Summary and highlights of the work

Urate oxidase or uricase (EC 1.7.33) an enzyme that catalyzes the oxidation of uric acid to allantoin, occupies a pivotal position in the chain of enzymes responsible for the metabolism of purines. During primate evolution the inactivation of the hominoid urate oxidase gene was caused by mutations in the promoter and coding regions of the gene. As a result elevated basal levels of uric acid present in the blood of hominoid primates, compared to other mammals. When the concentration of uric acid exceeds the basal level, the condition is known as hyperuricemia, it crystallizes and deposits at the joints of the organism leads to gouty arthritis. Uricase is emerging as a potential drug for the tophaceous gout. Uricase had been cloned and characterized from different microbes. *C. utilis* is the only yeast from which uricase had been cloned and characterized which is under phase III clinical trials.

In order to find new sources of yeast uricases, thirty six yeast isolates belonging to 19 different genera and 32 species were screened on uric acid containing plates. Twelve of them belonging to 9 genera and 10 species could grow on the plates indicating they have the ability to produce uricase enzyme. Among these ten species, genome sequence of nine species is available in the nucleic acid databases. Based on the industrial importance and GRAS status we have selected the following 5 species for examining their uricases.

1. *Kluyveromyces lactis*
2. *Lachancea thermotolerans*
3. *Pichia segobiensis*
4. *Pichia stipitis*
5. *Yarrowia lipolytica*

Using sequence details, primers were designed to amplify and sequence the uricase genes from the selected organisms (except *Pichia segobiensis*). Uricase gene of *P. segobiensis* was amplified from genomic DNA using uricase gene primers of phylogenetically closely related species *P. stipitis*. Sequence analysis indicated that the five yeast species are phylogenetically very different from *C. utilis*. The two regions designated as region B and region A found in all the uricases of our study. While the region B was identical to the sequence of other organisms, minor variations were observed in the sequences at region A of the uricases examined in this study. Like uricases of all eukaryotes two motifs (motif 1 and motif 2) were also found in the

uricases of this study. Motif 1 of four uricases of this study was identical to that of *C. utilis* and motif 1 of *L. thermotolerans* was identical to that of *A. flavus*. Motif 2 of all the 5 species was identical to that of *C. utilis* and *A. flavus* sequence.

The peroxisomal targeting sequence (S/A-K-L) that was present in all other eukaryotic organisms are also present in five yeast species examined indicating that the uricases of all these species are directed to peroxisomes. Variation in number of cysteine residues was found among the species examined while *L. thermotolerans* has five cysteines, *K. lactis* has four residues, *P. segobiensis* and *P. stipitis* have two residues and *Y. lipolytica* has only one cysteine residue. It may be interesting to examine the biological significance of this variation.

We have successfully cloned, overexpressed and purified uricase enzyme from the five yeast species. The genes of all the species were cloned in pET28c vector at *Nco*I and *Xho*I site which results in His-tag at the C-terminus of the recombinant protein. Uricase of *L. thermotolerans* was cloned at *Nde*I and *Xho*I sites of pET28c vector which yields protein with N-terminal His-tag. The cloned plasmid was transformed into *E. coli* strain BL21 (DE3) and the genes were overexpressed by inducing 1 mM IPTG. The pI of uricases of *K. lactis*, *L. thermotolerans*, *P. segobiensis*, *P. stipitis* and *Y. lipolytica* deduced by protparam Expasy tool were found to be 7.08, 8.36, 7.71, 8.56 and 7.19 respectively. The estimated size of uricases on SDS-PAGE was found to be approximately 30 to 40 kDa.

All five uricases were characterized biochemically and compared with the properties of uricases of *C. utilis*, *A. flavus* and *A. globiformis*. It was reported in literature that *C. utilis* was known to be better enzyme in terms of its activity and efficiency and it is in phase III clinical trials. Optimum pH of all uricases of this study was found to be at pH 7.0 which is close to the biological pH (7.35 to 7.45). Thus these uricases may have an edge as drugs in terms of activity over the uricases of *C. utilis* and *A. flavus* whose optimum pH found to be 8.5 and 8.0 respectively. Optimum temperature of *Kl*UOX, *Yl*UOX and *Lt*UOX was found to be 35°C, whereas optimum temperature of *Pst*UOX and *Pse*UOX was 40°C. The pH stability of all uricases examined in this study ranged from pH 5.0 – 11.0. All the uricases examined in this study were found to be stable up to 55°C. Kinetic studies showed that the uricase of *C. utilis*, used as control, found to be better among all the uricases in our experiments. Uricase of *C. utilis*

has higher affinity and catalytic efficiency, closely followed by *Kl*UOX and *Lt*UOX. The value of k_{cat}/K_M gives the enzyme efficiency in the reaction. The higher the k_{cat}/K_M value higher is the enzyme efficiency in the reaction. The k_{cal}/K_M value was higher for the uricase of *C. utilis* (0.315) which was followed by *Kl*UOX (0.288), *Lt*UOX (0.21), *Yl*UOX (0.066), *Pst*UOX (0.065), *A. flavus* (0.044), *Pse*UOX (0.039) and *A. globiformis* (0.004).

Biophysical characterization of *Kl*UOX and *Lt*UOX were performed by determining molecular weight and the oligomeric state by MALDI-TOF and Gel filtration profile respectively. Molecular weight of the enzymes *Kl*UOX and *Lt*UOX were found to be 36.388 kDa and 37.322 kDa respectively. Gel filtration profile showed that the enzyme of *K. lactis* exists as a tetramer in the solution which was found in case of all uricases described so far. The active enzyme exists as tetramer in the solution. Through CD studies it was found that the β sheets were predominantly present in the enzyme. During the initiation of this work, the crystal structure of *C. utilis* uricase was not available, and the same was published at a resolution of 1.93 Å in 2011. In the present study, we report the crystal structures of *K. lactis* uricase in both apoform and U-8aza complex, at 1.8 Å and 1.9 Å resolutions respectively. It was found that the uricase was tunnel shaped from top view and the residues at the active site were found in the crystal structure of U-8aza complex. Both the subunits were found to be contributing residues for the active site. Amino acid residues surfacing the active site were found to be T62 of one subunit and the residues F167, R184, Q240 and N266 of another subunit. This suggests that these five amino acid residues may play important role in the enzyme affinity and activity either by directly interacting with the substrate or by indirectly affecting the interaction of residues and substrate. Refinement of the structure is still in process..

The uricases of *K. lactis* and of *L. thermotolerans* were also cloned and expressed in *A. adeninivorans* strains G1212 in both HIS tagged and Tag free forms. The enzyme was under the strong constitutive promoter TEF and terminated by PHO5 terminator. ALEU2 and ATRP1m genes were used as selection markers in strains G1212. The clones were inoculated in YMM broth. The cultures were subcultured for ten generations to stabilize the integration of the genes in genomic DNA of *A. adeninivorans*. A total of 764 (382 clones of *Kl*UOX*Aad* and 382 clones of and *Lt*UOXA*Aad*) clones of all types of constructs were screened. The expression was found to be very low (0.5 mg-1mg/L) and optimization of the conditions may help in improving the

expression levels. The enzymes were characterized and the biochemical and biophysical parameters were found to be almost similar to that of the uricases expressed in *E. coli*.

Three different types of mutations were performed to understand their effect in the enzymes and to select the enzymes with enhanced enzyme activity. Among the 12 mutants obtained by SDM, 9 mutants showed decrease in enzyme activity. The decrease ranged from more than 100 fold less activity to 5 fold less activity compared to wild type. Two mutants F184H and R184A showed lowest catalytic efficiency among all the mutant uricases. Three mutants showed enhanced enzyme activity compared to wild type. It was found that the efficiency of the mutant enzyme T62S was 3 fold higher and F167W showed 1.5 times to that of the wild type *Kl*UOX. The mutant F167Y showed activity almost equal to wild type *K. lactis* and *C. utilis* uricases in terms of catalytic efficiency. The catalytic efficiency of the mutants was calculated by k_{cat}/K_M. Three mutants T62S followed by F167W and F167Y were found to be having better efficiency than the wild type of *K. lactis* and *C. utilis*.

In the site directed mutational study on uricase of *L. thermotolerans,* 10 out of 12 mutants showed decrease in enzyme activity which ranged from 50% to several hundred folds. Two mutants showed enhanced enzyme activity. The mutant Q239A had showed better catalytic efficiency than all other *L. thermotolerans* mutant uricases. The efficiency of the mutant enzyme Q239A was more than 2 fold higher than that of both wild type and the commercial uricases. Another mutant enzyme T61S was found to have catalytic efficiency marginally more than that of the wild type uricase of *L. thermotolerans*. The catalytic efficiency of mutant Q239A was found to be high when compared to both wild type and commercial uricases.

Random mutations were done in two ways, using chemical mutagen and mutazyme. Approximately 3,000 mutants each from *K. lactis* and *L. thermotolerans* uricases were screened for enhanced enzyme activity. None of the clones showed enhanced activity compared to wild type.

As mutant T62S of *K. lactis* showed higher catalytic efficiency among all the mutants, saturation mutagenesis was performed at this site. Ninety two clones were screened and none of the mutant showed better activity compared to wild type. Among the 16 randomly picked clones, 10 were found to be wild type and other 6 mutants showing less activity were found to have Alanine, Arginine, Glycine, Methionine, Proline, and Tyrosine substitutions. It will be

interesting to examine role of these amino acids using molecular modeling studies and future studies on these aspects were planned.

Even though there were reports of mutational studies in the uricase gene of different sources, there were no reports of mutational studies on all the residues at the active site. In this study we carried out mutational studies at all the residues of the active site of uricases of both *K. lactis* and *L. thermotolerans*. Three types of mutations SDM, saturation mutations and random mutations were performed in the uricase gene of *K. lactis*. Two types of mutations SDM and random mutations were performed in the uricase gene of *L. thermotolerans*. Both saturation and random mutations did not yield mutants with better activity than that of the wild type of uricases of respective strains. Using SDM approach in both *K. lactis* and *L. thermotolerans* we could obtain uricase mutants having better efficiency than wild type uricases as well as uricase of *C. utilis*. It was also observed that incorporation of similar amino acids at some of the active sites results in decreased enzyme activity. Modeling studies can be performed using the data obtained in these studies to understand the mechanism of action of different residues. The data generated may be useful for improving/designing enzymes with better properties.

Future directions

- Molecular modeling studies are planned to understand the structural modifications in the mutants which lead to the difference in enzyme activity compared to wild type.
- Cloning and expression of selected mutants in *Pichia pastoris* expression system.
- Scaling up of the clones to the fermenters and standardize the growth and protein expression conditions to obtain good yield of the enzyme.
- As part of this work we did not evaluate the potential of these uricases for their pharmacological properties. It is planned to evaluate the pharmacological properties of wild type and mutant uricases as potential drugs for hyperuricemia.