Summary

Being humans, we eat a diet rich in purines and consumption of alcoholic beverages is also increasing, making us overweight. These factors increase the risk of developing gout, as human body is incapable of degrading the uric acid. The excess uric acid crystallizes in and around joints leading to an inflammatory condition, gout. The risk of developing gout can be reduced and it can be managed by simply maintaining a healthy weight through dietary modifications and drinking plenty of water. However, over time this condition may worsens, if left untreated, and thus can cause permanent damage to joints and nephorpathies like kidney stones. Gout is a neglected disease and lesser attention has been paid for the economic development of therapeutics in concordance with its globally increasing trend. In developing countries like India, most of the people are not even aware of the severity of this disease. Moreover, people are taking pain killers and NSAIDs (non-steroidal anti-inflammatory drugs) by their whims to get relief from the pain of gouty attacks.

The conventional therapies to treat hyperuricemia and gout are xanthine oxidase inhibitors like allopurinol and uricosurics like probenicid etc. On the other hand, therapeutic potential of uricase against gout and hyperuricemia is not a new thing to the world now. The available approved enzyme-based therapies for uricase are: uricase from *A.flavus* expressed in *S.cerevisiae* (Rasburicase), which is used in its native form and has been approved by US FDA for its use against pediatric tumor lysis syndrome and the other uricase is a porcine-baboon chimera (Krystexxa®), which is a pegylated form of uricase and got a US FDA approval (2010) and European Medicines Agency (EMA) approval (2013) for the treatment of refractory or treatment failure gout. These chemical drugs, especially allopurinol, serve as a corner stone in present day treatment for hyperuricemia and gout. These drugs are incapable of degrading the existing uric acid or deposited urate crystals from the body. Nonetheless, these drugs have various side-effects like rash, fever, hypersensitivity reactions, gastrointestinal problems, blood dyscrasias, bone marrow suppression, liver and kidney problems. On the contrary, high immunogenicity of uricase-based therapies due to their parenteral use; and high cost are the limiting factors for their general use. There is an urgent need to explore and develop new uricase based formulations with a potential to be administered through alternate routes of body minimizing the immunogenicity and enhancing the treatment efficacy.

There exists a huge window of opportunity to reduce the progression of gouty arthritis in a developing country like India, where the risk factors are high. If successful, it will surely improve the quality of life and the future costs of disability due to gout will not become an unendurable burden, which is a genuine problem in the current day. Unfortunately, India is one of the countries where people are not competent to afford costly treatments like Krystexxa®, around USD 14000 for a single 8 mg vial to be administered every 2-3 weeks; and Rasburicase around INR 8000 for a single 1.5 mg vial which is administered in a dose of 0.2 mg/kg as a 30-minute IV infusion once a day for up to 5 days. However, the cost of Rasburicase is not that high but its high immunogenicity, short circulating half-life and repeated administration are some of the problems associated with its limited use. For the cost-effectve development of a protein (enzyme) therapeutic, its soluble and high expression in native form (free of any affinity or reporter tag etc.) is the first crucial step. Also, it should have excellent *ex-vivo* and *in-vivo* activity and a potential for administration through different routes of body. Our research group has previously screened 36 different yeasts available in Microbial Type Culture Collection (MTCC) for their uric acid utilization. The uricases of *Kluyveromyces lactis* followed by *Lachancea thermotolerans* were found to be most active. The uricase of *K.lactis* was cloned (with a C-terminal polyhistidine-tag encoding region) and expressed in *E.coli*. In the present work, detailed studies on production, purification and comparative characterization of a non-tagged uricase from recombinant bacteria, *Escherichia coli* BL21 (λDE3) and yeast, *Arxula adeninivorans* G1212 were undertaken. Also, attempts were made to carry out derivatization of uricase and to characterize its potential for therapeutic use.

The uricase gene of *K.lactis* (*Kl*UOx) was successfully cloned and expressed without a tag in *Escherichia coli* BL21 (λDE3) and yeast, *Arxula adeninivorans* G1212. The uricase was expressed intracellulary in the soluble fraction. Different purification matrices for ion-exchange chromatography (IEC) and hydrophobic interaction chromatography (HIC) were screened and a two-step based purification approach was designed. In the first step we used ion-exchange chromatography followed by hydrophobic interaction chromatography as a second step. We speculated elution of the bound uricase using a pH based gradient as it will prevent the use of salt in the purification procedure. The use of salt in the purification procedures increases the overall cost as additional steps of desalting and effluent treatment are required. The elution was carried out at very slow flow rates, in the

same buffer by changing the pH from 7.4 to 8.5 resulting in low elution volume and high concentration of the protein. The eluted protein appeared as a single predominant band on the SDS-PAGE. After HIC the homogeneity of the final product was found to be >90% and no other band was observed on SDS-PAGE. The overall yield of the purification process was found close to 65% in two-steps, proving the process to be cost-effective.

*Kl*UOx expressed in both *E.coli* and *A.adeninivorans* showed similar biochemical properties and kinetic properties. In both the cases, the optimum pH for was found to be 8.0 and were able to work across pH range of 5-11. Similarly, the optimum temperature was 35□ and temperature stability was found across a range of 5-55□ for both *Kl*UOx expressed in *E.coli* and *A.adeninivorans*. Metal ions like Cu^{2+}, Zn^{2+} and Mn^{2+} showed an inhibitory effect on the uricase activity and Fe^{3+} was found to completely inhibit the uricase activity and Na^{2+} and Mg^{2+} were found as weak enhancers for uricase activity. Surfactants like DTT, PMSF and metal chelator EDTA showed a stimulatory effect on the uricase activity. Uricase activity was inhibited by organic solvents like acetaldehyde and β-mercaptoethanol. However, methanol, ethanol and acetone did not show any inhibitory effect on uricase activity. The oligomeric state of the uricase determined using size exclusion chromatography and analytical ultracentrifugation showed its tetrameric state in solution.

The optimization of key parameters for the cell growth and uricase expression from *E.coli* was carried out at shake-flask level using LB medium. Different factors like inoculum properties, induction conditions, flask to growth medium volume ratio, agitation rate, temperature, and incubation time were optimized using one factor at a time (OFAT) approach. The maximum yield of uricase at shake flask level was found to be 11275 U/l of the culture, using LB medium. The specific productivity of UOx was found to be 5694 U/gDCW biomass. To increase the uricase production further we used rich media for its cultivation and a two-fold icrease in the specific productivity of UOx was found with 11239 U/gDCW using SB (super broth) medium. In order to achieve higher cell density with maximum volumetric production of uricase, fed batch fermentation was carried in out at lab scale fermenter level which resulted into ~10 fold increase in cell biomass (19.1 gDCW from 1.9 gDCW in LB medium at shake flask level) and volumetric production of uricase (128950 U/l from 11275 U/l of the culture in LB medium at shake flask level).

The uricase was evaluated for its safety in human blood and cell cultures. Hemolysis assay showed that uricase did not cause any hemolysis or erythrocyte agglutination in human blood samples and MTT assay showed that it is not cytotoxic to human derived cell lines. The *ex-vivo* stability and activity of uricase evaluated and compared against commercially available uricase drug (Rasburnat, Natco Pharma), showed a reduced residence time in human blood. The uricase was successfully encapsulated in the cationic liposomes (CLSU). *In-vitro* characterization showed better thermal and hypothermal stabilities for CLSU as compared to the free uricase. The CLSUs also showed higher activity at pH below and above its optimal pH indicating a better stability to acid-base transitions. Compared to the free uricase, CLSU took almost half time to bring uric acid levels from high to normal *in-vitro*. Also, CLSU showed higher stabilities against proteolytic cleavage and extended life in human plasma. The *in-vitro* release profile showed a burst release of uricase from CLSUs in initial phase of incubation (~ 46 % within 2-3 h) and a sustained release thereafter (upto 90% in next 48 h). The pegylation was attempted for uricase, however a drastic reduction in the activity was observed. A more in-depth study is required to optimize the pegylation conditions for uricase. Overall this study provides an optimized platform for production of recombinant uricase using *Escherichia coli* BL21 (λDE3) expression system at shake-flask as well as mini-scale fermenter level. Our work is in accord with ongoing efforts to develop cost-effective approaches for the production of uricase as well as development of therapeutic formulations with potential for alternate routes of administration in human body.

Future directions:

- Based on the crystal structure, we have created some mutations at the active site residues. The mutants were successfully cloned and expressed in *E.coli*. The kinetic studies after the purification of these mutant uricases can be carried out to design catalytically efficient candidates.
- We have also successfully cloned the uricase in pPIC9k vector for its expression in *Pichia pastoris* GS115 expression system. Unfortunately, we were able to achieve very low expression levels. Optimization studies can be carried to improve the expression levels.
- Optimization studies on derivatization of uricase like pegylation etc.
- *In-vivo* studies can be carried to evaluate the pharmacological properties of CLSUs in animal model.