4. MATERIALS AND METHODS

4.1 Description of the study area

The study area, Pichavaram mangrove forest (Lat. 11°20’ N; Long. 79° 47’ E) is located between the Vellar and Coleroon estuaries (Plate 1). The forest includes 51 islets, ranging in size from 10 m\(^2\) to 2 km\(^2\), separated by intricate waterways that connect the Vellar and Coleroon estuaries. The southern part near the Coleroon estuary is predominantly of mangrove vegetation, while the northern part near the Vellar estuary is dominated by mud-flats. The Vellar estuary opens into the Bay of Bengal at Parangipettai and links with the Coleroon River, which is the distributary of River Cauvery. The Pichavaram mangrove is influenced by mixing of three types of waters: 1. neritic water from the adjacent Bay of Bengal through a mouth called ‘Chinnavaikkal’, 2. brackish water from the Vellar and Coleroon estuaries and, 3. fresh water from an irrigation channel (‘Khan Sahib canal’), as well from the main channel of the Coleroon river. The mangrove covers an area of about 1100 ha, of which 50% is covered by forest, 40% by water-ways and the remaining filled by sand-flats and mud-flats (Krishnamurthy and Prince Jayaseelan, 1983). The year for convenience is arranged into four seasons: 1. post-monsoon: January–March; 2. summer: April–June; 3. pre-monsoon: July–September; and, 4. Monsoon (north-east monsoon): October–December. The tides are semi-diurnal and vary in amplitude from about 15 to 100 cm in different regions during different seasons, reaching a maximum during monsoon and post-monsoon and a minimum during summer (Muniyandi, 1986). The rise and fall of the tidal waters is through a direct connection with the sea at the Chinnavaikkal mouth and also through the two adjacent estuaries. The depth of the water-ways ranges from about 0.3 to 3 m (Muniyandi, 1986).
4.2 Collection of mangrove sediment samples

Sediment samples were collected from Pichavaram mangroves, Tamilnadu, South India by employing an alcohol-sterilized small Peterson grab at about 2m depth. The samples collected were aseptically transferred in to sterile polythene bags. Samples were transferred to the laboratory in an icebox maintained at 4ºC and were processed immediately for the strain isolation.

4.3 Isolation of fungi and screening for uricase production

Exactly 1gm of sediment sample was mixed with 100ml of sterilized 50% aged seawater which was used as the dilutent. Screening for uricolytic potency of the fungi from the sediment samples were done by direct plating of serially diluted samples on Czapek-Dox agar medium which was supplemented with 0.3% uric acid using spread plate technique. Plates were incubated at room temperature for 3-7 days. The formation of clear zone of clearance around the colonies was considered positive for uricase production. Isolate showed maximum zone of clearance was selected as the potent uricase producer and it was used for further study.
4.4 Composition of Czapek-Dox Agar (CDA)

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>30.000</td>
</tr>
<tr>
<td>Sodium nitrate</td>
<td>3.000</td>
</tr>
<tr>
<td>Dipotassium phosphate</td>
<td>1.000</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>0.500</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>0.500</td>
</tr>
<tr>
<td>Ferrous sulphate</td>
<td>0.010</td>
</tr>
<tr>
<td>Final pH (at 25°C)</td>
<td>7.3±0.2</td>
</tr>
</tbody>
</table>

Note: The alone medium was supplemented with 0.3% uric acid.

4.5 Selection of potential strain (Well diffusion assay)

4.5.1 Extracellular uricase production

The potential uricase producing strain was selected based on the measurement of zone of clearance using well diffusion assay. Each fungal isolate was inoculated in Czapek-Dox broth and were incubated for 3-7 days at room temperature. Then 50µl of cell free extract from each fungal broth was loaded into 8mm diameter well on the uric acid (0.3%) incorporated agar plates. The inoculated plates were incubated for 3-7 days at room temperature. The diameter of the zone of clearance was measured with calipers in millimeter from the edge of zone to the edge of the well.

4.5.2 Screening for intracellular uricase production

For testing the intracellular uricase production the harvested mycelia were washed thoroughly with distilled water and blotted dry with absorbent paper. These mycelia were
ground with washed cold sand in porcelain mortar and extracted with 0.05 M potassium phosphate buffer (pH-7.4). The slurries were removed by centrifugation at 7000rpm for 20 min. and the supernatants were used as the crude enzymes. All these procedures were done in a cold condition.

4.6 Identification of fungus by using lactophenol cotton blue staining

Lactophenol cotton blue stains the fungal cytoplasm and provides a light blue background against which the walls of hyphae can readily be seen. It contains four constituents (i.e.) phenol, which serve as fungicide, lactic acid, which act as clearing agent; cotton blue; which stains the cytoplasm of the fungus; and glycerin which gives a semi permanent slide preparation. A loopful of culture was placed on the clean glass slide containing few drops of lactophenol cotton blue stain. Mix gently with sterile needle. A clean cover slip was placed over the stain and care was taken to avoid the formation of gas bubbles. The slide was observed under the microscope (400x) and the image was photographed. The identification has been done by referring the standard manual (Ainsworth et al., 1973).

4.7 SUBMERGED FERMENTATION

4.7.1 Optimization of cultural conditions of Aspergillus niger for biomass and uricase production

Incubation period

Aspergillus niger spores (1 ml of 1.0x 10^6 spores/ml) were inoculated in the medium and incubated for different time interval to achieve higher biomass and high rate of uricase production. The incubation period was kept as 168 hrs with an interval of 24hrs. As highest biomass and uricase production was observed at 96hrs of incubation further optimization for each parameter were kept at 96hrs.
Enzyme assay

Uricase activity was measured according to the procedure described by Adamek et al., 1989. To 2ml of a solution containing uric acid (10µg per ml of borate buffer 0.2M, pH 8.5), 0.8ml of water and 0.1ml of crude enzyme at 25°C were added. After 10 min. 0.2ml of 0.1M potassium cyanide solution was added to the mixture to stop the enzyme reaction. In the reference sample, the solution of potassium cyanide was added to the mixture before the addition of the crude enzyme. The absorbance of samples was measured at 293nm. The difference between absorbance of the sample and reference is equivalent to the decrease in uric acid during the enzyme reaction. One unit of uricase enzyme was equal to the amount of enzyme which converts 1µmol of uric acid to allantoin per min. at 30°C.

Agitation

Aspergillus niger spores (1 ml of 1.0x 10^6 spores/ml) were inoculated in the medium and kept in different agitation speed to achieve higher biomass and higher rate of uricase production. The different agitation speeds kept were 0 rpm (static), 50 rpm, 100 rpm, 150rpm and 200rpm. All the flasks were incubated for 96hrs.

pH

Optimum pH was studied by varying the medium pH such as 4, 5, 6, 7, 8, 9, 10, 11 and 12. The following buffers were used: 100 mM citrate for pH 4.0–6.0, 100 mM phosphate for pH 6.0–8.5 and 100 mM borate for pH 8.5–12. Aspergillus niger spores (1 ml of 1.0x 10^6 spores/ml) were inoculated in the production medium and kept in an incubator shaker at 150 rpm for 96hrs. Biomass and uricase enzyme activity were assessed.

Temperature

Optimum temperature was studied by varying the incubation temperatures at 20°C, 25°C, 30°C, 35°C, 40°C and 45°C. Aspergillus niger spores (1 ml of 1.0x 10^6 spores/ml) were
inoculated in the medium and kept in an incubator shaker at 150 rpm for 96hrs. Biomass and uricase enzyme activity were assessed as before.

**Salinity (NaCl concentration)**

Different salinity ranging from 0.5 – 3% (at the interval of 0.5%) were maintained in the medium and incubated. *Aspergillus niger* spores (1 ml of $1.0 \times 10^6$ spores/ml) were inoculated in the medium and kept in an incubator shaker at 150 rpm for 96hrs. Biomass and uricase enzyme activity were assessed.

**Carbon sources**

*Aspergillus niger* spores were inoculated in the selected medium and kept in an incubator shaker at 150 rpm for 96hrs. Various carbon sources used in the medium were glucose, fructose, sucrose, lactose, maltose, cellulose and starch at the rate of 30% concentration. Biomass and uricase enzyme activity were assessed.

**Concentration of carbon source**

In the medium, the ideal carbon source (sucrose) was added at varied concentration. Various concentration of the carbon source kept for optimization study were 5%, 10%, 15%, 20% and 25% (w/v). *Aspergillus niger* spores (1 ml of $1.0 \times 10^6$ spores/ml) were inoculated in the medium and kept in an incubator shaker at 150 rpm for 96hrs. Biomass and uricase enzyme activity were assessed.

**Nitrogen Sources**

Nitrogen sources used in the medium were peptone, yeast extract, beef extract, casein, uric acid, $\text{NaNO}_3$, $\text{NH}_4\text{NO}_3$, $\text{KH}_2\text{PO}_4$ and $\text{Na}_2\text{HPO}_4$. After inoculation of spores in the medium it was kept in an incubator shaker at 150 rpm for 96hrs. Biomass and uricase enzyme activity were assessed.
Concentration of nitrogen source

Concentration of nitrogen source was kept varied from 0.2, 0.3, 0.4, 0.5 and 0.6% (w/v). Other aspects were as given above.

Cheaper substrates

Different substrates such as, used waste motor oil, molasses, whey, poultry waste at 10% concentration was tried.

4.7.2 Mass scale culture in shake flask

Using all the optimized parameters (i.e.) Incubation time – 96hrs, Agitation speed – 150 rpm, pH - 8, Temperature – 30°C, Salinity – 2%, sucrose – 10% (w/v), uric acid - 0.4% (w/v), Mass scale culture was done in shake flask. Estimation of uricase was performed as previously.

4.7.3 Mass scale culture in fermentor

The parameters optimized for shake flasks were used for mass scale production in a 3L lab scale fermentor and incubated at 150rpm agitation for 96 hrs, which was decided based on trial runs. DO (Dissolved oxygen) was maintained at 60% using air compressor based on trial runs in the fermentor. Fermentor study was done separately for C and N sources as well as for the ideal cheaper substrate (i.e) 1% poultry waste. Biomass and enzyme activity were assessed.

4.7.4 Mass scale using cheaper substrates

Shake flask

Using all the optimized parameters (i.e.) Incubation time – 96hrs, Agitation speed – 150 rpm, pH - 8, Temperature – 30°C, Salinity – 2% and 10% poultry waste as an ideal
cheaper substrate. Mass scale culture was done in shake flask. Estimation of uricase was performed as previously.

**Fermentor**

The parameters optimized for shake flasks using cheaper substrate were used for mass scale production in a 3L lab scale fermentor and incubated at 150rpm agitation for 96 hrs. Estimation of uricase was performed as previously.

**Measurement of fungal Biomass**

In order to measure the fungal biomass dry weight, mycelial balls were filtered through Whatman filter paper No. 1 and dried at 60°C for 72 h in a hot air oven (Singh *et al.*, 2012).

**4.8 Solid state fermentation using agriculture residues as cheaper substrates**

Agriculture residues locally available in abundance such as rice straw, corn straw and sugarcane bagasse were used in the present study. The residues were oven-dried in a hot air oven at 60°C until a constant weight was obtained. Then the moisture free residues were ground and sieved to reduce the particle size. The samples were stored in plastic containers in dry place until further use.

**4.8.1 Alkali pretreatment**

In separate shake flask, about 50g milled dried rice straw, corn straw, sugarcane bagasse was suspended in 1N NaOH in ratio of 1:10 (w/v) substrate and NaOH. After that the samples were incubated in water bath at 85°C for 1hr (Yoswathana and Phuriphipat, 2010). The samples were washed repeatedly with water to remove the solvents used for pretreatment and then dried at 60°C to obtain a constant weight.
4.8.2 Acid pretreatment

About 50g chopped dried substrates was suspended in acid solution (1% sulfuric acid) in ratio of 1:10 (W/V) substrate and sulfuric acid. The mixtures were autoclaved at 121°C for 20 min. (Yoswathana and Phuriphipat, 2010). The samples were washed repeatedly with water to remove the solvents used for pretreatment and then dried at 60°C until constant weight was obtained.

4.8.3 SEM (Scanning Electron Microscopy) Analysis

To visualize the structural changes in agri residues used SEM analysis was done. The untreated and pretreated agricultural samples were placed over the glass slide, dried, and coated with gold film using a SC7620 “mini” polaron sputter coater and analysed under the field emission scanning electron microscopy (FESEM). The images were obtained for the untreated, acid treated and alkali treated samples.

4.8.4 Inoculum preparation

Spores from A. niger was obtained from 7 days old PDA/ Che agar slants which were dislodged in to 10ml of sterile 50% aged sea water containing 0.1% Tween 80 and used as an inoculums after adjusting the desired spore count using hemocytometer (Agarwal et al., 2003).

4.8.5 Uricase production under solid state fermentation

Uricase production was carried out in 250ml Erlen Mayor flask containing 5g of substrate (untreated or treated with acid or alkali) and moistened with 50% sea water in a ratio of 1:1.5. and supplimented with 0.1% uric acid. The initial pH was fixed at pH-8 by adding few drops of 1N NaOH and sterilized at 121°C for 20min. After cooling 1 ml of 1.0x 10^6 spores/ml was added and the flasks were incubated at 35°C for a period of 4 days. After
fermentation 50ml of sterile distilled water was added to each flask and shaken on a rotary shaker (180 rpm for 1hr). The mixture was centrifuged at 5000g for 10min. and the supernatant was further filtered using cheese cloth and used for assay the enzyme activity.

4.8.6 Fungal biomass estimation

Duplicate samples of 1g fermented substrate from each flask were transferred to a pre-weighed centrifuge tubes and 5ml of sodium sulphate (150g/L) was added to each tube. The tubes were centrifuged at 12000rpm for 15min. the procedure was repeated thrice to achieve complete separation of fungal biomass from solid substrates. At the end of the centrifugation process, fungal mass floating on the top should be transferred to a pre-weighed filter paper and dried in hot air oven for 72hrs at 85°C (or) at 60°C for extended period to obtain a constant weight.

4.9 Purification of uricase enzyme

The uricase from *Aspergillus niger* was purified as per the standard protein purification procedures which involves various steps such as centrifugation, ammonium sulphate precipitation, and ion exchange chromatography. Uricase obtained through mass scale production in fermentor using selected C and N sources was used for this purpose.

4.9.1 Ammonium sulphate precipitation

Ammonium sulphate precipitation is a convenient and effective method because of its high solubility, cheapness, lack of toxicity to most of the enzymes (Dixon and Webb, 1979). It forms dense solution enhancing protein collection, which may be precipitated by centrifugation.
Procedure

The aliquots of the extract was taken and pre-chilled at 4°C. The amount of ammonium sulphate required to give different saturation levels (40, 50, 60, 70 and 80%) as per standard chart was added slowly to each aliquot while stirring (using a magnetic stirrer) (Green and Hughes, 1955). The aliquot stirred for overnight at 4°C was centrifuged at 10,000 rpm for 30 min. The supernatant was removed and the precipitate was dissolved in buffer (e.g. 50mM Tris - HCl pH-8-10). The enzyme solution thus obtained was dialyzed using a dialysis membrane No.150 (Himedia) against the same buffer for 24 hrs with several intermittent buffer changes. The supernatant was assayed for total protein concentration and enzyme activity. Based on the result 70% ammonium sulphate was used for the precipitation of mass scale culture filtrate.

The purification of uricase was done through three steps including ammonium sulfate precipitation, ion exchange and gel filtration chromatography. Ammonium sulfate was added to the crude uricase until it reached to 70 % saturation. Precipitation of the protein was carried out by leaving the solution for 24 h at 4 °C. A column of DEAE- (diethylaminoethyl) cellulose was prepared and the desalted uricase was poured on the surface of column (2 x 50 cm). The elution of sample was carried out with 100mM phosphate buffer (pH 6.0).

4.9.2 Purification of uricase enzyme by using DEAE- Cellulose column

(1) Selection of column

The column was selected with a height and internal diameter of 30cm and 1.5cm respectively and was gradually filled with the suspension of DEAE- cellulose.

(2) Packing of DEAE-Cellulose column
The dialyzed sample was then applied to a DEAE-cellulose column (1.5 by 30 cm) which had been pre-equilibrated with 50 mM Tris-HCl (pH 8.0) buffer. The cellulose column matrix was packed without any air bubble. Air from the column was eliminated by gently flushing the end pieces with buffer.

(3) Equilibration of the column

The column was equilibrated with 100ml of equilibration buffer (50 mM Tris-HCl (pH 8.0 buffer) until the conductivity or pH of the effluent become same as that of in-going solution.

(4) Sample preparation

0.25g of partially purified sample was dissolved in 2ml of binding buffer (20mM Tris buffer pH 8.2). When the buffer reached the gel bed 2ml of sample was loaded on the top of the column.

(5) Elution of samples

The column was eluted using elution buffer (50 mM Tris-HCl (pH 8.0) buffer containing 0.1, 0.2, 0.3, 0.4, and 0.5 M NaCl). 50 fractions (of 2 ml) were collected. The active fractions were pooled together and it was precipitated with 70% ammonium sulfate. The enzyme precipitate was dissolved in 50 mM Tris-HCl (pH 8.0) and it was dialyzed against the same buffer as previously. The concentrated sample of 2ml was further purified in a second column (i.e.) by using DEAE Sephadex A-50. Protein concentration and uricase activity were assayed for the fractions. The protein contents were determined by measuring the absorbance at 280 nm or by the method Lowry et al. 1951.
4.9.3 Purification of Uricase enzyme by using DEAE Sephadex A-50 column

(1) Selection of column

The column was selected at a height and internal diameter of 30cm and 1.2cm respectively and was gradually filled with the suspension of DEAE Sephadex A-50.

(2) Preparation of DEAE Sephadex A-50 medium

DEAE Sephadex A-50 and DEAE Sephadex A-25 are weak anion exchangers. The ion exchange group is diethylaminoethyl which remains charged and maintains consistently high capacity over the entire working range, pH 2-9. Sephadex is a hydrophilic ion exchange matrix and hence it shows very low non-specific adsorption. Proteins, nucleic acids and other labile biological molecules are not adsorbed or denatured by the medium.

DEAE Sephadex A-50 slurry was prepared by mixing 0.5g of dry powder DEAE Sephadex A-50 with binding buffer (50 mM Tris-HCl (pH 8.0)) at a ratio of 75% settled medium to 25% buffer. Swelling factor depends on the buffer used. Complete swelling takes 1-2 days at room temperature or 2 hrs at 100°C in boiling water with vigorous stirring.

(3) Packing of DEAE Sephadex A-50

Air from the column dead space was eliminated by flushing the end pieces with buffer. The slurry was poured into the column in one continuous motion without any air bubble. The remainder of the column was filled with buffer.

(4) Equilibration of the column
The column was equilibrated with 100ml of binding buffer (50 mM Tris-HCl (pH 8.0)) until the conductivity or pH of the effluent become same as that of ingoing solution.

(5) Sample Loading

Active DEAE-Cellulose purified fractions were combined, concentrated (by lyophilization) and further purified in DEAE-Sephadex A-50 column. 2ml of concentrated sample from previous purification was loaded on the top of the column and eluted with 50 mM Tris-HCl (pH 8.0).

(6) Elution of samples

The column was eluted with elution buffer (50 mM Tris-HCl (pH 8.0)). Fifty 2ml fractions were collected and analyzed for enzyme activity and protein concentration. The resolution of purification was checked on 12% SDS-PAGE gel.

4.10 Specific activity

Specific activity of the sample was calculated by dividing the enzyme units with the protein content and was expressed as U/mg protein.

\[
\text{Specific activity (U/mg)} = \frac{\text{Enzyme activity (U/ml)}}{\text{Protein (mg/ml)}}
\]

4.11 Enzyme assay and protein measurement

Uricase activity was measured as previously according to the procedure described by Adamek *et al.*, 1989. Protein concentration was measured by the Folin-phenol method (Lowry *et al.* 1951).
4.12 CHARACTERIZATION OF ENZYME

4.12.1 Stability of purified uricase

pH

To study the effect of pH on the activity of uricase, the enzyme was assayed at different pH in the range from 4.0 to 12.0 with an interval of 1.0. The following buffers were used: 100 mM citrate for pH 4.0–6.0, 100 mM phosphate for pH 6.0–8.5 and 100 mM borate for pH 8.5–12. The pH stability was studied by incubating the purified enzyme solution in the corresponding buffers in the range from 4.0 to 12.0 at 25°C for 30 min and the residual activity was measured (Hesham et al., 2004 and Kai et al., 2007). The maximum activity at the optimum pH (i.e.) pH-8 was considered as 100%.

Temperature

To study the effect of temperature on the uricase activity, the standard enzyme reaction solution was incubated at temperature range of 20–70°C with 10°C intervals for 30 min. and measured its activity. For stability studies enzyme solution after DEAE-Cellulose purification was used. The residual activity was calculated as before.

Metal ions

Effect metal ions on enzyme activity were measured using metals namely ZnCl₂, CuSO₄, FeCl₂, MnCl₂, CaCl₂, MgCl₂, HgCl₂ and AgCl₂ of 10mM concentration for 2hrs. The activity of enzyme assayed without metal was considered as control and the activity was taken as 100%.

Proteolytic enzymes

The conjugated and free uricase were treated with proteolytic enzymes like trypsin and pepsin in 0.05 mmol/L borate buffer at 30°C and pH 8.0 at the final concentrations
of 0.05mg/mL. The reduction in uricase activity was observed for 2hrs (Tan et al., 2009).

4.13 PEGylation

**Preparation of uricase-PEG-8000 conjugates**

PEGylation was performed according to the method of Nanda and Babu (2012). Pure uricase isolated in the present study from *Aspergillus niger* (1 mg/mL) was allowed to react with linear PEG-8000 (Sigma Aldrich) in the concentration ratio of 1:40 in 100mM sodium borate buffer solution having pH 8.0, as the reaction medium in a final volume of 1 mL. These components of the mixtures were allowed to react at 30°C with slight agitation for 2 hrs.

**Uricase enzyme assay and protein measurement**

The enzymatic assay for uricase was carried out as previously. The activity of the enzyme before and after bioconjugation was determined using this method.

4.14 Purification of PEGylated uricase

A column of Sephadex G-100 (3 x 50 cm) was prepared and the eluent from the previous step was added. Elution was then carried out by 100mM phosphate buffer (pH 8.0). A total of 20-fractions of three-ml each were collected. The enzyme activity was assayed and the protein estimation was carried out. Fractions containing higher enzyme activity were pooled and used for enzyme assay.

4.15 SDS-PAGE analysis for the confirmation of PEGylation

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method described by Laemmli (1970) for the determination of molecular weight of the conjugates formed. Staining and detection of protein bands was done
using Coomassie blue stain and later de-stained. Pooling of fractions was guided by both higher activity of the enzyme as well as based on SDS-PAGE result.

**SDS-PAGE analysis**

The purified uricase sample and the PEGylated samples were analyzed by SDS-PAGE (Laemmli, 1970) and the molecular weight of proteins were determined.

**Gel casting**

**Chemistry involved in gelling**

Polyacrylamide gel results from the polymerization of acrylamide monomers into long chains and cross linkages are brought by N-N-methylene bisacrylamide. Polymerization of acrylamide is initiated by the addition of either ammonium per sulphate or riboflavin. TEMED (N-N-N-N) Tetra methylene diamine acts as an accelerator of polymerization. Effective pore size of polyacrylamide gel is greatly influenced by the total acryl amide concentration in polymerization mixture. Buffer system in PAGE is designed in such a way that the protein is separated into individual polypeptide.

To study the homogeneity of the proteins, polyacrylamide gel electrophoresis was carried out for the separation of proteins according to their electric charges. Most commonly the strong anionic detergent SDS is used in combination with a reducing agent and heated to disassociate the proteins before they are loaded on the gel. The amount of SDS bound is always proportional to molecular weight of the poly peptides and is independent of their sequence. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (i.e.) SDS-PAGE is an excellent tool to identify and monitor proteins during purification and to access the homogeneity of the purified proteins.
Sample preparation and electrophoresis

The samples were mixed with equal volume of sample buffer (2X Laemmli buffer) and were boiled in a boiling water bath for 5 min. Then the samples were loaded into the wells and allowed for electrophoresis at 50V initially. After the dye front had reached the end of the stacking gel, the voltage was increased to 100V and proteins were allowed to migrate through resolving gel.

Procedure

The casting apparatus was assembled and the gel volume was determined using distilled water. Then the solution for the resolving gel of 12% concentration was prepared. The solution was poured into the gel plate and overlaid it with a layer of butanol:water (1:1) to prevent exposure of the gel to air and allowed to solidify and then the solution for the stacking gel was prepared. The butanol layer was removed and washed twice or thrice to remove traces of butanol. The components of the stacking gel (5%) were added; the gel solution was poured over the separating gel and was allowed to solidify after placing the comb.

Loading of the Sample

Protein marker and samples were loaded into the separate well of the stacking gel. The electrophoresis buffer was added in to the top and bottom reservoir. The samples were electrophoresed at 100V until the tracking reached 0.5cm from the bottom of the plate. Power supply was turned off and the gel was kept in the staining solution.

Detection of proteins in gel

Protein detection was done in many ways. Widely used staining method is coomassie brilliant blue staining.
Coomassie brilliant blue staining

This is the standard method of protein detection. Easy visibility requires the order of 0.1-1mg of protein per band. After electrophoresis, the gel assembly was removed and the glass plates were separated. The gel was soaked in the staining solution and left overnight for staining and then the gel was destained with the destaining solution. The protein bands were observed in the Gel-documentation system (DGelDAS, biotech, Yercaud).

4.16 Stability of PEGylated uricase

The stability of the unmodified uricase and the bioconjugates was tested at different pH values by maintaining them in the following buffers solutions for an hour: for pH 6-7 sodium phosphate buffer, for pH 8- 9, Borate buffer and for pH 10 Tris-HCl buffer. The enzymatic activities of the conjugates were determined after one hour of incubation. The pH at which the unmodified uricase showed the highest activity, was considered as 100% activity. Residual activities at other pH values were evaluated with respect to this value. Likewise stability of PEGylated uricase at different temperatures and proteolytic enzymes were carried out as previously done for normal uricase.

4.17 Location of gene responsible for uricase production using PCR

4.17.1 DNA extraction

50mg of mycelia was ground to a fine powder in 2ml of extraction buffer (0.1M - Tris-HCl, .01M - EDTA, 1M - NaCl, 1% - SDS, 0.05 mg/ml - I-proteniase K, pH – 8.0) with the micro pestle in a micro centrifuge tube. Samples were vortexed and incubated at 65°C for 30 min. After incubation, samples were centrifuged at 10,000 rpm for 10 min. and the supernatant was transferred in to a new tube. To the supernatant, equal volume of isopropanol was added and the tubes were incubated at -20°C for 20min. Samples were centrifuged at 10,000 rpm for 10min. After discarding the supernatant, the pellet was washed with 0.2ml of
70% ethanol. Tubes were centrifuged briefly. The pellet was air dried and dissolved in sterile distilled water. This serves as a template DNA for PCR analysis (Sambrook and Russel, 2001).

**4.17.2 Amplification of uricase gene of \textit{A.niger}**

The genomic DNA extracted from \textit{A.niger} was PCR amplified for the detection of uricase genes using uricase gene specific primers. UOX-Forward - 5′-TCCGCAGTTAAAGCTGC-3′ and UOX – Reverse - 5′-CAATT TAGACTTCAGAGAGG-3′ (Fazel \textit{et al.}, 2014). This primer combination amplifies a 980bp uricase gene specific fragment. Amplification reaction was performed in a 0.2 mL optical-grade PCR tube (Tarsons, India). 50 nanogram of DNA extract was added to a final volume of 50µL of PCR reaction mixture containing 1.5mM MgCl$_2$, 1X Reaction buffer (without MgCl$_2$) (Fermentas), 200µM of each dNTPs (Fermentas), 100pM of each primer and 1.5U Taq DNA polymerase (Fermentas). PCR was performed in an automated thermal cycler with an initial denaturation at 95°C for 5min. followed by 30 cycles at 95°C for 30sec. (denaturation), 53°C for 45sec. (annealing), 72°C for 60 sec. (extension) and 72°C for 10min (final extension). PCR product was run on 1% agarose in TAE buffer [40mM Tris, 20mM Acetic acid, 1mM EDTA (pH8.0)] to confirm that the product (980bp) formed.

**4.17.3 Detection of PCR products by agarose gel electrophoresis**

Electrophoresis is a technique used to separate charged molecules. DNA is negatively charged at neutral pH and when electric field is applied across the gel, DNA migrates towards the anode. Purified agarose is a powder insoluble in water or buffer at room temperature but dissolves on boiling. Molten solution is then poured into a mould and allowed to solidify. As it cools, agarose undergoes polymerisation i.e., sugar polymers cross-link with each other and
make the solution to form gel, the density or pore size of which is determined by concentration of agarose.

Matrix of agarose gel acts as a molecular sieve through which DNA fragments move on application of electric current. Higher concentration of agarose gives firmer gels, i.e., spaces between cross-linked molecules is less and hence smaller DNA fragments easily crawl through these spaces. As the length of the DNA increases, it becomes harder for the DNA to pass through the spaces, while lower concentration of agarose helps in movements of larger DNA fragments as the spaces between the cross-linked molecules is more. The progress of gel electrophoresis is monitored by observing the migration of a visible dye (tracking dye) through the gel. Two commonly used dyes are xylene cyanol and bromophenol blue that migrate at the same speed as double stranded DNA of size 5000 bp and 300 bp respectively. These tracking dyes are negatively charged, low molecular weight compounds that are loaded along with each sample at the start of run, when the tracking dye reaches towards the anode, run is terminated.

Visualization of DNA fragments

Since DNA is not naturally coloured, it will not be visible on the gel. Hence, after electrophoresis, the gel was stained with a dye specific to the DNA. Discrete bands are observed when there is enough DNA material present to bind the dye to make it visible, otherwise the band can be not detected. The intercalating dye like ethidium bromide was added to agarose gel and to locate the DNA; which will fluoresces when observe the gel under UV light.

Preparation of 1% agarose gel

1% agarose gel was prepared by adding 0.5g of agarose in TAE buffer and it was boiled to dissolve the agarose completely. When the solution was cooled to 55-60°C, 2μl of
10mg/ml stock of ethidium bromide dye was added and poured in to the gel boat. After the solidification of the gel 20µl of the PCR product was loaded and was electrophoresed at 50-100V for 30-45 min. The result was observed under UV transilluminator.

4.18 APPLICATION STUDIES

4.18.1 Diagnosis of uric acid levels in blood samples

Evaluation of uric acid produced in the present study was done by comparing the values obtained with other diagnostic methods in use. Blood samples were collected from volunteers in a blood donating camp, and uric acid content was estimated using Rasburicase kit and using laboratory auto analyzer. The purified lyophilized uricase enzyme was diluted with phosphate buffer and used spectrophotometric method to estimate the serum uric acid level.

4.18.2 Control of serum uric acid level using animal model

Study design

Male albino rats, ten weeks old, weighing 80 grams were purchased from National Institute of Nutrition, Hyderabad and maintained in the Central Animal House, Rajah Muthiah Medical College and Hospital, Annamalai University. The animals were housed in polypropylene cages at room temperature. The animals were provided with standard pellet diet (Laboratory Animal Feed Limited, Bangaluru, India) and water. The animals were maintained as per the principles and guidelines of the ethical committee for animal care of Annamalai University. Animals were maintained on a 12hrs light and 12hrs dark cycle. They were housed 6 rats/cage.

Grouping was done as follows.

1. Hyperuricemia – Control
2. Hyperuricemic + Allopurinol (5mg/kg)

3. Hyperuricemia + uricase (*A. niger*)

Control animals in normal model were orally administered with 0.9% saline solution. Regarding allopurinol, the tablet available was dissolved in distilled water and given water orally to study groups. The volume of suspension administered was based on body weight measured immediately prior to each dose.

Animals were divided into different groups. Acclimatization was done for a week time after which treatment procedures were initiated. To induce the experimental rat model with hyperuricemia, and uricase inhibitor potassium oxonate was given orally using gastric gauge at a dose of 250 mg/kg/day (Mahdabadi *et al.*, 2013). Blood samples from control and test groups were collected via cardiac puncture under diethylether anesthesia (Yoburn *et al.*, 1984). Serum was obtained by centrifuging blood samples at 3000 rpm for 10 min. and the serum was separated and transferred into a fresh tube and were stored at -20°C until use (Hall *et al.*, 1990).

**Total antioxidant capacity assay**

The total antioxidant capacity of serum was determined by measuring its ability to reduce ferric ions (Fe$^{3+}$) to ferrous form (Fe$^{2+}$) by the FRAP (Ferrie Reducing Ability of Plasma) test. The FRAP assay measures the change in absorbance at 593 nm owing to the formation of a blue colored Fe$^{2+}$- tripyridyltriazine compound from Fe$^{3+}$ by the action of electron donating antioxidants. The FRAP reagent consists of 300 μmol/ml acetate buffer (pH 3.6), 10 μmol/ml tripyridyltriazine in 40 μgmol/ml HCl and 20 μmol/ml FeCl$_3$ in the ratio of 10:1:1. Briefly, 30 μl of serum was added to 1.0 ml freshly prepared and prewarmed (37°C) FRAP reagent in a test tube and incubated at 37°C for 10 minutes. The absorbance of the blue colored complex was read against a reagent blank (1.0 ml FRAP reagent + 30 μl distilled
water) at 593 nm. Standard solutions of Fe$^{2+}$ in the range of 10-1000 μmol/L were prepared from ferrous sulphate in water. The FRAP values were expressed as μmol Fe$^{3+}$ reduced to Fe$^{2+}$ per liter (Benzie and Strain, 1996).