Summary

Purine metabolism is one of the essential pathways of all living organisms and is broadly divided into three different categories: synthesis, salvage, and catabolism. The Purine catabolic (degradation) pathway is regulated by a series of reactions which degrade various purines into different end products in different organisms. Purine degradation occurs in all living organisms, but the end product may vary. Different organisms contain different sets of enzymes for the purine degradation, resulting in complete or partial degradation of purines.

Adenine deaminase (ADA) that deaminates the adenine and converts into hypoxanthine is found only in prokaryotes and lower eukaryotes. Guanine deaminase (GDA) converts guanine into xanthine and ammonia is found in all organisms (prokaryotes, eukaryotes, plants, and fungi). Purine nucleoside phosphorylase (PNP) converts nucleosides into corresponding purine bases with the release of ribose 1 phosphate. Xanthine dioxygenase (XanA) is a homolog of XDH and XO found only in fungal kingdom oxidizes xanthine into uric acid. Uricase converts the uric acid into allantoin. In case of humans, uric acid is the end product of the purine degradation pathway. It is well known and established that purine degradation pathway plays important role in purine homeostasis and various disorders. Enzymes of purine degradation pathway play role in immune-deficiency, renal failure, cardiovascular diseases, neurological disorder, gout, and hyperuricemia.

An elevated level of uric acid is known as hyperuricemia, which finally leads to gout. High uric acid is also an important risk factor for cardiovascular, renal, and metabolic diseases. Gout is the most common inflammatory arthritis, characterized by deposition of uric acid crystal predominantly in synovial fluid. Gout is always associated with lifestyle and is known as the disease of rich man and upper-class. It was confirmed that dietary intake is associated with the increased prevalence of gout and hyperuricemia. High prevalence of gout is reported from developed countries: New Zealand (6.1%), US (3.2%), UK (1.4-2.5 %), Germany (1.4 %), and France (0.9 %).

Various uricosuric, uricostatic, and uricolytic drugs are available to cure gout, but they are associated with the side-effects, immunogenicity, and cost-effectiveness. Although, many therapeutics and drugs are available to treat gout, low purine diet is also recommended to the gouty patients. Physician always recommends gouty patients to avoid alcohol consumption because of high purine content. Amongst all alcoholic beverages, beer has the highest content of purines.

Summary

Alcohol intake is highly associated with the increased risk of gout. Alcohol contains high purine content and some mechanisms proposed to explain the pathogenesis of alcohol-induced hyperuricemia suggest that ethanol ingestion increases the urate production and decreases the urate excretion. Several reports revealed that beer drinkers have more chances to increase risks of gout in comparison to wine and spirit drinkers. Studies also reported that two and more beer drinks per day increase risk of gout by 2.5 fold as compared to non-beer drinkers. One of the possible reasons for the beer associated with gout is the presence of high level of guanosine.

The enzymes of the purine degradation pathway well studied in bacteria and humans, but few studies are reported from the yeasts. Our lab has earlier worked on the uricase of *Kluyveromyces lactis*, a biotechnologically important yeast. It has been approved by Food and Drug Administration (FDA) as generally regarded as safe (GRAS) status organism and is widely used in food industries and large-scale therapeutic protein productions. It was also reported to contain one novel enzyme XanA, a homolog of XDH and XO. In the present study we have focused on characterization of other important enzymes (ADA, GDA, PNP, and XanA) involved in the purine degradation pathway of *K. lactis* and demonstrate their application in the reduction of purine content in a beverage.

ADA is one of the important enzymes of the purine degradation pathway and plays important role in adenine utilization. *Klac*ADA is 1,068 base pairs in length and codes for 356 amino acids. *Klac*ADA showed the high degree of similarity with ADA of *K. marxianus* (84%) followed by *S. cerevisiae* (67%), human (24%), *E. coli* (28%), bovine (24%), and murine ADAs (24%). It was cloned and overexpressed in *E. coli* expression system with N-terminal His-tag and yeast expression system (*A. adeninivorans*) with C-terminal His-tag. Molecular weight of purified *Klac*ADAs was found to be ~41 kDa on SDS-PAGE gel, which was further confirmed by the MALDI-TOF analysis.

*Klac*ADAs expressed in both *E. coli* and *A. adeninivorans* showed the optimum pH 6.0, but were able to work across pH range 4.0-9.0. The optimum temperature of *Klac*ADA was 30 °C and it exhibited moderate thermostability as it was able to work up to 40 °C with > 70% activity. Adenine was the preferred substrate of *Klac*ADA however, it showed broad substrate specificity and consumed various purines and their analogs e.g. 2, 6-diaminopurine, 6-chloropuine, hypoxanthine, guanine, 8-azaxanthine, and 8-azaguanine. Metal ions Fe^{2+}, Zn^{2+}, Cu^{2+}, Mg^{2+}, Co^{2+}, and Ni^{2+} were weak inhibitors, and Ca^{2+} was weak enhancer for *Klac*ADA activity. Surfactants like DTT and PMSF showed inhibitory effect on *Klac*ADA activity, whereas metal chelator EDTA did not show any inhibitory effect on its activity.

Kinetics study of *Klac*ADA showed that *E. coli* expressed protein had higher K_M value compared to the eukaryotic expressed protein, but both had comparable catalytic efficiencies. CD analysis of *Klac*ADA revealed that it had 56.26% of alpha helix and 5.67% of the beta sheets. Oligomeric state of *Klac*ADA determined by gel exclusion chromatography showed its dimeric state in solution. Attempts made to obtain the crystals of *Klac*ADA were not successful.

GDA is an important enzyme of the purine degradation pathway of humans and plays important role in humoral and innate immunity, neural development, a promising drug target for liver and cognitive disease. *Klac*GDA is 1,491 bp in length and codes for 496 amino acids. *Klac*GDA showed high degree of similarity with guanine deaminase of *K. marxianus* (69%) followed by *S. cerevisiae* (51%), human (37%), and *E. coli* (35%). *Klac*GDA was cloned and overexpressed in *E. coli* and yeast expression systems with N and C-terminal His-tags, respectively. The molecular weight of purified *Klac*GDAs determined through SDS-PAGE gel was ~57 kDa, which was further confirmed by MALDI-TOF analysis.

The optimum pH and temperature of *Klac*GDA purified from *E. coli* and *A. adeninivorans* were 7.0 and 25 °C, respectively. Enzymes purified from both prokaryotic and eukaryotic expression systems were moderately thermostable and were able to work up to 40 °C with 50% and 75% activity, respectively. Besides guanine *Klac*GDA was also able to consume 8-azaxanthine, 8-azaguanine, 2-amino-6-chloropurine, 2-amino-6-bromopurine, guanosine, adenosine, inosine, xanthosine, and adenine. This is the first report of a GDA catalyzing the xanthosine. Metal ions Zn^{2+}, Mg^{2+}, Co^{2+}, Fe^{2+}, Ca^{2+}, and Ni^{2+} had weak negative effect on *Klac*GDA activity, whereas detergent DTT showed weak positive effect on *Klac*GDA activity.

*Klac*GDA purified from *E. coli* and *A. adeninivorans* had comparable catalytic efficiencies and higher substrate affinity compared to the human and *A. adeninivorans* GDA. CD analysis of *Klac*GDA revealed that it was composed of the 60.44% of alpha helix and 4.69% of beta sheets. Gel exclusion chromatography showed that *Klac*GDA exists as a dimeric state in solution. Several trays set up for obtaining crystals of *Klac*GDA didn't yield diffractable crystals.

The putative PNP sequence containing the complete *Klac*PNP gene (921 bp) was retrieved from GenBank. Sequence analysis revealed that *Klac*PNP shares sequence homology with *S. cerevisiae* PNP (66%), human PNP (47%), calf spleen PNP (47%), *Escherichia coli* PNP (29%), *Bacillus cereus* PNP (27%), and *Bacillus subtilis* PNP (27%). The presence of a catalytically active Asn or Asp residue at the structurally equivalent position in trimeric and hexameric PNPs, respectively, is known to play an important role in substrate specificity. On

the basis of multiple sequence alignment and structural analysis, we identified the residue dictating substrate specificity and created *Klac*PNP^{N256D} and *Klac*PNP^{N256E} point variants. *Klac*PNP, *Klac*PNP^{N256D}, and *Klac*PNP^{N256E} were overexpressed in *E. coli* expression system and wild-type *Klac*PNP was overexpressed in yeast expression system, and purified by Ni-NTA chromatography. Fractions containing purified *Klac*PNPs, *Klac*PNP^{N256D}, and *Klac*PNP^{N256E} were pooled, concentrated, and further purified by size exclusion chromatography. The protein could be purified to homogeneity with >95% purity, as inferred from SDS-PAGE analysis. The molecular weight of protein was further confirmed by MALDI-TOF analysis, which matched very well with theoretical molecular mass. The protein could be concentrated to >20 mg/mL and was stable for months when stored at 8 °C in an elution buffer (25 mM Tris-HCl, 100 mM NaCl, 10% glycerol, pH 7.0).

*Klac*PNPs purified from the *E. coli* and *A. adeninivorans* were optimally active at pH 7.0 and 25 °C. They were moderately thermostable and retained ~40% of activity when incubated at 45 °C and 35 °C, respectively for 2 h. *Klac*PNPs purified from both sources were able to work at pH conditions of 5.0-10.0 and 5.0-8.0, respectively retaining ~40% activity. *Klac*PNP optimum pH was 7.0, whereas *Klac*PNP^{N256D} and *Klac*PNP^{N256E} optimum pH was 6.0 and 7.0, respectively. Both point variants showed optimum temperature 25 °C as showed by *Klac*PNP. pH stability of *Klac*PNP^{N256D} and *Klac*PNP^{N256E} was comparable with *Klac*PNP purified from the *E. coli*, whereas both the mutants were less thermostable compared to wild type *Klac*PNP.

*Klac*PNP was able to consume inosine, guanosine, xanthosine, deoxyguanosine, but unable to catalyze adenosine. This result indicated that *Klac*PNP may fall in homotrimeric class that utilizes only 6-oxopurines. The catalytic efficiency of *Klac*PNP purified from *E. coli* was higher than the *Klac*PNP purified from *A. adeninivorans*. The mutant *Klac*PNP^{N256D} accepted adenosine with the higher affinity and catalytic efficiency. Interestingly, there was no significant change in the k_{cat} for inosine, but a 10-fold decrease in the K_M and catalytic efficiency. Engineered *Klac*PNP^{N256D} was able to consume all nucleosides (6-oxo and 6-aminopurines), whereas *Klac*PNP^{N256E} became highly selective for inosine with a 7-fold increase in K_M and a 10-fold decrease in the catalytic efficiency, in comparison to wild-type *Klac*PNP purified from the *E. coli*.

The effect of additives on *Klac*PNP activity revealed that Zn^{2+}, Mg^{2+}, Co^{2+}, Fe^{2+}, and Cu^{2+} had no effect, whereas Ca^{2+}, Ni^{2+}, DTT, and PMSF showed the weak inhibitory effect on *Klac*PNP. Ribavirin and uridine were the potent inhibitors of *Klac*PNP. Sequence and kinetics results revealed that *Klac*PNP belongs to the homotrimeric class of NP-1 family. Same conclusion

was obtained from the gel exclusion chromatography, which dictated the trimeric form of *Klac*PNP in solution. *Klac*PNP crystal structure has been solved at 1.9 Å and compared with the human and *E. coli* PNP structures. The most distinguishing feature of *Klac*PNP is the presence of a long loop connecting β9 and α7, which participates extensively in establishing intermolecular interactions. Out of 24 hydrogen bonds at the interface, this loop region contributes to an average of 5 hydrogen bonds and several non-bonded interactions. This loop may probably play an important role in stabilizing *Klac*PNP. Interestingly, with crystal structure found the intrinsically bound compound. Adenine and hypoxanthine both the ligands fit according to the electron density. Since, there is a mass difference of only 1 Da in both ligands, ESI-MS analysis was used to confirm the identity of the bound ligand. Based on the observed mass, we could unambiguously identify the bound ligand as hypoxanthine. The presence of hypoxanthine is logical, as *Klac*PNP utilizes inosine as a preferred substrate and produces hypoxanthine as one of the end products. The average B-factor of the hypoxanthine molecules (B-factor 26.6) and the surrounding residues (B-factor 22.94) is comparable, suggesting a near unit occupancy and, hence, high affinity of the ligand. High-affinity hypoxanthine, observed to be intrinsically bound to the active site, forms extensive interactions *in* the active site. In addition to 7 conventional hydrogen bonds, we also observed two potential C–H···O interactions that have not been reported earlier in the ligand bound PNP structures. Although the occurrence of such weak non-classical hydrogen bond interactions and their role in the structural stability and molecular recognition is well documented, these interactions are not so commonly reported in the context of protein-ligand interactions.

XanA is a novel enzyme reported from only fungal kingdom so far. *Klac*XanA is homolog of XDH and XO, these enzymes play important role in various disorders (renal failure, gout). *Klac*XanA is 1,122 bp in length and codes for 373 amino acids. *Klac*XanA showed high degree of similarity with xanthine dioxygenases of *Y. lipolytica* (66%), followed by *A. nidulans* (48%), *N. crassa* (47%), *S. pombe* (44%), *P. brasilianum* (39%), and *S. lycopersici* (35%). *Klac*XanA was cloned and overexpressed in *E. coli* expression system with the N and C-terminal His-tags and in yeast expression system (*A. adeninivorans*) with C-terminal His-tag. Purified *Klac*XanAs showed the band on SDS-PAGE gel corresponded to ~43 kDa. Gel exclusion chromatography indicated that purified *Klac*XanA from both *E. coli* and *A. adeninivorans* elute as monomeric forms. Interestingly, native-PAGE showed that *Klac*XanA exists as trimeric form. The small angle X-ray scattering (SAXS) analysis used to resolve the issue showed that *Klac*XanA exists as tight trimeric state in solution. SAXS data analysis also revealed that *Klac*XanA is tightly packed as trimeric form that elutes as monomeric form through the gel

filtration chromatography. *Klac*XanA is the first enzyme of dioxygenase family, whose oligomeric state in solution is described by the SAXS data analysis.

The optimum pH of *Klac*XanA from both *E. coli* and *A. adeninivorans* was 8.0, whereas the optimum temperature of both enzymes varied. *E. coli Klac*XanA was stable only at 4 °C and *A. adeninivorans Klac*XanA showed maximum activity at 25 °C. *E. coli Klac*XanA and *A. adeninivorans Klac*XanA were pH stable enzymes and able to work across pH 6.0-8.0 and 6.0-10.0 respectively, with ~40% activity. *Klac*XanA purified from *A. adeninivorans* was able to work up to 30 °C with >60% activity. *Klac*XanA was able to consume the different purine and their analogs in order xanthine, guanine, hypoxanthine, adenine, 1- methylxanthine, and 7-methylxanthine. *A. adeninivorans Klac*XanA was more catalytic efficient compared to the prokaryotic expressed *Klac*XanA. Effect of additives on *Klac*XanA activity revealed that Fe^{2+} had enhancer effect, whereas Co^{2+}, Ni^{2+} showed an inhibitory effect on its activity. Allopurinol, DTT, and PMSF showed inhibitory effect towards the *Klac*XanA.

All purine degrading enzymes characterized in this study were examined for their ability to reduce the purine content in alcoholic beverage beer. To the best of our knowledge, there are no reports on purine content analysis of beers available in India. Our analysis of 16 different beers showed the presence of various purines e.g. hypoxanthine, adenine, guanine, xanthine, guanosine, inosine, xanthosine, and uric acid. It was reported previously that beer contains high amount of guanosine, but high content of hypoxanthine was also found in Indian beers. Hypoxanthine is considered to be most harmful purine as it is directly converted into uric acid. Beer sample (brand KS) treated with individual enzymes and the cocktail of enzymes (*Klac*ADA, *Klac*GDA, *Klac*PNP, and *Klac*XanA, 1.0 U), along with the commercial *C. utilis* uricase (0.2 U) and showed decrease in purine content, indicating that purine degrading enzymes are useful for reducing the purine content in beer. This approach may be applicable to other varieties of beverages and foods.

Currently, our group is exploring strategies to make low purine content foods and beverages affordable. Yeast strain harboring engineered *Klac*ADA, *Klac*GDA, *Klac*PNP, and *Klac*XanA for the production of beer could be an attractive approach for creating low purine content beer. Alternatively, the recombinant enzymes can be immobilized on columns for processing the beverages and foods. More work needs to be done to exploit potential of enzymes in producing low purine content foods and beverages at industrial scale. Our work is in line with ongoing efforts to develop alternate approaches that can help reduce uric acid levels in the body and provide safer and easy way to control a number of diseases associated with hyperuricemia.