyces spp.*, the *xylA* and *xylB* genes are transcribed divergently on different strands, whereas in *E. coli*, *Lactobacillus spp.*, and *Bacillus spp.*, they are transcribed from the same strand. The analyses of *xyl* genes therefore from a variety of organisms will yield consensus opinion about the genetic organization and regulation of the *xyl* genes.

1.7 Applications of xylose isomerase

1.7.1 In the production of HFCS from starch.

The primary importance of the enzyme xylose isomerase is in the industrial glucose – fructose conversion process. Additional use of this enzyme activity is in the production of HFCS from starch. This comprises of three enzymatic process steps namely liquefaction of

wet-milled corn using α-amylase, Saccharification using glucoamylase and optionally a debranching enzyme and in an isomerization process utilizing xylose isomerase.

In early industrial processes isomerization was catalyzed in batch reactors with a soluble enzyme. An improved method was described using heat treated *Streptomyces albus* containing the enzyme in a packed bed reactor through which glucose was passed. Entrapment of cells in gels or fibres has been widely employed for many xylose isomerase containing organisms. The cells of several *Streptomyces spp.* were reported to be entrapped in polyacrylamide gels and used in a continuous or batch process. A stable immobilized cell system was obtained by adsorption of cells of *Streptomyces* on DEAE-Sephadex A-50. Eighty percent of the original activity was retained in a column after operating 40 days at 60°C (Ge et al., 1999).

Industrial isomerization processes are usually run at temperatures around 60°C and at pH 6.8–7.5 resulting in 45% fructose and 55% glucose. High fructose corn syrup production is initiated by hydrolysis of corn starch at acidic pH. This pH is too low for the isomerization reaction and the solution may contain high concentrations of Ca^{2+} ions. Therefore the hydrolysate is ion exchanged and neutralized before isomerization. Under alkaline conditions D-psicose is formed in hot glucose and fructose solutions. Therefore a low pH optimum is preferred for the enzyme function in this application (Chen, 1980).

Various HFCS products are available commercially. The standard HFCS containing 42% fructose is equivalent to sucrose syrup in sweetness and has no side flavours or odours. Some HFCS contains fructose up to 90%. HFCS has several commercial applications in foods. The desired fructose content, 55%, used in soft drinks may be obtained in theory by the use of either high concentration of ethanol (85-90%) or by the use of high temperature (88°C) in the

isomerization process (Tewari et al., 1985). The drawbacks with these methods are the low solubility of glucose at high ethanol concentration and the increased risk of chemical side-reactions at high temperature. Therefore neither of these conditions is applied in industrial processes. The highest reported conversion of glucose to fructose is 88-90 %. This high fructose yield was achieved by adding tetraborate into the reaction medium to form complexes with fructose (Takasaki, 1971).

In industrial use and especially in food manufacturing it is important that all the materials used are safe. Xylose isomerase from *S. murinus* has been tested for its safety properties. These tests revealed that it was not mutagenic nor did it provoke chromosomal damage. It did not have antimicrobial activity. It had no teratogenic activity, either when administered to pregnant rats at 100000 ppm in the diet (Ashby et al., 1987).

Xylose isomerases are usually used in industrial applications as immobilized catalysts. Several methods for immobilization have been developed. Immobilized xylose isomerase from a *Streptomyces spp.* had a 240 day half-life under continuous operation at 50°C. For maximal activity and stability, the presence of activating metal ions in the medium is necessary. Generally, immobilized enzymes have a narrower pH optimum than free enzymes while operating temperature can be increased. The pH optimum can be slightly shifted if the enzyme is immobilized on charged supports. In general, the apparent pH for optimal activity shifts toward the acidic side when an enzyme is bound to a polycationic support, and to the alkaline side if bound to a polyanionic support. Shifts in pH optima are observed in solutions of high ionic strengths. Immobilized xylose isomerases usually have higher K_m values than soluble ones indicating that bound enzymes are somewhat less effective catalysts than free enzymes (Ge et al., 1999).

1.7.2 In ethanol production

Xylose isomerase can also be used in ethanol production. The principal agricultural residue, xylose, is converted to xylulose by this enzyme and then to ethanol by fermentation. Bioconversion of renewable biomasses to fermentable sugars and ethanol may become important in future due to depletion of fossil fuels. The economic feasibility of biomass utilization depends on the hydrolysis of cellulose and hemicellulose to glucose and xylose and their subsequent fermentation to ethanol by yeasts. So far, no industrial applications exist of xylose isomerase for ethanol production (Bhosale et al., 1996).

1.7.3 For analytical applications

Xylose isomerase has been used in analytical applications in combination with other enzymes and cofactors. A coupled enzyme system for detection of D-xylose and D-xylulose had been developed. This system is based on three consecutive enzymatic steps. The enzymes xylose isomerase, mutarotase and glucose dehydrogenase are co-immobilized in a packed bed reactor. The key reaction is the conversion of β-D-xylose with glucose dehydrogenase to δ-xylonolactone in the presence of NAD^+ that is converted simultaneously to NADH. NADH is then analyzed by UV spectroscopy at 340 nm. D-xylulose can be analyzed if it is produced by xylose isomerase and further when converted to β-D-xylose by mutarotase (Dominguez et al., 1994; Kersters-Hilderson et al., 1977).

1.7.4 XI gene as a marker in transgenic plants

During transformation of plant tissues only a limited number of cells are transformed. Selection of the transformed plant cells has traditionally been accomplished by introduction of an antibiotic or herbicide resistance gene, enabling transformed cells to survive on medium containing the inhibitor compound. The debate continues worldwide, regarding the safety of

these markers in genetically modified food organisms (Flavell et al., 1992; Fuchs et al., 1993; Miller, 1994; Nap et al., 1992). The controversy over the environmental effects of the use of antibiotic resistance markers have triggered the scientific community working with genetically modified food organisms to probe for an alternative.

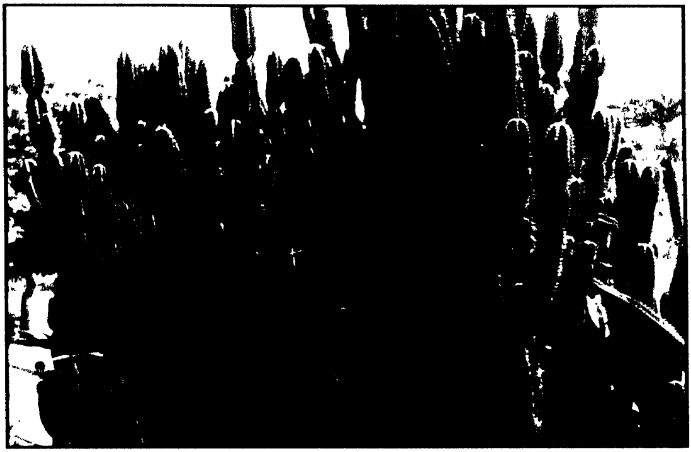
Haldrup et al., (1998) have reported a new method for the selection of transgenic plants which involves selection of transgenic plant cells expressing the *xyl*A gene from *Streptomyces rubiginosus,* which encodes xylose isomerase on medium containing xylose. The XI selection system was tested in potato and the transformation frequency was found to be approximately ten fold higher than with kanamycin selection. The level of enzyme activity in the transgenic plants selected on xylose was 5- to 25-fold higher than the enzyme activity in control plants. This novel selection system is more efficient and is independent of antibiotic or herbicide resistance genes, but dependent on an enzyme that is generally recognized as safe for use in the starch industry, as well as being extensively utilized in specific food processes.

Scope of the present investigation

The present study aimed at the purification and characterization of thermophilic xylose isomerase from the xerophytic species *Cereus pterogonus* and *Opuntia vulgaris,* found commonly growing in the coastal areas of Puducherry. Specific objectives of the study included investigations on the Biochemistry, Biophysics, Immunology, Molecular Biology and Bioinformatics of the enzyme protein from these two species. Critically, they are as follows -

- ✓ Isolate, purify and characterize thermophilic xylose isomerase enzyme activity from the xerophytic eukaryotes, *Cereus pterogonus* and *Opuntia vulgaris* plant species.
- ✓ Biochemical characterization through, pH and Temperature studies, normal and denaturation kinetics, Gel filtration, SDS-PAGE, Activity gel assay.
- ✓ Estimate catalytic rate constant, reaction free energy, activation energy, enthalpy and entropy.
- ✓ Investigate the purified enzyme for endogenous cations employing Electron Paramagnetic Resonance Spectrometry, thermal denaturation/aggregation using Spectrofluorimetry, and Tm using Differential Scanning Calorimetry.
- ✓ Generate polyclonal antibodies to XI and study cross-reactivity with other thermophilic and mesophilic XI species.
- ✓ Isolate genomic DNA, mRNA, synthesize cDNA, PCR amplification of xylose isomerase gene.
- ✓ Use bioinformatics tools for comparative analysis of thermophilic and mesophilic bacterial and plant XI enzymes, and also use information for primer design.

Cereus pterogonus



Opuntia vulgaris