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SUMMARY OF THE THESIS

The pentose sugar D-xylose is the predominant hemicellulosic compound which comprises about one third (25-35%) of the total carbohydrates present in the lignocellulosic biomass (Hinman et al, 1989). Lignocellulose is renewable and this low cost carbohydrate is potentially attractive for the production of useful chemicals and biofuel e.g. ethanol. Recently, a combination of ever-increasing energy costs and global warming concerns has created an impetus for new alternative fuels that are renewable and can be produced in a sustainable way. Efficient conversion of xylose from lignocellulosic material is required for the large scale production of ethanol. In recent past, there has been tremendous progress in the understanding of xylose metabolism and in the identification, characterization and development of improved strains with xylose fermenting properties. Understanding the xylose metabolism also includes the detailed investigation of the components (enzyme systems) of it. As the genes encoding these enzymes can be used as tool for the engineering of microorganism and develop new strains, thus studying the enzymology of the initial xylose assimilation pathway is important. However, yeasts are more exploited organisms for fermentation process than bacteria, due to some of its physiological characteristics. So enzymes involved in metabolism of xylose have been investigated in a number of in yeasts. Most xylose-assimilating yeasts, utilize D-xylose via two enzymatic oxido-reductive reactions, with xylose reductase (XR; EC1.1.1.21) and xylitol dehydrogenase (XDH; EC 1.1.1.9), to form D-xylulose, which is converted to xylulose 5-phosphate by xylulose kinase (XK) and then enters the pentose-phosphate pathway. The XR and XDH enzymes were studied in detail in various yeast species like- *C. tenuis, C. shehatae, C. tropicalis, P. stipits, P. tannophilus, G. mastotermitis* etc. However very little information is available about these enzymes from extremophilic yeasts e.g. *D. hansenii* and *P. angusta*. The halotolerant, osmotolerant yeast *D. hansenii* has capability of metabolising xylose and produces xylitol as a major fermentation product. *P. angusta* on the other hand is a thermotolerant, methylotropic yeast which is able to ferment glucose, xylose etc. at higher temperature (48-50°C). Characterization of the XR and XDH enzyme from these yeast species could furnish basic understanding of the xylose metabolism in these organisms as well as provide a new tool for the engineering of microorganism.

The enzyme xylose reductase (XR) is a member of aldo/keto reductase family and is the first enzyme in the xylose metabolic pathway which acts upon xylose and converts it into xylitol. Xylose reductase gene of *D. hansenii* was identified by 'tblastn' search in *D. hansenii* genome using the protein sequence of *P. stipitis* XR as a query. The DhXR gene was cloned in pET28c expression vector and expressed in *E. coli* BL21 (DE3). Size exclusion chromatography experiment indicated that, the enzyme exist as a homo-dimer in solution. The enzymatic assay was performed to determine pH optimum (7.0) and temperature optimum (45°C). The thermal stability of the enzyme was also measured by monitoring remaining activity after incubating it at different temperatures- 25°C to 55°C. The enzyme showed maximum activity when incubated at 40°C and beyond this temperature activity was declined. Thus, the routine enzyme assays were performed at 40°C and pH 7.0. Like other AKR members, DhXrp shows substrate promiscuity and catalyse several pentoses (xylose, ribose) as well as hexose sugars (glucose, galactose). The K_m for xylose was found to be 28mM in DhXrp which is lower than XRs of other yeasts *C. tenuis* (87mM), *P. stipitis* (90mM), *C. shehatae* (160mM). As the xylose reductase enzyme is associated with pentose sugar (xylose) metabolism, The *DhXR* gene expression as well as enzyme activity has been observed to be induced with pentoses. The qRT-PCR result suggested *DhXR* gene was differentially expressed according to the carbon source; 4 fold higher expression of gene was found in xylose than in glucose media. Equilibrium binding studies were performed to investigate the affinity of the enzyme towards substrates and co-enzyme. DhXrp was observed to follow a negative cooperativity for ligand binding where binding in one subunit perturbs the binding in other subunit. Binding studies of DhXrp with various pentose and hexose sugars show that, DhXrp has higher binding affinity with xylose, galactose and sucrose than with arabinose, ribose and rhamnose. When catalysis was compared, ribose (~95%) and galactose (~50%) showed higher activity than arabinose, rhamnose and sucrose (2-10%). Thus, binding-affinity of the substrate to the apo-enzyme is not correlated with its activity toward it. Next, competition titration assay was performed, where activity of xylose (100mM) was measured keeping varying concentrations (25mM to 400mM) of D-arabinose and L-rhamnose in the reaction mixture. Specific activity of xylose did not change even with increasing concentration of the competing sugars. This experiment reflected that, NADPH provides specificity to

e enzyme molecule and cofactor bound holoenzyme could bind only to its preferred ıbstrate. To further confirm this result, binding studies were performed with NADPH ɔund enzyme. L-rhamnose did not bind with the holoenzyme where as sucrose, D-abinose and galactose showed reduced binding affinity than the holoenzyme. From ese experiments, it can be concluded that cofactor modulates the substrate binding to a eat extent. The cofactor NADPH showed ~1000 higher binding affinity than the ıbstrates. Five active site mutants (D42A, Y47A, K76A, H109A, and N305A) were enerated by replacing the residues with alanine. The activity assay of the enzyme ıowed that, except N305A which shows ~5% activity, other mutants lost their activity ɔmpletely. Ligand binding experiments were performed with all the mutants. D42A ıd N305A did not lose their affinity towards D-xylose, but mutation of Tyr47, His109 ıd Lys76 residues leads to ~2 fold reduced affinity for substrate. Therefore binding udies performed with mutant apoenzymes indicate that, the interaction is primarily ediated through Tyr47, His109, and Lys76.

Crystal structures of DhXrp were obtained in complex with the canonical ıbstrate D-xylose in closed pyranoid and open chain configurations. The overall ıbunit architecture of DhXrp suggests a typical $(\alpha/\beta)_8$ TIM barrel structure which is pical for aldo-keto reductases. As the substrate bound structure of DhXrp was not in ɔmplex with NADPH, thus it can be inferred that the co-factor is not required for ring ɔening of xylose. Proximity of the OH1 and O5 of the closed chain xylose with His109 ssigns that, this amino acid has crucial role in ring opening. The earlier studies on uman ALR in complex with inhibitors concluded that, His110 residue helps in ienting the substrate (Harrison 1994). But our observation assigns a more definite role f the histidine. Catalytic reaction mechanisms are not investigated in our study but ɔsitional conservation of the residues across the species, site directed mutagenesis and ctivity assay of enzymes confers that D42, Y47 and K76 are part of the catalytic ntre. Earlier literature suggests that Tyr47 act as a proton donor to the substrate and ys76 help to reduce the pKa of Tyr47. As the Tyr47 makes interaction with C2OH us it is believed to donate the proton in the hydroxyl group of second carbon. The role f Asn305 could not be assigned with certainty through ligand binding studies but ructural studies indicate that, Asn305 helps in substrate stabilization when the xylose ng structure opens in a linear configuration. In the Asn305 substituted mutant, the

substrate might be weakly stabilized and catalysis can progress very slowly. However, all of these residues are important for catalysis because mutation of any one of these residues leads to loss in activity. We can summarize the xylose binding and catalysis through following steps; first cyclic xylopyranose binds at the active site of holoenzyme, ring opening of the cyclic xylose occurs through the interaction of His109 residue, the open chain xylose stabilizes in an extended form through interaction of amino acid residues, catalysis takes place primarily with the interaction of Tyr47, Lys76 and Asp42.

The enzyme Xylitol dehydrogenase is a member of medium chain polyol dedydrogenase family and is the second enzyme in the xylose metabolic pathway of yeast which converts xylitol into xylulose. Xylulose enters into the PP Pathway and ultimately produces ethanol under micro-aerobic condition. *XDH* gene of *D. hansenii* was identified by 'tblastn' search using the protein sequence of *P. stipitis* XDH as a query. The *DhXDH* gene was cloned in pET28c expression vector and expressed in *E. coli* BL21 (DE3). The biochemical characters of DhXdhp are mostly in agreement with the characterized xylitol dehydrogenase enzymes in other yeasts and fungi. The native molecular mass of DhXdhp was found to be homo-tetrameric. The enzyme assays reveal that, the oxidative activity of DhXdhp is best at alkaline pH (8.8) and DhXdhp shows maximum activity at lower temperature range (20°C to 25°C). Optimum temperature for oxidation in other mesophilic yeasts was found to be at the range of 30°C to 45°C. Thus, in comparison to other mesophilic yeast and fungal XDH enzymes DhXdhp shows sensitivity towards temperature increment. The thermo-stability assay confirmed that, the enzyme is less stable at higher temperature. Upon 10 min of incubation at 42°C DhXdhp completely loses its activity. The thermal denaturation CD spectroscopy result showed that, the transition temperature (T_m) for thermal melting is 60°C. Thus the activity is lost before the denaturation of the structure. Probably the active site of the enzyme is affected by the higher temperature. In accordance with Zn containing MDR enzymes, DhXdhp also contains Zn atom which is found to be essential for the enzyme activity. We further investigated the ratio of Zn ion present per polypeptide chain in the enzyme. The atomic absorption spectroscopy result revealed that, the DhXdhp wild type enzyme contains one Zn atom per polypeptide which is found to be catalytic. Some members of this family possess another structural Zn in

addition to catalytic Zn, which was found to be absent in this protein. The DhXdhp shows activity with its canonical substrate xylitol (100%) and sorbitol (75%), no activity was found with the other substrates tested (D-mannitol, L-arabitol, D-arabitol). Reasonably good activity with sorbitol might be due to structural similarity of the XDH with sorbitol dehydrogenase (SDH). The kinetic parameters (Km) are also measured for DhXdhp with xylitol (12mM), sorbitol (24mM), and NAD^+ (0.91mM). Role of different carbon sources in inducing the *DhXDH* expression in *D. hansenii,* was investigated at the level of enzyme activity and mRNA expression. Xylose grown cells showed highest XDH activity (~2.5 fold) in comparison to sorbose, arabinose, cellobiose, mannose, dextrose, and fructose (~1.15 to 1.6 fold) grown cells keeping glycerol grown cells as control. Thus, the pentose sugars induce the XDH activity more than the hexoses. The level of mRNA expression of *XDH* under different carbon sources was measured by qRT-PCR. The xylose grown cells showed ~7.5 fold more mRNA expression than the glucose grown cells. These results affirm that the pentose sugar xylose modulates the expression pattern of *DhXDH* gene. To delineate the role of *DhXDH*, in its native host i.e. *D. hansenii*, the gene was disrupted by homologous recombination. The disruptants failed to grow normally in minimal medium where xylose was the sole carbon source thus proved that *XDH* was indispensable for growth in xylose media. To find out the level of xylitol accumulation in wild type and disruptants, fermentation experiments were carried out with xylose. HPLC analyses of the supernatants after fermentation showed that, the *XDH* disruptants accumulated ~3 fold more xylitol than the wild type. Structural neighbour search revealed that DhXdhp was structurally related to sorbitol dehydrogenases which catalyze the oxidation of sorbitol to fructose using NAD^+ as a coenzyme. Sequence alignment also showed that DhXdhp has the typical signature of alcohol dehydrogenases belonging to the MDR family and the binding sites for NAD^+ and one zinc ion. A model was build for DhXdhp using the template of human sorbitol dehydrogenase (e-value 2e-67). Metal detecting server predicted Cys41, His66, and Cys67 residues are involved in Zn ion binding. Mutating these residues with alanine resulted in the loss of activity in the enzyme. Thus Cys41, His66, and Cys67 residues might be playing very crucial e.g. coordinating the catalytic Zn atom. As, it has been reported earlier that, the differences in co-enzyme specificity of the two subsequent enzymes XR and XDH causes redox imbalance in cells, so attempts have been made to

change the co-enzyme specificity of the *DhXDH* with specific mutations at the Rossmann fold region. As the Asp207 provides NAD^+ specificity to the protein, so aspartic acid residue was replaced by the smaller uncharged residues like alanine and serine along with arginine that forms a positive binding pocket for the 2'-phosphate group of $NADP^+$. Two mutants of DhXdhp were generated and named as ARSAR and ARSeR. Activity assay showed that, these mutants did not show any detectable activity with NAD^+ while ARSAR and ARSeR showed equal affinity with $NADP^+$ (0.96mM and 1.1mM, respectively) as wild type shows with NAD^+ (0.91mM). In PsXdh the quadruple mutant ARSdR also showed comparable K_m for NAD^+ (1.38mM), but the affinity for xylitol was reduced significantly in this mutant (72mM) (Watanabe et al, 2005) than the DhXdhp mutants (25mM for ARSAR and 29mM for ARSeR). Our result showed that DhXdhp was thermo sensitive enzyme as it lost its activity rapidly with the increase in temperature. Attempts were made to reinforce the structure of the protein against thermal denaturation, by replacing two serine and one tyrosine residue with three cysteine residues to incorporate structural Zn atom in the protein. The C_4DhXdhp mutant protein was cloned and expressed and enzyme assay revealed that, the temperature optima of the mutant enzyme is shifted to 35°C from 25°C. The thermal-stability of the C_4DhXdhp protein was measured by monitoring remaining activity at 35°C. It was interesting to find that, the mutant enzyme showed 70% activity even after incubating at 50°C in contrast to wild type enzyme which loses activity after incubating it at 35°C. It can be concluded that, the basic biochemical properties of *D. hansenii* xylitol dehydrogenase were in agreement with the other characterised enzymes of yeast and fungi. Attempts were made in this study to engineer the protein for co-factor reversal and thermo-stabilization. These engineered proteins can be introduced into *S. cerevisiae* to improve the efficiency of ethanol production by xylose fermentation.

Pichia angusta is able to grow at high temperature and endowed with the unique feature of fermentation of xylose at elevated temperature (45-48°C). Thus it is important to study the enzymes of xylose metabolism of in this thermotolerant yeast as they might be useful for metabolic engineering of other organisms for xylose fermentation. *P. angusta* is reported to possess two ORFs encoding xylitol dehydrogenase enzyme (Dmytruk et al. 2008b). However no information is available regarding biochemical

properties of these two proteins. In this study, ORFs encoding both XDH (*PaXDH1* and *PaXDH2*) were identified by *in silico* method, cloned in expression vector and proteins were purified and characterized. Sequence alignment showed that, these two proteins are quite different with respect to their primary sequence (~40% identity). Biochemical properties showed some similarities and dissimilarities with each other. Both the proteins PaXdh1p and PaXdh2p showed optimum activity at alkaline pH above 10. Size exclusion chromatography revealed the tetrameric organization of the two enzymes which was also observed in the xylitol dehydrogenase of yeasts. Similar to other orthologues, Zn atom was found to be present at the catalytic site in both the enzymes. Interestingly, PaXdh2p also contained a structural Zn atom which most likely provided structural stability against thermal denaturation at higher temperature. With respect to the substrate specificity, both PaXdh1p and PaXdh2p showed higher activity only with xylitol (100%) and sorbitol (~90%). PaXdh2p also showed some activity (40%) with L-arabitol. Kinetic parameter shows PaXdh1p exhibited much lower K_m (22mM) and higher K_{cat} (16 s^{-1}) for xylitol than PaXdh2p (K_m148mM and K_{cat} 2.85 s^{-1}). Thus, PaXdh1p appeared to be more efficient than PaXdh2p in utilizing xylitol as substrate. Both enzymes (PaXdh2p and PaXdh1p) showed higher thermal tolerance. The optimum temperature was found to be 50°C and 55°C for PaXdh2p and PaXdh1p, respectively which was much higher than that of XDHs reported from other xylose utilizing yeasts. Further the thermostability assay showed that both the enzymes could retain maximum activity after incubation at 50°C for 10 min. Thermostable nature of PaXdh2p and PaXdh1p was also examined by estimating the thermal unfolding transition temperature (T_m). The PaXdh2p showed the T_m at 60°C and PaXdh1p showed 62°C. Site directed mutagenesis was performed to delineate the role of conserved residues of PaXdh2p in substrate binding and catalysis. Characterization of the mutants clearly indicated a crucial role of Glu155 in the catalytic activity of PaXdh2p. Ser44 and Arg308 appeared to be very for important for substrate binding as the mutations in these residues resulted in 10 fold decreases in the affinity for the substrate. In this regard Thr121 appeared to have a limited role in PaXdh2p. Recently, for glucose dehydrogenase from halophilic archaeon *H. mediterranei* which is also a MDR family enzyme, mechanism for catalysis has been proposed (Baker et al. 2009) where, amino acid residues equivalent to Glu155 appeared to play a vital role in coordinating Zn atom

active site as well as in the catalysis. A similar role for Glu155 in PaXdh2p has envisaged and therefore the mutant E155A was completely inactive.

Present study on the components of xylose metabolism pathway in two mophilic yeasts, *D. hansenii* and *P. angusta* entails detail characterization of its mes. This study could be beneficial for further improvement of organisms own bolism as well as provide a new tool for the engineering of other microorganisms.