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Review paper

XYLOSE ISOMERASES FROM THERMOTOGALES

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ABSTRACT

Biotechnology has been directed primarily towards reproductive technology been employed for improvement of industrial important enzymes which are foremost concern over the years for researchers. This review paper discusses the exciting scientific and technical advances in molecular biology for the genetic improvement in thermotogales, the hyperthermophiles with respect to xylose isomerase to meet the industrial demands. A thermo-acid stable enzyme possesses neutral or slightly acidic pH optima and a higher affinity for glucose have a potential for industrial applications for the production of high fructose corn syrup (HFCS). Xylose isomerases from *Thermotoga* sp are class II enzymes, utilize a 1, 2 hydride shift catalytic mechanism, active only in the presence of Mn^{+2}, Co^{+2} and Mg^{+2}. Recombinant xylose isomerases from *T. neapolitana* existed both as homodimer as well as homotetramer have been produced under mesophilic fermentation conditions, with a maximal activity at 97°C. Mutant enzyme in addition to this catalytically active at pH 5.5 and showed 3.1 fold increased catalytic efficiency towards glucose. The addition of the carbohydrate biding domain to *Thermotoga*'s xylose isomerase successfully immobilized the enzyme to chitin beads. The turnover numbers (k_{cat}) for glucose to fructose conversion for both unbound and immobilized mutants was greater than the wild-type enzyme.

Key words: xylose isomerases, thermotogales, hyperthermophiles

INTRODUCTION

Xylose/glucose isomerase (EC 5.3.1.5) is one of the enzymes related to xylose metabolism, encoded by gene *xyl-A*. It used *in vivo* to convert xylose to xylulose, which is then phosphorylated and transferred to the pentose-phosphate pathway as well as it also catalyses the conversion of D-glucose to D-frutose *in vitro*. This latter activity is used in industry for the production of high fructose corn syrup (HFCS). Hence, xylose isomerase (XI) is one of the largest volume commercial enzymes used today. Most industrially used xylose isomerases (XIs) are isolated from mesophilic organisms (e.g. *Streptomyces* spp and *Actinoplanes* spp). The reaction temperature used in the current industrial glucose isomerization process is limited to 60°C because of by-product and color formation that occur at high temperature and alkaline pH, because the isomerases themselves are not highly thermostable (Lee and Zeikus, 1991; Vieille and Zeikus, 2001). Equilibrium for the isomerization reaction is shifted toward fructose at high temperatures. Currently operated at 58 to 60°C with moderately thermostable XIs, the industrial process gives rise to 40 to 42% fructose syrup, requiring an additional chromatography step to achieve the 55% fructose concentration needed for high-fructose corn syrup. The isomerization reaction taking place at 85 to 95°C would permit the direct production of 55% fructose syrups (without the last chromatography step). Thermostable XIs with neutral or slightly acidic pH optima have a potential for industrial applications. Performing isomerization at higher temperature and neutral or slightly acidic pH with thermo-acid stable XIs would allow faster reaction rates, higher fructose concentrations at equilibrium, higher process stability, decreased viscosity of substrate and product streams, and reduced byproduct formation (Lee and Zeikus, 1991). An ideal XI should also have a resistance to inhibition by Ca^{+2} and a higher affinity for glucose than do presently used enzymes. Introduction of all these properties into a single protein is a herculean task, which has been an obstacle in the development of an economically feasible commercial process for enzymatic isomerization of glucose to fructose. Advances in recombinant DNA technology and protein engineering have opened new and encouraging possibilities of combining the desirable properties in a single organism to produce a tailor-made protein (Bhosale *et al.*, 1996). Brown *et al.* (1993) and Starnes *et al.* (1993) in fact, isolated and characterized XIs from the hyperthermophilic eubacteria *Thermotoga maritima* and *Thermotoga neapolitana*. Since these hyperthermophilic enzymes are active above 95°C, they have been proposed for high-fructose corn syrup production.

Thermotogales are of huge significance because of their ability to metabolized simple sugars and complex carbohydrates such as sucrose, starch, cellulose, ribose, maltose and xylose for the industrial production of renewable carbon and energy sources (Van-Ooteghem *et al.*, 2002). They are rod shaped cells surrounded by a proteinaceous sheath-like structure and balloons over the ends, termed as "toga" hence the designation "toga" which plays a role in breaking down a wide range of

complex polysaccharides (Huber *et al*., 1986; Fardeau *et al.,* 1997). The toga containing genera include, *Fervidobacterium, Thermosipho, Geotoga, Petrotoga, Marinitoga, Thermopallium* and *Thermotoga* (Fardeau *et al*., 1997). Among the *Thermotogales,* there are four species of *Fervidobacterium,* eight members of the genus *Thermosipho,* four species of *Geotoga,* four species of *Petrotoga,* two species of *Marinitoga,* and one species of *Thermopallium*. The genus *Thermotoga* consists of 9 members: *T. maritima* (Huber *et al*., 1986), *T. neapolitana* (Jannasch *et al*., 1988), *T. thermarum* (Windberger *et al*., 1989), *T. subterranean* (Jeanthon *et al*., 1995), *T. elfii* (Rovot, 1995), *T. hypogea* (Fardeau *et al*., 1997), *T. petrophila* and *T. naphthophila* (Takahata *et al*., 2001), and *T. lettingae* (Balk *et al*., 2002). In the past few years, this innovative group of organisms has attracted fabulous interest among biochemical and biomolecular researcher.

Reduction of enzyme cost by amplification of the XI gene may cause an increase in fermentation productivity. Isolation of a mutant for the constitutive production of XI and elimination of the requirement of metal ions will contribute significantly to the improvement of the existing processes for HFCS production. Combination of saccharification of starch by glucoamylase (pH 4.5-5.5) with isomerization will result in shortening of reaction time and lead to a major saving in terms of equipment cost. However, the major drawback in the development of the uni-pH process is that the wide difference in optimum reaction conditions for the two enzymes tends to lower the efficiency of a simultaneous system. Efforts to produce thermostable and acid-stable XI with higher affinity for glucose by site-directed mutagenesis of the XI gene are already under way, with a view to evolving a XI preparation suitable for biotechnological applications (Bhosale *et al.,* 1996). This review aims at presenting updated information on the biochemical and genetic aspects of XI with a view to identifying important problems faced in its commercial application and evolving potential solutions.

APPLICATIONS

High Fructose Corn Syrup (HFCS) production: The largest industrial usage of XI occurs in the production of HFCS due to its ability to catalyze the conversion of glucose to fructose. For this reason, it is also known as glucose isomerase. This process was the first large-scale industrial use of an immobilized enzyme (Vieille and Zeikus, 2001).

Ethanol production: The second largest use is in the production of bio-ethanol from xylose. The pentose fraction constitutes as much as 40% of the hemicellulose, the majority of which is xylose. The preferred organism for bio-ethanol production is the yeast *Saccharomyces cerevisiae*, since it can withstand high concentrations of ethanol (Chandrakant and Bisaria, 2000). This yeast cannot metabolize D-xylose, however it can utilize D-xylulose, the isomerization product of xylose (Wang and Schneider, 1980).

Xylose isomerase gene as a marker in transgenic plants: Most plant species are unable to metabolize D-xylose, while they can utilize D-xylulose because they express a xylulokinase. Expression of a functional XI would enable the plants to be selected by providing D-xylose as the sole carbon source (Joersbo, 2001). The XI system is also independent of antibiotic or herbicide resistance genes, hence ultimately eliminating their drawbacks. Instead, it depends on an enzyme that is generally recognized as safe for use in the starch industry and which is already utilized in the food industry. This makes XI an attractive candidate for transgenic plant selection (Haldrup *et al.,* 1998).

Analytical applications: XI has also been used in analytical applications in combination with other enzymes and cofactors (Dominguez *et al.,* 1994; Kersters-Hilderson *et al.,* 1977).

CLASSIFICATION OF XYLOSE ISOMERASES

XIs are divided into two classes based on their sequence homology, Class I and Class II (Vangrysperre *et al.,* 1990). The Class II enzymes contain an additional 50 amino acid residue insert at the N-terminus, that are lacking in the Class I enzymes which are consisted of approximately 390 amino acids. There is also another short (10-14 residue) insertion that forms a short 3/10 helix and extends the first helix in triose-phosphate isomerase (TIM) barrel or $(\alpha/\beta)_8$ barrel. The remaining differences occur in the C-terminal loop helices, which primarily contact the surface of the adjacent subunit. The Class I enzymes all have a high degree of homology between them, and are similar in catalytic activity and temperature optima, while the Class II enzymes have a wider range of sequence homology and show more variability in catalytic activity and temperature optima (Hartley *et al.,* 2000). The class II XIs consists of approximately 440 amino acids and varies more in their sources, containing enzymes from mesophiles, thermophiles, and hyperthermophiles. However, the amino acids involved in substrate and metal binding, as well as catalysis, are completely conserved in both classes (Bhosale *et al.,* 1996).The *Thermotoga* XIs are all class II enzymes, have an N-terminal 50-amino acid insert (Vieille *et al.,* 1995). The functional role of the N-terminal insert in *Thermotoga neapolitana* xylose isomerase (TNXI) has been examined using several mutants indicated that the N-terminus might be involved in correctly folding of the protein. Further, the crystal structure reveled that those residues 1-12 are situated outside the protein structure and do not have any contact with the other subunits (Fig. 1). Around residue 16-20

there are more hydrophobic interactions with the surface of the $(\beta/\alpha)_8$ fold and the surface of other subunits, which corresponds to the point where the enzyme will no longer properly fold (Harris *et al*., 2010).

STRUCTURE

XIs from *T. maritima* and two strains of *T. neapolitana*, strains 5068 and 4359, in their native forms are all type II homotetramers. The recombinant form appeared to be predominantly dimeric, however, in contrast to the tetrameric native form, consisting of a TIM barrel, which contains the catalytic and metal binding sites, and a C-terminal helical domain that forms a loop that interacts with the surface of the adjacent subunit. The tetramer structure is composed of a dimer of dimers that can be represented by an A-B/B*-A* symmetry (Fig. 2). Three theoretical dimer configurations are also shown in Figure 2. The ying-yang (B-B*) dimer appears to be the most stable, but is lacking the subunit interactions around the active site, whereas the butterfly (A-B*) dimer retains these interactions. However, the structure of the active dimer is still unknown (Starnes *et al*., 1993; Vieille *et al*., 1995; Hartley *et al.* 2000). Gel filtration chromatography also showed that the recombinant enzyme existed both as homodimer as well as homotetramer, with the dimer being the more abundant form. The ration of dimer to tetramer is approximately 20:1. The purified native enzyme, however, has been shown to be exclusively tetrameric. The biochemical and biophysical properties of the two recombinant TNXI forms were investigated. The two forms had similar pH and temperature optima (7.0 and 95°C, respectively). Inactivation at 95°C did not follow a first-order decay profile for either form. After an initial rapid decrease in activity, the inactivation rate decreased considerably. The tetramer lost more activity in the initial phase than the dimer lost. Thus, it is possible that disassociation of the tetrameric TNXI may result in a mixture of dimers, a minority of which are inactive or unstable (Hess *et al.*, 1998).

ACTIVE SITE

Each monomer contains an active site that features two distinct metal binding sites. Metal M1 is coordinated by four carboxylate groups (Fig. 3), while metal M2 is coordinated by one imidazole and three carboxylate groups (Collyer *et al.*, 1990). All XIs are known to be active only in the presence of divalent cations (i.e. Mn^{2+}, Co^{2+}, and Mg^{2+}), mutating either metal binding site inactivated the TNXI. In addition to maximizing activity, the presence of both Mg^{2+} and Co^{2+} also enhanced the thermostability of all the XIs studied when compared with Mg^{2+} or Co^{2+} alone. TNXI exhibits two melting transitions in the presence of any two of the metals studied. Further investigations showed that the relative size of the transitions changed when the pH was increased from 7.0 to 7.9 (Hess and Kelly, 1999), suggesting that the unusual melting behavior is related to metal binding affinity. To confirm that this melting behavior is due to the presence of two different metals in TNXI's metal binding sites, mutations to site M1 (E232K) and site M2 (D309K) have been introduced in TNXI. In either case, the enzyme contained at most one metal per active site. Both TNXI mutants showed single melting transitions. The TNXI E232K mutant would therefore have higher affinity for Co^{2+} as only the M2 site is intact, while the D309K mutant would have higher affinity for Mg^{2+} for similar reasons. In the presence of Mg^{2+} and Co^{2+}, D309K TNXI has a T_m (95.5°C) slightly lower than that of the apo-enzyme, and E323K TNXI has the same T_m as TNXI in the presence of a single metal. These results suggest that M2 is the only metal important for TNXI stability, while both metals are needed for activity (Epting *et al.*, 2005).

CATALYTIC MECHANISM

The mechanism for XI has been the subject of many studies. The enzyme is thought to utilize a 1, 2 hydride shift mechanism (Collyer and Blow, 1990). Hartley *et al*., (2000) summarized the steps of this catalytic mechanism as follows. The first step is the binding of the α-D-pyranose form of the substrate, with O3 and O4 coordinated with M1 (The residues involved with substrate binding are highlighted (Blue) on Fig. 3). This orients the substrate so that His-53 can form a hydrogen bond to the carboxyl hydrogen of C1 and transfer it to O5, which opens the ring (Fig. 4A). The substrate unfolds to an open chain where O2 and O4 coordinate with M1. The hydrogen on the C2 hydroxyl group is removed by the water molecule coordinated with the M2 metal sight (Fig. 4B). The isomerization occurs via an anionic transition state formed by the movement of M2 to the position M2´ (Fig. 4C). The consolidation of positive charges of M1, M2´, and the nearby Lys-182, which has a H-bond to O1, causes the hydrogen of O2 to shift into a position between C1 and C2. Four hydrophobic residues, Trp-136, Phe-93, Trp-15, and Phe-25* (from the other monomer of the "tight" dimer) shield the C1-C2 transition state from solvent (Hydrophobic Shield highlighted (Gray) on Fig. 3). When M2´ returns to its original position, the transition state collapses and the hydrogen is transferred to C1. The proton from the ε NH^{3+} group of Lys-182 donates a proton to form the C1 hydroxyl group after the hydride shift (Hu *et al*., 1997). The chain returns to a cyclic position with M1 again coordinating with O3 and O4. His-53 catalyzes the ring closure, essentially the reverse of the ring opening (Hartley *et al*., 2000).

PRODUCTION OF XYLOSE ISOMERASE FROM THERMOTOGALES

The enzyme generally produced by anaerobic cultivation of XI producing strains of *Thermotoga* sp (*T.*

maritima, *T. neapolinata*), in submerged aerated fermentation, followed by recovery and purification of the desired enzyme by conventional means. Cells grown on suitable nutrient medium, containing carbon, nitrogen sources and inorganic salts (NaCl, Na_2SO_4, KCl, $NaHCO_3$, KBr, H_3BO_3, $MgCl_2.6H_2O$, $CaCl_2$. $2H_2O$, $SrCl_2.6H_2O$, Tryptone, Yeast Extract, xylose as inducer). Xylose generally sterilized separately. pH adjusted to 6.0 to 6.5 with H_2SO_4 at room temperature. $Na_2S.9H_2O$ (0.5 g/l) generally added before inoculation. The fermentation has been proceeded at 80°C, 100 rpm, with an N_2 sparge of about 0.05 vvm. An 8% inoculum used, on the same medium, and the run least about 8 hours, to an $O.D_{600}$ of about 0.3. The enzyme can also be obtained by recombinant DNA technology (Starnes *et al.,* 1993).

GENETIC ENGINEERING OF THERMOTOGALE'S XYLOSE ISOMERASE

Because of difficulties in cultivating hyperthermophilic microorganisms (e.g., unusual fermentation conditions, low cell yields, toxic and/or corrosive metabolites) (Adams *et al.,* 1993; Kelly *et al.,* 1992), obtaining large amounts of a potentially useful thermostable biocatalyst from the natural host is often impractical. Today's molecular biology tools allow the expression of a desired gene product in a foreign host i.e., expressing enzymes from hyperthermophiles in mesophilic hosts. The genes (*xylA*) encoding the XI from hyperthermophilic eubacteria *T. neapolitana* and *F. gondwanense* have been cloned, sequenced and overexpressed in *Escherichia coli* (Vielle *et al.,* 1995; Hess *et al.,* 1998; Kluskens *et al.,* 2010).

PURIFICATION OF XYLOSE ISOMERASE

XIs from *T. maritima* (Brown *et al.,* 1993) and two strains of *T. neapolitana*, strains 5068 and 4359, have been purified and characterized. *T. neapolitana* recombinant XI has been purified from *E. coli* [HB101 (DE3), BL21 (DE3)], carrying plasmid (pTNE2-kan, pET23a, $pET22b^+$). The cells have been grown in Terrific/LB Broth supplemented with kanamycin and harvested by centrifugation. The cell extracts have been prepared by passage through a French pressure cell or sonication. For purification, the cell extract was heat treated at 90°C for 2.5 h (*T. neapolitana*), 30 min at 65°C (*F. gondwanense*), 20 min at 80°C (*T. maritima*) in an oil bath with shaking. An $(NH_4)_2SO_4$ fractionation step was added after the heat treatment then ion-exchange chromatography was performed on Q-Sepharose Fast Flow, and proteins were eluted with a linear NaCl gradient (0.0 to 0.3 M) (Vieille *et al.,* 1995; Sriprapundh *et al.,* 2000; Sriprapundh *et al.,* 2003; Epting *et al.,* 2005; Kluskens *et al.,* 2010).

PROPERTIES OF XYLOSE ISOMERASE

Optimum Temperature and Thermostability: The recombinant XIs from *T. maritima, T. neapolitana* are optimally active a temperature range of from below 60°C to above 98°C. The temperature optimum is at above 90°C, more precisely at 95°C for *T. maritima* and at 97°C for *T. neapolitana* and shows more than 90% of maximal activity between 94 and 100ºC. In the absence of a substrate, the enzyme retains 40% of its activity after incubation at 90°C for 2 h and 90% of its activity after incubation at 50°C for 4 h. The native enzyme shows the same half-life at 95°C as the recombinant enzyme. The logarithmic representation of the residual activity after thermo-inactivation does not appear to be linear, indicating that inactivation is probably a multistep process. The optimal temperature of XI from *F. gondwanense* is 70°C. The enzyme retained 80% of its activityfrom 66 to 85°C. Compared with its orthologs from the hyperthermophiles *T. maritima* and *T. neapolitana*, the XI from *F. gondwanense* is less thermoactive and thermostable (Starnes *et al.,* 1993; Vieille *et al.,* 1995; Kluskens *et al.,* 2010).

Optimum pH and pH stability: The XIs from *T. maritima* and *T. neapolitana* are optimally active at pH 6 to 7, around pH 6.6, when measured in a MOPS (3-(N-morpholino)-propanesulphonic acid) buffer system, but it retains a high percentage of activity over a wide pH range. The enzyme shows more than 80% of its maximum activity between pHs 6.1 and 7.6. The enzyme retains 100% of its activity over a pH range of 6.8 to 7.3 for at least 30 min when incubated at 90°C in the absence of a substrate. The remaining activity as measured at pH 6.3 after 30 min at 90°C and pH 6.95 or 7.08 is 10% higher than that of the positive, non-pre-heated control. The *F. gondwanense* XI also showed a broad pH optimum, with maximal activity at pH 7.3. At least 80% of activity has been observed in the pH ranged 7.0–7.6, however, *F. gondwanense* XI retained at least 40% of its activity from pH 5.0 to 8.0 (Starnes *et al.,* 1993; Vieille *et al.,* 1995; Kluskens *et al.,* 2010).

Kinetic Properties: The kinetic properties of TNXI on xylose and glucose have been determined at 90 and 98°C, and the kinetic properties on fructose were determined at 60, 75, 85 (the growth temperature of the organism), 90, and 98°C. As do other XIs, the TNXI has a lower K_m for xylose (15.9 mM at 90°C) than for glucose (88.5 mM at 90°C). Kinetic parameters defined for the TNXI are comparable to the kinetic parameters defined for the other XIs. In comparison with values obtained for *T. maritima* XI, *F. gondwanense* XI had lower K_m values at 85 °C, whereas V_{max} values overall were equal or slightly higher. The results suggested a higher catalytic efficiency (k_{cat}/K_m), especially for xylose, but also for glucose (Vieille *et al.,* 1995; Kluskens *et al.,* 2010).

Metal Ion Requirement: Metal cations (Co^{2+}, Mg^{2+}, and Mn^{2+}) play an important role in the catalytic mechanism and stability of XIs; this role was closely examined using differential scanning calorimetry (DSC) and site directed mutagenesis (SDM) to investigate differences between the hyperthermophilic (from *T. maritima* and *T. neapolitana*) and the other enzymes. Co^{2+} best activate the class II enzymes for activity on glucose; Mn^{2+} is preferred for activity on xylose (Van-Bastelaere *et al.,* 1991). However, Mn^{2+} or Co^{2+} provides superior thermal stabilization (Bogumil *et al.,* 2000). Metal specificity appears to be related to the residues surrounding the metal binding sites. XIs from *T. maritima* and *T. neapolitana* stabilizes by Co^{+2} and Mg^{+2}. The stabilization of *F. gondwanense* XI is mainly increased by Co^{+2} (1 mM of $CoCl_2$) than with 10 mM of $MgCl_2$ (Starnes *et al.,* 1993; Kluskens *et al.,* 2010).

GENETIC IMPROVEMENT OF XYLOSE ISOMERASE

Mutations Pro58Gln, Pro62Ala and double mutation Pro58Gln/Pro62Ala were introduced in TNXI by site-directed mutagenesis decreased the optimum temperature for enzyme activity. Mutation Pro58Gln decreased it's half-life by 29% (from 69.3 to 49.5 min). Both mutations (Pro62Ala and Pro58Gln/Pro62Ala) decreased half-life to 11.6 min (83%). As all these reverse counterpart mutations destabilizing TNXI thus confirming that Pro58 and Pro62 play important roles in thermostability of the enzyme. Although, Trp138Phe mutation decreased TNXI's optimum temperature to 87°C, However, the mutant enzyme still retained almost 80% of its activity at 97°C. With the half-life of 69.3 min, the Val185Thr mutation did not affect enzyme's stability at 95°C. The Trp138Phe mutant (half-life of 87 min) and the Trp138Phe/Val185Thr double mutant (half-life of 99 min) enzymes are 25 and 43%, respectively, more stable than wild-type.

XI displays higher affinity for xylose than for glucose. TNXI's active site has been engineered to improve its catalytic efficiency towards glucose in view of its application in the production of HFCS. Mutation (Trp138Phe) significantly increased its catalytic efficiency on glucose. Similarly, Val185Thr mutant is the most efficient mutant derivative has a 3.1-fold increased catalytic efficiency towards glucose, with a maximal activity at 97°C of 45.4 U/mg on glucose. This mutant derivative is the most active type II xylose isomerase ever reported. Double mutant (Trp138Phe/Val185Thr) also had a higher catalytic efficiency for glucose than wild-type. These increases mainly resulted from a much lower K_m for glucose than that of wild-type. On the other hand, these entire mutant TNXIes showed a lower catalytic efficiency for xylose (Sriprapundh *et al.,* 2000).

Although TNXI V185T is highly thermostable and highly active at 97°C, it is very poorly active (10% of maximal activity) at the current industrial isomerization temperature (60°C) and it requires a neutral pH for optimal activity. To further optimize TNXI's potential industrial utility, random mutagenesis using the TNXI V185T-encoding gene as the template was performed and two highly active mutants 3A2 (V185T/L282P) and 1F1 (V185T/L282P/F186S) were obtained.

1F1 was more active than 3A2, which in turn was more active than TNXI V185T at all temperatures and pH values tested. 3A2 and 1F1's high activities at low temperatures were due to significantly lower activation energies (57 and 44 kJ/mol, respectively) than that of wild type TNXI and mutant V185T (87 kJ/mol). Mutation L282P introduced a kink in helix α_7 of 3A2's $(\alpha/\beta)_8$ barrel. Surprisingly, this mutation kinetically destabilized 3A2 only at pH 5.5. 1F1 displayed kinetic stability slightly above that of TNXI V185T (Sriprapundh *et al.,* 2003).

IMMOBILIZATION OF XYLOSE ISOMERASE

For optimal performance of the isomerzation process, the xylose isomerase can be immobilized. The isomerization process can then be carried out as a continuous, fixed-bed reactor process. In addition to the convenience of continuous operation, the fixed-bed process permits a short reaction time thereby minimizing by product formation. The potential of the two *Thermotoga* enzymes (from *T. maritima* and *T. neapolitana*) was evaluated for use in HFCS production at elevated temperatures and compared with an industrial immobilized Class I XI from *Streptomyces* (Sweetzyme T). The extended N-terminus of the class II XIs makes it an attractive target for attaching a carbohydrate-binding domain (CBD) for immobilization. The CBD from *Pyrococcus furiosus* chitinase ChiB (PF1233) was selected to create a fusion protein (TNXI-CBD) at the N-terminus of TNXI. The addition of the CBD to TNXI successfully immobilized the enzyme to chitin beads. This is the first report of a hyperthermophilic CBD being employed to immobilize an enzyme for an industrial process. The turnover numbers for glucose to fructose conversion for both unbound and immobilized CBD-TNXI were greater than the wild-type enzyme: k_{cat} (min^{-1}) was approximately 1000, 3800, and 5800 at 80°C compared to 1140, 10350, and 7000 at 90°C, for the wild-type, unbound, and immobilized enzymes, respectively. These k_{cat} values for the glucose to fructose isomerization measured are the highest reported to date for any XI at any temperature. Enzyme kinetic inactivation at 100°C, as determined from a bi-phasic inactivation model, showed that the CBD-TNXI bound to chitin had a half-life approximately three times longer than the soluble wild-type TNXI (19.9 hours vs. 6.8 hours, respectively). Surprisingly, the unbound soluble CBD-TNXI had a significantly longer half-life (56.5 hours) than the immobilized enzyme. Molecular modeling results suggest that the N-terminal

fusion impacted subunit interactions, thereby contributing to the enhanced thermostability of both the unbound and immobilized CBDTNXI. These interactions likely also played a role in modifying active site structure, thereby diminishing substrate-binding affinities and generating higher turnover rates in the unbound fusion protein (Harris *et al*., 2010).

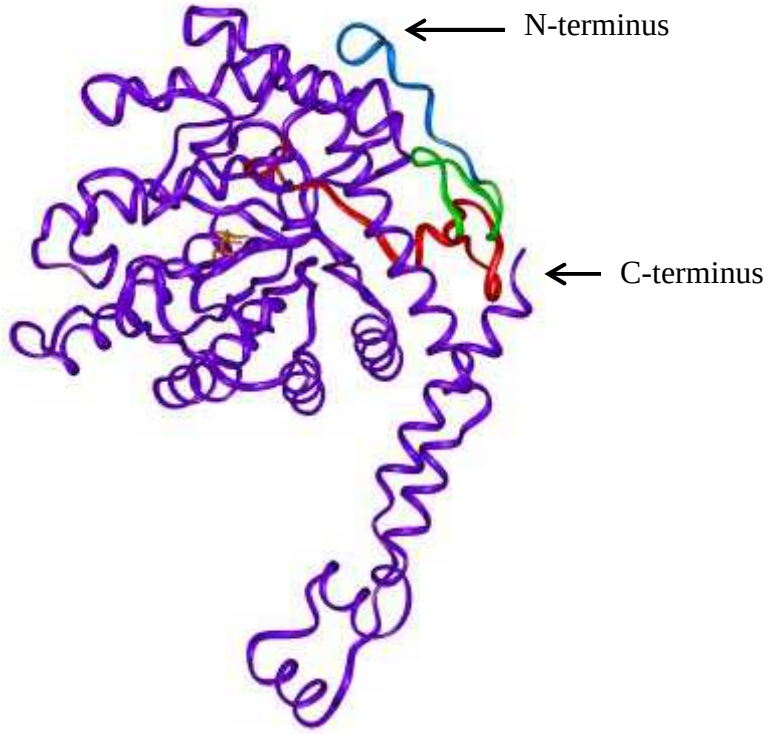

Fig. 1. Structural analysis of the N-terminus of TNXI. Residues 1-12 Blue; Residues 13-24 Green; Residues 25-50 Red (Vieille, personal communication)

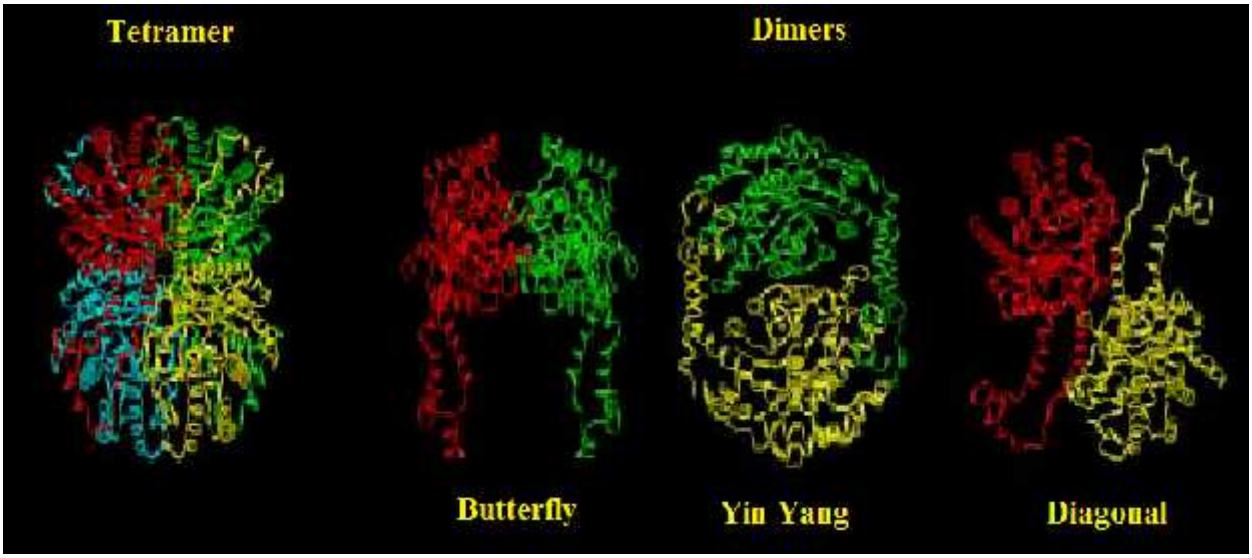

Fig. 2. Subunit structure of XI. Tetramer and theoretical dimer configurations. Red = subunitA; Blue = sununit A*; Yellow = subunit B; Green = subunit B* (Vieille, Personal communication)

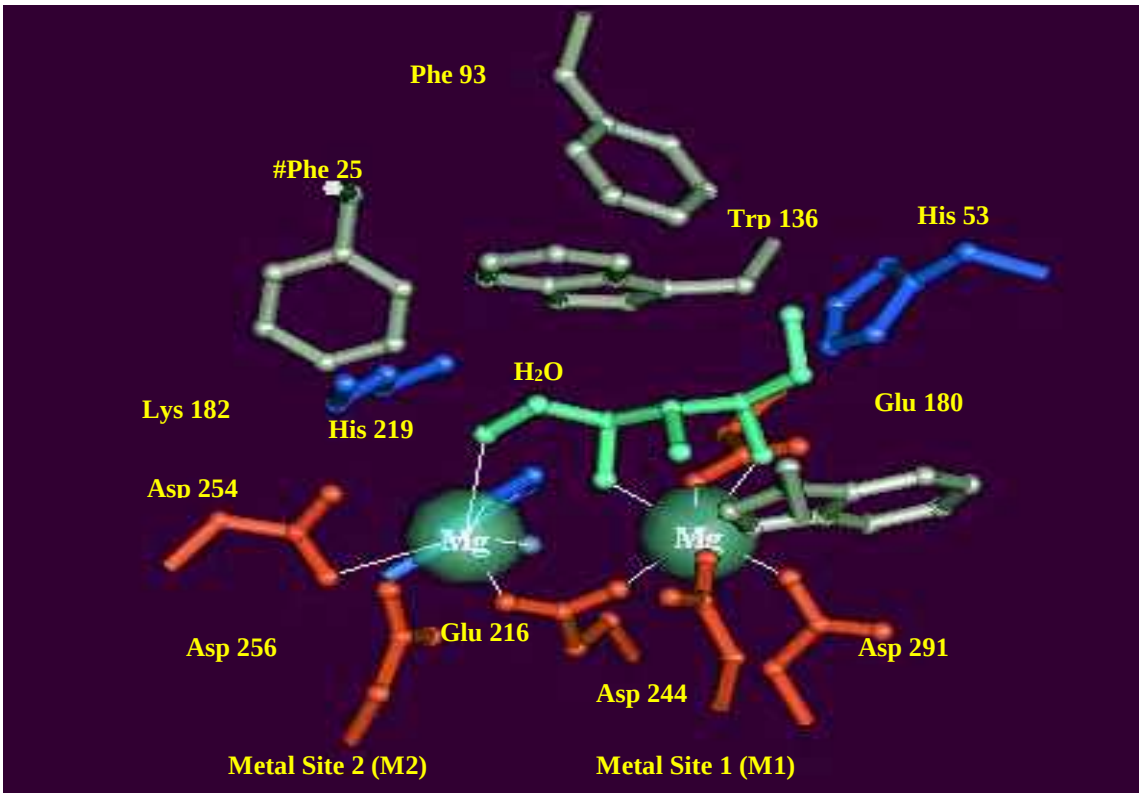

Fig. 3. Catalytic site of Xylose Isomerase coupled with D-xylose (green); Negatively charged metal binding amino acids residues (Red); Positively charged substrate binding amino acids residues (Blue); Hydrophobic Shield (Gray) Vieille, personal communication.

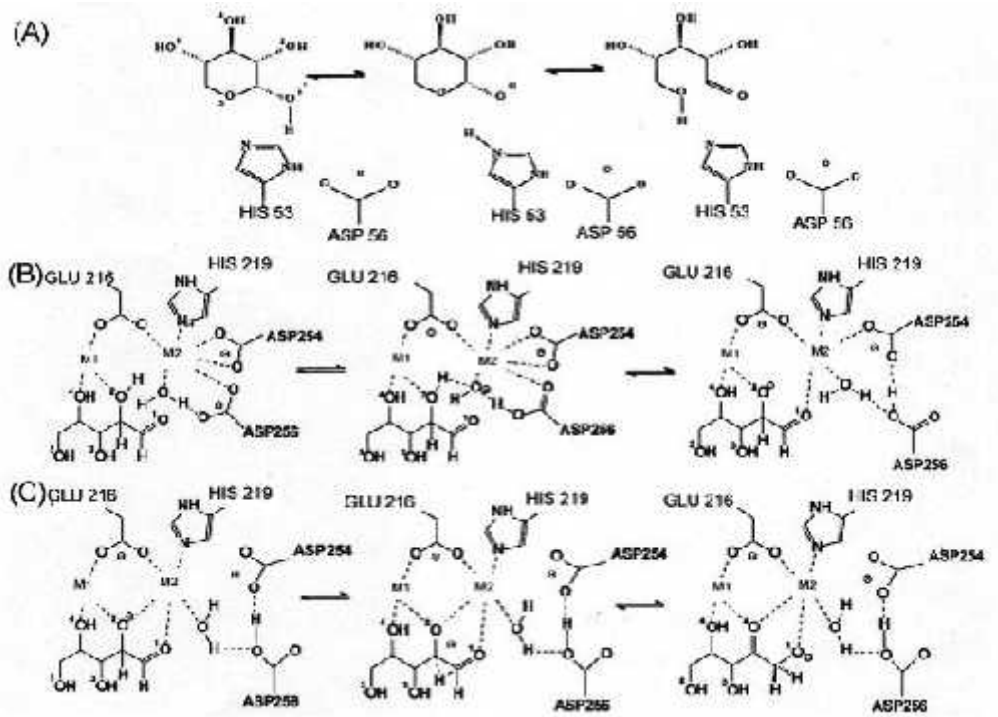

Fig. 4. Steps in catalytic mechanism of xylose isomerase: (A). Ring opening catalyzed by His 53; (B). Proton transfer from hydroxyl group on C2 to M2 water molecule; (C). Hydride shift- movement of M2 forms transition state. Figure from Gerczei *et al.* (1999).

Conclusions: It is concluded that recombinant DNA technology and advances in molecular genetics enabled the researchers not only to express hyperthermophilic gene *xyl-A* in mesophilic organism but also by genomic biotechnology they genetically improved the gene product which is catalytically active at low temperature and pH 5.5, can be employed for uni-pH process as there is no wide difference in optimum reaction conditions of glucoamylase and xylose isomerase tends to improve the efficiency of simultaneous system. The mutant showed 3.1-fold increased catalytic efficiency towards glucose, with a maximal activity at 97°C of 45.4 U/mg on glucose which produced 55% fructose syrups, hence eliminating the additional expensive chromatographic steps for the enrichment of fructose is suitable for biotechnological applications.

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