7. SUMMARY

The present study dealt with production, characterization and application of uricase enzyme from *A. niger*, a fungal strain isolated from sediment sample of Pichavaram mangrove environment.

The density of uricolytic fungi was found to be $1.0 \times 10^4$ CFU/g. Among 98 different fungal strains tested 7 were found to be highly potent and identified as *Alternaria alternate*, *Rhizoctonia solani*, *Aspergillus niger*, *Aspergillus flavus*, *Fusarium oxysporum* and *Penicillium citrinum*. Based on well assay done in uric acid incorporated Czapak Dox agar, *A. niger* was selected as the most potential strain.

When optimized, incubation time – 96 hrs, agitation speed – 150 rpm, pH - 8, temperature – 30°C, salinity – 2%, sucrose – 10% (w/v) as carbon source, uric acid - 0.4% (w/v) as nitrogen source were found to be optimum for biomass production and uricase activity.

In this study, regarding incubation period the maximum uricase production was observed at 96 hrs (148U/ml/min.), where the biomass was found to be 6.4g/L.

Regarding agitation, maximum biomass and uricase activity of 6.3 g/L and 141 U/ml/min. respectively was observed at 150 rpm.

At pH-8 maximum biomass and uricase production were observed and the values were 6.2g/L and 143 U/ml/min. respectively.

30°C favored maximum biomass (5.8 g/L) and uricase production (140 U/ml/min.). 2% salinity favored maximum biomass and uricase enzyme production respectively of 6.3g/L and 140U/ml/min.
In the present, study sucrose was found to be the best C sources for uricase production where 6.1g/L of biomass and 144U/ml/min. of uricase production were observed. Regarding the concentration of the ideal carbon source, 10% sucrose favored maximum biomass (6.2g/L) and uricase activity (151 U/ml/min.).

Uric acid as a nitrogen source resulted in maximum biomass (6.4g/L) and uricase activity (146 U/ml/min.). Regarding the ideal nitrogen source concentration 0.4% favored a maximum biomass (6.5g/L) and uricase activity (152 U/ml/min.).

10% poultry waste as a cheaper substrate favored respectively 6.7g/L and 135U/ml/min of biomass and uricase activity.

When mass scale was done with optimized parameters in shake flasks biomass and uricase activity were observed as 7.18g/L and 162U/ml/min. When poultry waste was used as a cheaper substrate for mass scale production, the biomass 6.7g/L and enzyme activity found as 135U/ml/min.

When mass scale was done in fermentor using above parameters and 60% of oxygen, the biomass yield was 9.6g/L and 232U/ml/min. When standard carbon and nitrogen sources were replaced with the cheaper substrate (i.e.) the biomass yield was 8.4g/L and the enzyme activity was 189U/ml/min. This result indicated the possibility for the economic production of uricase.

Solid state fermentation with cheaper agri-residues like sugarcane bagasse (SCB), rice straw and corn straw, after pretreatment with alkali and acid showed surprisingly higher uricase enzyme activity in their mycelium free fermentation broth obtained. At the end of 4 days incubation period alkali treated sugarcane bagasse resulted in 250U/ml/min. of enzyme activity with a biomass of 10.0g/L. whereas acid treated SCB showed 186U/ml/min. with 8.1g/L of biomass. Untreated substrate supported only 4.0g/L of biomass with an enzyme
activity of only 40U/ml/min. Rice straw (6.2g/L, 150U/ml/min.; 5.0g/L, 132U/ml/min.;
2.9g/L, 32U/ml/min.) and corn straw (4.2g/L, 80U/ml/min.; 3.8g/L, 62U/ml/min.; 1.6g/L,
28U/ml/min.) showed varying biomass and uricase activity in alkali, acid and untreated
forms.

The strain did not produce any intracellular uricase. When the cell free extract
containing extracellular uricase was precipitated with varying saturation level of ammonium
sulphate, 70% resulted in maximum total enzyme activity of 216U/ml/min. and 1.4 fold
purification with 96.0% of recovery where specific activity increased from 18.8U/mg protein
to 26.34 U/mg.

After dialysis, the enzyme precipitated was further purified using DEAE-cellulose and
DEAE Sephadex A-50, during which the specific activity increased from 18.8 U/mg (crude)
to 425U/mg with 22.60 fold of increase in purification.

Purification with DEAE-cellulose column resulted in 9.84 fold of purification and
81.85% of recovery with a specific activity of 185U/mg protein. Second level of purification
with DEAE-Sephadex A-50 column resulted in specific activity increased to 425U/mg with a
purification fold of 22.6 and recovery of 75.22%.

The fungal uricase gene was amplified with the oligonucleotide primers specific to
Aspergillus sp. showed the presence of uricase gene 980bp.

With trypsin, native uricase reduced to 27% at 20 min. 18% at 40 min. and 6% at 60
min. When it was treated with pepsin for 20, 40 and 60 min. respectively the residual activity
was respectively 48%, 31% and 0%. Pepsin treatment likewise showed 76% at 20 min. 74%
at 40 min. and 74% at 60 min. It further reduced and reached 70% at 120 min.

When native enzyme was PEGylated using 8K linear PEG, the conjugated enzyme
was found to be more stable towards pH, temperature and proteolytic enzymes like trypsin
and pepsin. Stability and higher activity at human physiological conditions showed that both native and conjugated uricase were preferable and PEGylation had improved these properties.

Regarding stability to proteolytic enzyme, trypsin treatment of PEGylated uricase was observed to be 62% at 20 min, 57% at 40 min. and 52% at 60 min. It further reduced and reached 6% at 120 min. The study clearly indicated that PEGylation increased the stability which may be due to structural modification as PEG is a multi agent and might have led to formation of strong covalent bonds in many sites. Hence protein digestion was resisted.

Regarding metal ions on uricase stability the observed residual activities for the tested metal ions such as CaCl$_2$, MgCl$_2$, CuSO$_4$, ZnCl$_2$, MnCl$_2$, FeCl$_2$, HgCl$_2$ and AgCl$_2$ were 96%, 89%, 47%, 52%, 23%, 41%, 17% and 24% respectively. PEGylation did not improve the stability towards metal ions.

The purified uricase was found to be a 34kDa protein. As no other protein bands were found, the extend of purity was confirmed. The bioconjugated uricase with PEG-8000 has showed an increased molecular weight of around 67kDa. This showed a successful enzyme conjugate development.

The purified enzyme was evaluated for its diagnostic potential in finding out serum uric acid level in 40 samples. The values obtained were comparable to that analyzed using commercially available kit as well as by the auto analyzer. The study clearly indicated the potential of this enzyme as a diagnostic agent.

The purified uricase also been evaluated in hyperuricemic mouse model and surprisingly the uric acid reducing level was comparable to the allopurinol, the drug in use. A 14 days period of experiment, clearly indicated on the 1$^{st}$ day of experiment itself reduction from 3.5mg/dL to 2.6mg/dL of uric acid. The result indicated that when the allopurinol as well as the uricase obtained in the present study was administered to normal rats. In control
rats, the serum uric acid level maintained at 1.70-1.80mg/dL. When allopurinol as well as uricase were injected to normal rats, the reduction in uric acid level was not much. With allopurinol injection the uric acid level dwindled around 1.30-1.77 and 1.20 to 1.80mg/dL uric acid in 14 days period.

In hyperuricemic model, in control animal uric acid level increased from 1.80 to 3.61mg/dL over 14 days period. When allopirinol was injected along potassium oxonate it was reduced from 3.5mg/dL to 2.49mg/dL in the first day itself, which was further reduced to 1.22mg/dL in 14 days period. When uricase was injected along with potassium oxonate in 14 days period, the serum uric acid level was reduced to 1.4mg/dL where the control animal maintained at 3.61mg/dL.

In normal mice, the allopurinol and uricase enzyme increased the antioxidant potential in blood serum compared to the control (i.e) 250μM/L is the antioxidant potential in control whereas 310μM/L and 300μM/L were that related to allopurinol and uricase injected samples. In hyperuricemic mouse also total antioxidant potential was found to be higher in blood serum in allopurinol and uricase injected forms compared to PO (Potassium oxonate) injected animals. However the FRAP values were slightly lower in hyperuricemic model. Uric acid though acts as an antioxidant at higher concentration that property seemed to be reassessed.

Thus the research work done in the present study on uricase was found to be holistic, covering isolation and screening of potential strain, production of uricase, characterization of the enzyme produced, modification of the same using PEG for the enhanced stability followed by application of the same in diagnostic and therapeutic purposes.