Chapter 2 REVIEW OF LITERATURE

2.1. Uricase (Urate oxidase EC 1.7.3.3)

The historical origin of uricase enzyme is in 19th century found in prokaryotes and eukaryotes. Wiechowski in 1907 found that the uricase enzyme played a significant role in the oxidation of insoluble uric acid to highly soluble allantoin. The finding of Wiechowski was re-established by Batelli & Stern (Battelli and Stern 1909), who had first isolated the uricase enzyme in the purified form and determined its fundamental properties and mechanism of action. Further, the purification of uricase was improved by many scientists including Keilin & Hartree (Keilin and Hartree 1936), Davidson (Davidson 1942), Holmberg (Holmberg 1939) etc.

2.2. Sources of uricase

Uricase enzyme can be obtained from plants, animals, and microbial sources. Shigeyuki Tajima in 1975 had obtained the uricase enzyme from soybean plants and found that root nodules have a maximum concentration of uricase enzyme from that of the rest of the parts of plant (Tajima and Yamamoto 1975). Further, Karen Lucas et al in 1983 achieved 45-folds of purified uricase enzyme by using ammonium sulfate precipitation, gel filtration, and affinity chromatography from soybean root nodules (Lucas, Boland et al. 1983). In addition to this, uricase was found in higher plants also, obtained in purified form from leaves of chickpea (*Cicer arietimum*), broad bean (*Vicia faba*), and wheat (*Triticum aestivum*) by P. Montalbini et al (Montalbini, Redondo et al. 1997). The uricase enzyme is localized in the glyoxysomes of the plant cells was studied by RR Theimer (Theimer and Beevers 1971). The major uricase

enzymes obtained from plants sources are mentioned in Table 2.1. The gene responsible for uricase production is expressed in animals except in hominoids. Although the gene responsible for uricase expression is present in the human genome, it got silenced during evolution. The lack of uricase enzyme in humans and chickens results in the condition of hyperuricemia which is associated with gouty arthritis. Keilin in 1959 observed that the concentration of the end product of purine metabolism i.e. uric acid in the human blood is 50 times higher than that of other mammals due to the lack of expression of uricase gene (Keilin 1959). However many researchers proposed the evolutionary advantage of uricase gene mutation because of uric acid act as a strong antioxidant and oxygen scavenger which may help in prolonging the lifespan of hominoids by reducing the number of free radicals (Glantzounis, Tsimoyiannis et al. 2005; Johnson, Titte et al. 2005).

Table 2.1: Major sources of uricase production

Sr. No.	Sources	References
	Plant source	
1	Soybean plant	(Tajima and Yamamoto 1975)
2	Soybean root nodules	(Lucas, Boland et al. 1983)
3	Cowpea	(Lucas, Boland et al. 1983)
4	Castor bean	(Smith and Atkins 2002)
5	Bean leaves	(Montalbini 1991)
6	Wheat	(Montalbini 1992)
7	Pigeonpea	(Luthra, Sheoran et al. 1983)
8	*Glycine max*	(Tajima and Yamamoto 1977)
9	Algae	(Stabenau and Beevers 1974)

10	*Pistacia integerrima*	(Ahmad, Farman et al. 2008)
11	*Coffea Arabica*	(Waller, MacVean et al. 1983)
12	*Rhizopus oryzae*	(Farley and Santosa 2002)
1	*Rattus norvegicus*	*(Goldman and Blobel 1978; Motojima and Goto 1990)*
2	*Rana catesbeiana*	*(Fujiwara, Ohashi et al. 1987)*
3	*Equus ferus*	*(Truszkowski and Goldmanówna 1933)*
4	*Silurus glanis*	*(Kinsella, German et al. 1985)*
Animal Sources		
1	*Mus musculus*	*(Lee et al. 2006)*
2	*Sus scrofa*	*(Tsuji et al. 1985)*
3	*Bos taurus*	*(Usuda et al. 1988)*
4	*Oryctolagus cuniculus*	*(Motojima et al. 1989)*
Microbial Source		
1	*Bacillus subtilis*	(Meraj, Rahman et al. 2012)
2	*Microbacterium spp.*	(Zhou, Ma et al. 2005)
3	*Pseudomonas aeruginosa*	(Abdel-Fattah, Saeed et al. 2005)
4	*Streptomyces exfoliates*	(Aly, Tork et al. 2013)
5	*Bacillus cereus*	(Nanda and Babu 2013)
6	*Bacillus thermocatenulatus*	(Lotfy 2008)
7	*Pseudomonas aeruginosa*	(Amirthanathan 2011)
8	*Gliocladium viride*	(Nanda, Babu et al. 2012)
9	*Candida utilis*	(Yokoyama, Ogawa et al. 1988)
10	*Aspergillus niger*	(Geweely and Nawar 2011)
11	*Aspergillus niger*	(Geweely and Nawar 2011)
12	*Mucor hiemalis*	(Yazdi, Zarrini et al. 2006)

13	*Candida tropicalis*	(Tanaka, Yamamura et al. 1977)
14	*Saccharopolyspora sp.*	(Khucharoenphaisan and Sinma 2011)
15	*Micrococcus luteus*	(Snoke, Risley et al. 1977)
16	*Streptomyces exfoliates*	(Aly, Tork et al. 2013)
17	*Sphingobacterium thalpophilum*	(Ravichandran, Hemaasri et al. 2015)

2.3. Production of microbial uricase

For the commercial purpose, among the all sources mentioned above the uricase production from microbial source is the best choice. The production process varies from microbe to microbe and that relies on physical parameters, nutritional components and of course the potential of strain. So to improve the production process, one needs to optimize these parameters effectively. The production of uricase by submerged fermentation (SMF) and solid-state fermentation (SSF) was studied. Uricase production was obtained from various microbial sources under optimally varying conditions. Uricase production was reported by Tanaka et al in 1976 using *Candida tropicalis* on n-alkane as a substrate (Tanaka, Yamamura et al. 1977). Various amino acids were found to stimulate the microbial growth and uricase production (Atalla, Farag et al. 2009). Atalla M. M. et al had screened 19 different microbial strains and found that *Gliomastix gueg* showed a higher degree of uricase production under optimum conditions. The enhanced microbial production of uricase was found to be reported as inducer dependent and various purine metabolites including adenine, guanine, xanthine, hypoxanthine, and uric acid was used in the production media. Among the used metabolites, uric acid was found to be significantly involved in the induction of uricase enzyme (Machida and Nakanishi 1980). However, Yasuto Watanabe (2014) was examined that among the various

purine metabolites, hypoxanthine significantly enhanced the uricase production using *Streptomyces sp.* (Watanabe 1971). Further, Vladimir et al also reported having hypoxanthine as the best inducer for the uricase production by *Candida utilis* strain (Adámek, Suchová et al. 1990). The release of extracellular biochemicals can be enhanced by using surfactants in the media. The surfactants tend to increase the pore size of the cell membrane which in turn increases the cell permeability and hence the release of biochemicals into the extracellular environment is significant. The time of addition of surfactant into the medium is not significantly important. Usually, the surfactant was added into the medium and thereby sterilized togetherly. In 1977, D. Sternberg studied the extracellular production of β-glucosidase using various surfactants such as Tween-80, poly (acrylic acid), Triton-X-100, polyethylene glycol etc and found that poly (acrylic acid) was significantly enhanced the release of β-glucosidase than other surfactants (Sternberg, Vuayakumar et al. 1977). Using surfactant, the extracellular production of enzyme was also reported in solid state fermentation. In 2002, Nutan et al examined the extracellular production of acidic lipase by *Aspergillus niger* using Triton-X-100 surfactant under solid state fermentation (Mahadik, Puntambekar et al. 2002). The hydrolysis of malt starch to alcohol was significantly reported by the improved production of α-amylase from *Bacillus sp.* using Tween-40 under solid state fermentation (Sodhi, Sharma et al. 2005). Around 35% of improvement in the saccharification of cellulose was reported using Tween-80, cetylpyridinium chloride, and cetyl trimethylammonium bromide surfactants (Geddes, Nieves et al. 2011).

The production of uricase was carried out by different microorganisms under different set of conditions. Earlier studies of uricase production by optimization were investigated using *Pseudomonas aeruginosa (Abdel-Fattah, Saeed et al. 2005).*

2.4. Mode of uricase action

Wiechowski in 1907 inferred that enzyme uricase is an oxidoreductase enzyme which consecutively oxidizes and hydrolyzes uric acid to allantoin, carbon dioxide, and hydrogen peroxide. Further, Batelli & Stern (1909), Keilin & Hartree (1936), Holmberg (1939), Davidson (1942) reported and described the uric acid degradation by uricase (Battelli and Stern 1909; Keilin and Hartree 1936; Holmberg 1939; Davidson 1942). Uricase catalyzes the following reaction:

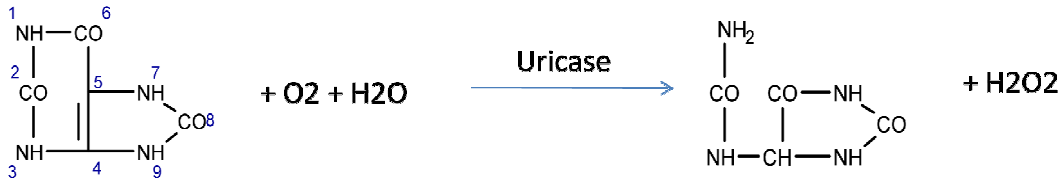

Fig. 2.1: Mechanism of uricase action (Ad⇐ek, Králová et al. 1989; Jiang, Wang et al. 2007; Akgöl, Öztürk et al. 2008)

2.5. Measurement of uricase activity

For the measurement of uricase activity, researchers have tried many ways to assay the uricase and its activity. However, the researchers have developed efficient ways to measure the uricase activity either by substrate uric acid degradation or the product allantoin or H2O2 measurement under the optimized conditions. The measurement of H2O2 produced during the oxidoreduction of uric acid by rat and guinea pig uricase

was reported (GRAHAM JR and KARNOVSKY 1965). This was further supported for the measurement of H2O2 by the colorimetric method using Peroxidase enzyme which measures H2O2 produced after the oxidation of uric acid by uricase (Domagk and Schlicke 1968). By this method, the amount of uric acid was assayed by measurement of the formation of quinoneimine dye, using the Peroxidase-phenol-4-aminoantipyrine as the H2O2 acceptor. The amount of quinoneimine dye formed is directly proportional to the uric acid degraded (Machida and NAKANISHI 1980). The determination of H2O2 concentration using sodium 2-hydroxy-3, 5-dichlorobenzenesulfonate and 4-aminoantipyrine were reported to be sensitive than the phenol-4-aminoantipyrine systems (Artiss and Entwistle 1981; Ensor, Clark et al. 2005; Kotb 2015). Also the purified uricase from *Comamonas sp*. was measured by assaying the concentration of H2O2 produced after oxidation of uric acid (Ghosh and Sarkar 2014).

In addition to the measurement of uricase activity by determining the concentration of H2O2 produced, the activity can also be measured by measurement of uric acid degraded by uricase. The H2O2 produced during the uricase treatment of gout or hyperuricemia is being scavenged by the catalase system of RBC's whereas, CO2 produced during the action of uricase is gets dissolved in the blood plasma in addition to the conversion to bicarbonates ($HCO3^-$) which also gets dissolved in blood plasma. The uric acid concentration was measured by using spectrophotometer with the decrease in absorbance of uric acid at 293 nm as adapted by Mahler et al (Mahler, Hübscher et al. 1955). Further, a novel uricase assay was developed to assay uric acid at 340 nm; the hydrogen peroxide produced reacts with the ethanol in the presence of catalase enzyme to form acetaldehyde and water. The acetaldehyde produced was

reduced by NADH in the presence of alcohol dehydrogenase to ethanol. The decrease in absorbance at 340 nm caused by the oxidation of NADH is directly proportional to the uric acid concentration in the mixture (Trivedi, Rebar et al. 1978). The Mahler et al protocol was followed to measure the uricase activity from the isolated uricolytic bacteria from the gut of *Reticulitermes flavipes* termites (Potrikus and Breznak 1980). Further, the purified extracellular uricase from *Pseudomonas aeruginosa* was assayed by the disappearance of uric acid measured at 293 nm (Saeed, Yousry et al. 2004) and also the purified uricase from *Gliocladium viride* (Nanda, Babu et al. 2012)

The measurement of uric acid concentration present in human blood is the direct application of the uricase. The glassy carbon electrode modified with Nafion and methyl viologen was used to prepare uricase and bovine serum albumin with glutaraldehyde as a cross-linker based biosensor for the measurement of uric acid concentration in human serum (Jin, Ye et al. 1993). The uricase immobilized on ZnO nanorods have reported being thermostable up to 85^{o}C with retaining the uricase bioactivity and the transfer of an electron between uricase and electrode was found to be enhanced proving the better efficiency (Zhang, Wang et al. 2004). In addition to this, the qualitative as well as a quantitative basis of uric acid measurement using the infringement of polyaniline-uricase immobilized in the biosensor (Kan, Pan et al. 2004; Arora, Sumana et al. 2007)

Table 2.2: Uricase Sources and activity

Sr. No.	Sources	Opt. pH	Opt. Temp.	Enzyme activity	Reference

1	*Bacillus subtilis*	8.5	35	42.77 U/ml	(Meraj, Khalil-ur-Rahman et al. 2012)
2	*Microbacterium spp.*	8.5	37	1.0 U/ml	(Zhou, Ma et al. 2005)
3	*Pseudomonas aeruginosa*	5.5	30	7.1 U/ml	(Abdel-Fattah, Saeed et al. 2005)
4	*Streptomyces exfoliates*	8.0	45	0.5 U/ml	(Aly, Tork et al. 2013)
5	*Bacillus cereus*	8.5	30	15.43 U/ml	(Nanda and Jagadeesh Babu 2014)
6	*Bacillus thermocatenulatus*	8.5	25-80	1.25 U/ml	(Lotfy 2008)
7	*Gliomastix gueg*	8.5	25	275.98 U/ml	(Atalla, Farag et al. 2009)
8	*Gliocladium viride*	7.5	30	63.14 U/ml	(Nanda, Babu et al. 2012)
9	*Candida utilis*	8.5	25	70.24 U/ml	(Itaya, Yamamoto et al. 1967)
10	*Aspergillus niger*	9.0	30	47.40 U/ml (extracellular)	(Geweely and Nawar 2011)
11	*Aspergillus niger*	9.0	30	12.90 U/ml	(Geweely and

(intracellular) Nawar 2011)

2.6. Effect of pH on uricase production

The growth of microorganism and production of biochemicals is highly dependent on the culturing habitats. The specific pH of the production medium for the uricase production is vital in the growth of microbe and induction of the enzyme. Vladimir in 1989 produced uricase under controlled conditions by maintaining the pH at 5.0 from *Candida utilis* (Adámek, Suchová et al. 1990). The production of recombinant uricase was significantly improved by switching the culture pH from 5.5 to 6.5 during the induction phase using *Hansenula polymorpha* by Zhiyu Chen et al in 2008 (Chen, Wang et al. 2008). However, Atalla et al in 2008 produced uricase enzyme from *Gliomastix gueg* under the alkaline condition of pH 8.0 (Atalla, Farag et al. 2009). Therefore the optimum pH for the production of uricase varies with the microbe.

2.7. Effect of Temperature on uricase production

The microbial growth and production of biochemicals also vary with the temperature. Atalla et al in 2008 produced uricase enzyme at the optimum temperature of 30°C from *Gliomastix gueg* fermentation (Atalla, Farag et al. 2009). Neveen et al 2011 produced uricase enzyme from *Aspergillus niger* with the optimum temperature of 27°C.

2.8. Media design and process optimization

The proper choice of medium components based on both aspects of regulatory effects and economy is the objective in designing the chemical composition of the

fermentation media, where the nutritional requirement for growth and production must be appropriate. Rapid production and high concentration of the desired product are the criteria for the qualitative and quantitative addition of nutrients and other ingredients.

Different methods for media design have been described over the time period.

A] Classical approach

The classical approach to media designs is commonly employed where individual components such as nitrogen sources, carbon sources are alternatively used as a single variable. By this method, for the development of media, it is essential to understand the metabolic pathway in the producing microorganism. Frequently, these methods seek to define nutritional components that resemble the elemental composition of the cells to be grown. The choice of particular carbon and nitrogen sources may occur by trial-and-error or may depend on the understanding of metabolic pathways in the using microorganism. Attention may also be given to balance carbon, nitrogen, phosphate, sulfate, magnesium, and other salts present in the cell.

B] Statistical design of experiments

Statistical approaches have been used commonly to the first screen and to optimize the fermentation processes. The analysis of experimental results is now commercially available using software such as QUALITEK®, MINITAB®, Statistica® and Design-Expert®. The optimization of nutritional factors or physical parameters can be done with using Plackett-Burman screening design or a fractional-factorial design. The screen out of certain factors is possibly done due to the lack of

significant impact on a particular fermentation, and the rest of the factors are retained and examined more carefully. In order to surpass the old conventional methodology of optimization which consists of alteration of each parameter at a time by keeping rest of the parameters constant, due to these, the impact of the individual parameters can be evaluated. But these methodologies are time-consuming, cumbersome and also require more experimental data sets which cannot show the mutual interactions of parameters (Beg, Sahai et al. 2003).

To overcome this, Taguchi method of Orthogonal Array (OA) can be used to design the fractional factorial experiments (Azin, Moravej et al. 2007; Im, Song et al. 2009; Tripath and Srivastava 2011). Taguchi methodology of design of experiments (DOE) identifies the effect of the individual parameters and shows the relationship between the variables, also the desired design is obtained by selecting the highest performance (Bhatt and Srivastava 2008; Das, Das et al. 2014). Based on the concept of robustness and the S/N ratio, the optimal level of important controlling parameters is determined by this methodology.

2.9. Purification of uricase

The final cost of any product is based on the cost-effective downstream process, thus leads to commercial success. The yield of the product is inversely proportional to the number of sequential purification steps due to the loss of the product during handling. The commercial use of uricase generally does not require a substantial amount of purification of the enzyme, but uricase utilized in pharmaceutical and clinical sectors require a high degree of purity. The purified enzyme is being identified and structurally, functionally characterized.

The partial purification of protein using ammonium sulfate precipitation and other chromatographic techniques such as ion-exchange and size exclusion are the methods of choice for uricase purification. However, the protein can also be purified based on the hydrophobic interactions using hydrophobic chromatography. The folds of purification of the protein are calculated by using specific activities at each purification step.

Table 2.3: Purification of uricase from microbial source

Sl. No.	**Organism**	**Purification Strategy**	**Specific activity (U/mg)**	**Purification fold**	**References**
1.	*Microbacterium* sp.	$(NH_4)_2SO_4$ DEAE-Cellulose Toyopearl HW-65 Sephadex G-75	5.32	19.7	(Kai, Ma et al. 2008)
2.	*Enterobacter cloacae*	$(NH_4)_2SO_4$ DEAE-Cellulose Sephadex G-150	6.6		(Machida and NAKANISHI 1980)

		DEAE-Sephadex A-50			
3.	*Pseudomonas aeruginosa* 50071	$(NH_4)_2SO_4$ Sephadex G100 CM Sephadex C50	106	43	El-Bessoumy *et al*., 2004
4.	*Sphingobacterium thalpophilum*	CIM monolith column	7400	14.8	(Ravichandran, Hemaasri et al. 2015)
5.	*Neurospora crassa*	Protamine sulfate $(NH_4)_2SO_4$ Sephadex G150 DEAE- Cellulose Affinity chromatography.	18.5	1000	(Wang and Marzluf 1980)
6.	*Aspergillus niger*	$(NH_4)_2SO_4$ Sephadex G-200 Sephadex G-100	105.9	17.8	(Geweely and Nawar 2011)
7.	*Candida sp.*	$(NH_4)_2SO_4$	12.2	244	(Liu, Li et al.)

		Sephadex G-200 DEAE-Cellulose Sephadex G-200			
8.	*Bacillus fastidious*	$(NH_4)_2SO_4$ DEAE-Cellulose Sephadex G-200	44	-	(Zhao, Zhao et al. 2006)
9.	*Gliocladium viride*	$(NH_4)_2SO_4$ Three-phase partitioning	206.6	1.44	(Nanda, Babu et al. 2012)
10.	*Soybean*	$(NH_4)_2SO_4$ Sephadex G-200	15.6 µM/µg	45	(Lucas, Boland et al. 1983)

The purity of the uricase at each step during purification can be checked by SDS-PAGE analysis.

2.10. Characterization of the purified protein

The microbial produced protein and then purified by means of partial purification and chromatography-based techniques are identified with different techniques like protein sequencer, MALDI-TOF etc (Perkins, Pappin et al. 1999). The purified protein can be identified either by characterizing MALDI MS/MS or by means of MALDI-TOF

analysis of the excised purified band from the SDS-PAGE gel. Prior to the MALDI-TOF, the excised purified protein can be treated with Dithiothreitol (DTT)/ Iodoacetamide for reducing the disulfide linkage present in the protein and digested with trypsin in order to produce the protein in peptide forms. This protocol is referred to as in-gel trypsinization (Morris, Phatnani et al. 1999).

2.11. Molecular mass of uricase

The molecular mass of uricase can be determined on the basis of size by 1D gel or 2D gel electrophoresis. In addition to these methods, the molecular mass of the purified uricase protein can also be determined by gel filtration chromatography or MALDI-TOF analysis (Clauser, Baker et al. 1999). The molecular mass of the uricase varies from microbe to microbe such as *Arthrobacter globiformis* (33 kDa) (Suzuki, Sakasegawa et al. 2004); *Pseudomonas aeruginosa* (34 kDa) (Amirthanathan and Vijayakumar 2011); *Microbacterium sp.* (34 kDa) (Kai, Ma et al. 2008); *Bacillus subtilis* (58.9 kDa) (Pfrimer, Moraes et al. 2010); *Candida utilis* (34 kDa) (Chen, Wang et al. 2008) etc.

2.12. Physico-chemical properties of the purified uricase

The most of the uricase produced from microbial sources are reported to be extracellular in nature, hence the pH and temperature optima for the activity varies from uricase to uricase. The pH optima for maximum uricase activity are reported to be in the alkaline range such as uricase isolated from *Pseudomonas aeruginosa* (pH 9.0), (Saeed, Yousry et al. 2004), *Bacillus fastidious* (pH 9.2) (Zhao, Zhao et al. 2006), Candida utilis (pH 8.0) (Liu, Wen et al. 2011) etc. The enzyme activity of uricase is highly dependent on the pH of the buffer solution. Shigeyuki Tajima in

1975 isolated uricase enzyme from nodules and radicles of the soybean plant with a pH optimum of 9.5 and 7.0 respectively (Tajima and Yamamoto 1975).

The uricolytic degradation by uricase is highly dependent on the working temperature of the reaction mixture. Bongaerts in 1978 was purified the uricase enzyme from *Bacillus fastidiosus* having optimum temperature for activity at 30 to 35°C. The increase in temperature above 35°C has decreased the activity may be due to the conformational changes occurred with the loss of activity and no due to the denaturation of the enzyme, as stated by Bongaerts et. al (Bongaerts, Uitzetter et al. 1978). Jianguo et al in 1993, purified uricase enzyme from *Candida spp.* with the optimum temperature of 30°C (Jianguo, Gaoxiang et al. 1994).

The uricase stability at different temperature also varies from the habitat of the microbe. The uricase produced from the *Bacillus thermocatenulatus* strain was found to be thermostable and found to retain 100 % of its activity after heating the enzyme for 45 min at 75°C (Lotfy 2008). The uricase isolated from bean leaves of *Phaseolus vulgaris* was found to be homotetramer with four identical subunits with the molecular weight of 32 – 33 kDa and have *Km* values of 15 – 34 µM. with an optimum pH of 9.0 (Montalbini, Aguilar et al. 1999). The produced uricase from isolated soil bacteria *Arthrobacter pascens* exhibited the Michaelis constant (K_m) value of 0.2 mM which was found to have 10-fold higher than the isolated liver uricase of 0.02 mM with the optimal pH was 9.2 (Arima and Nose 1968). The recombinant uricase obtained from *Arthrobacter globiformis* displayed acidic in nature with pI 4.96 and the molecular weight of 33.8 kDa. The kinetic properties V_{max}

and K_m of this protein were found to be 208 µM/min and 75 µM respectively (Suzuki, Sakasegawa et al. 2004).

Further, the purified uricase was reported to be tested for the lowering of uric acid concentration in hyperuricemic and gout in animal models. In order to carry out the enzymatic treatment, the *in vivo* efficiency of the enzyme is necessary (Shi, Evans et al. 2003).

2.13. *In vivo* study of uricase

The efficiency of therapeutic protein inside the animal body is foremost important in order to use for the treatment of disease. In order to use purified uricase for the treatment of hyperuricemia in uricase deficient animals such as humans and higher primates, the *in vivo* efficiency and bioactivity of uricase protein is significantly studied.

2.13.1. Hyperuricemia: Uricase deficient animals

Many of the researchers have tried to develop hyperuricemic conditions in rat, dog, rabbit, but due to the expression of liver uricase enzyme had prevented the hyperuricemic conditions. So in order to study the efficacy of the external uricase enzyme, the uricase deficient animal model can be used either by inhibiting the uricase present in the animal by means of inhibitor or by blocking the expression of uricase at the genetic level. By these strategies, the uric acid concentration in the serum of that animal can be enhanced effectively.

2.13.2. Selection of inhibitor and animal model

The ideal uricase inhibitor for development of hyperuricemia should be irreversible, noncompetitive and nontoxic to the experimental animal, hence its activity would remain after the induction of uric acid levels and also the effective inhibition could be achieved with a minimal dosage. The s-Triazines based inhibitors such as oxonic acid, allantoxaidine are commonly used for the inhibition of uricase in the animals however, they are not ideal as such because they inhibit uricase by means of competitive mechanism with that of uric acid. Hence their effects would be minimized due to the removal from the body after eliciting the uric acid concentration to the considerable level. The efficacy of the oxonic acid could be increased by conjugating with the potassium to make potassium oxonate which is easily available and can be effectively used in the development of hyperuricemia by inhibiting the uricase enzyme.

Johnson et al in 1969 have used the rat as an experimental animal for toxicolological evaluation of the hyperuricemic conditions. In this study, they have used oxonic acid and allantoxaidine as potent inhibitors for the uricase present in the rats. The oxonic acids when fed to rats or administered intraperitoneally, displayed an increase in the concentration of uric acid in blood plasma. However, further plasma uric acid concentration was increased by 22-fold on day 23 after adding 5 % oxonic acids and 1 % uric acid in the diet (Johnson, Stavric et al. 1969). Stavric et al has induced nephropathy in rats by feeding a diet having uric acid and oxonic acid for inhibiting the uricase enzyme (Stavric, Johnson et al. 1969) and also other researchers (Bonardi and Vidi 1973; Stavric, Johnson et al. 1976) has effectively studied the inhibition

mechanism using potassium oxonate for the induction of hyperuricemia in rats (Norrlind and Kihlberg 1973; Waisman, Bluestone et al. 1974; Bluestone, Waisman et al. 1975), rabbits (Wildman and Philp 1970; Stavric and Nera 1978; Bacq, Deby et al. 1981).

2.13.3. Effects of drugs in Hyperuricemia: Uricase deficient animals

The elevated uric acid concentration in the animals after inhibition of uricase present in their body can be regulated either by means of removal of uric acid from the body using uricosuric drugs or by regulating the further synthesis of uric acid by inhibiting the purine metabolic enzyme xanthine oxidase with chemical drugs like allopurinol and Febuxostat. The uricosuric drugs work by enhancing the urinary excretion of uric acid with restraining the renal tubular reabsorption of the uric acid which indeed regulates the level of uric acid in serum. The use of uricosuric drug probenecid in the oxonate treated hyperuricemic rat was firstly reported by Stavric et al (Stavric and Nera 1978) where they observed the effects of probenecid on the increased urinary excretion rate of uric acid and found that in 60 to 70 % hyperuricemic rats the renal clearance of uric acid was decreased which then increased the plasma uric acid concentration by the formation of precipitates in the kidney tubule. However, in the rest of the 30 to 40 % rats, the urinary excretion of uric acid using probenecid was significant with no any other side effects. Also, there are no such severe side effects observed with using probenecid in humans however, the 10 to 20 % of the patients had developed gouty arthritis and 9 % suffered from urate kidney stones (Scott and O'Brien 1968; Kelley 1975).

So the other way to regulate the concentration of uric acid in the animal body is to block the activity of enzyme xanthine oxidase by means of chemical drugs such as allopurinol, febuxostat etc. This blocks the further synthesis of uric acid. The severe effect of allopurinol in humans was observed by depleting the erythrocyte phosphoribosylpyrophosphate (PRPP) in which the allopurinol gets converted into allopurinol ribonucleotide by the enzymatic way which then consumes PRPP (Fox, Wyngaarden et al. 1970).