Chapter 3 MATERIALS AND METHODS

3.1. Materials

3.1.1 Chemicals

Most of the chemicals used were procured locally from different companies such as: Himedia (Mumbai, India); Merck (India); Qualigens Fine Chemicals Ltd. (India); Sisco Research Laboratories (Mumbai, India); S.D. Fine Chemicals (India). Chemicals of high purity and analytical grades were procured from Sigma Aldrich (St. Louis, USA).

3.1.2 Microorganisms procured from culture collections

The enlisted microbial cultures in Table 3.1 were procured from MTCC, Chandigarh, India.

Table 3.1: List of microbial cultures procured from different culture collections

Sl. No.	Name of Microorganism	Culture identity
1.	*Bacillus cereus GHMS*	MTCC 8550
2.	*Bacillus cereus Akg-1*	MTCC 9817
3.	*Pseudomonas aeruginosa Ph3*	MTCC 7199
4.	*Pseudomonas aeruginosa 5Y2*	MTCC 7602
5.	*Saccharomyces cerevisiae*	MTCC 2376

3.1.3 Equipments/ Instruments

Following equipments and instruments were mainly used during experimentation and analysis of results:

- Gel Doc XR+ System (BioRad, Richmond, California)
- UV-1700 PharmaSpec Spectrophotometer (Shimadzu, Japan)
- *Up-right* polarized light microscopy (Olympus BX-63, USA).
- Laminar Air Flow (Narang Scientific, India)
- pH meter (Toshiwal, India)
- Cooling Centrifuge Biofuge Thermo Fisher Scientific
- Weighing Balance (JE303GE, Mettler Toledo, US)
- Shaking incubator (Remi, Mumbai, India)
- 7.5 L Bentchtop fermenter (BioFlo/Celligen 115, New Brunswick, USA)
- Electrophoresis (Genei, Bangalore, India)
- Circular Dichroism Spectropolarimeter (Model 500A JASCO, USA)
- Fast Protein Liquid Chromatography (FPLC) system (AKTA Prime, GE Healthcare, USA)
- MALDI TOF/TOF (5800system, Sciex)

3.1.4 Media

Sterilization of the media is accomplished by autoclaving at 15 psig pressure for 15 min unless otherwise specified.

3.1.4.1 Nutrient agar

Nutrient agar (NA) medium was used for the purpose of bacterial maintenance of cultures *Pseudomonas aeruginosa 5Y2, Bacillus cereus GHMS, and Bacillus cereus Akg-1* and their storage.

Table 3.2: The nutrient composition for the revival and storage of microbial cultures

Components	**Composition**
Peptone	5.0 g
Beef extract	1.0 G
Yeast extract	2.0 G
Sodium chloride	5.0 g
Agar	15.0 g
Distilled water	1.0 L
	pH =7.0

3.1.4.2 Luria Burtani Medium

LB medium was used for the purpose of bacterial maintenance of culture *Pseudomonas aeruginosa Ph3* and their storage.

Table 3.3: The LB medium composition for the revival and storage of microbial culture

Components	**Composition**
Tryptone	10.0 g
Yeast extract	5.0 g
Sodium chloride	10.0 g
Agar	15.0 g
Distilled water	1.0 L
	pH =7.0

3.1.4.3 Yeast Extract Peptone Dextrose (YEPD) Medium

YEPD medium was used for the purpose of maintenance of culture *Saccharomyces cerevisiae* and its storage.

Table 3.4: The YEPD composition for the revival and storage of yeast culture

Components	**Composition**
Peptone	10.0 g
Yeast extract	3.0 g
Dextrose	20.0 g
Agar	15.0 g
Distilled water	1.0 L
	pH =7.0

3.1.4.4 Uricase production medium

Production medium was used for the purpose of uricase enzyme production.

Table 3.5 The nutrient composition for the production of uricase enzyme

Components	**Composition**
Peptone	5.0 g
Beef extract	4.0 g
Yeast extract	5.0 g
Sodium chloride	5.0 g
Uric acid	1.0 g
Distilled water	1.0 L
	pH =7.0

3.1.5. Softwares used

For processing and analysis of the experimental results, the following softwares have been used: MS Excel 2007, OriginPro-8.0, OriginLab Corp., MA, USA; QUALITEK-4 (Nutek Inc., MI, USA); Mega software version 6.0 (http://www.megasoftware.net/index.php); and online K2D3 software (http://www.ogic.ca/projects/k2d2/). Endnote X3 software for citing the references; MASCOT software (http://www.matrixscience.com/); Biotools software (Brucker, Daltonics); PSIPRED v3.3 software (http://bioinf.cs.ucl.ac.uk/psipred/). Adobe Photoshop CS software, MathType software.

3.2. Analytical methods

3.2.1. Biomass estimation

The objective of this study was the effect of different concentrations of UA on the growth of the selected strains. Microbial biomass was measured in terms of dry cell weight. After centrifugation at 11962 *g* for 30 min. at 4°C., the pellets were collected and washed twice with distilled water. The pellets were dried in a hot air oven at 80°C for overnight and weighed (JE303GE, Mettler Toledo, US).

3.2.2. Determination of protein content

The protein content of the obtained crude enzyme was measured as per the standard protocol of Biuret et al with Bovine Serum Albumin (BSA) as standard (Zhou and Regenstein 2006). The protein concentration was quantified in terms of mg/mL.

3.2.3 Uricase activity

Uricase activity of the produced crude enzyme was determined either with the substrate UA utilization or product allantoin formation per unit time. In this study, the activity was measured on the basis of decrease in absorbance at 293 nm of UA per unit time. Uricase activity was measured as described by Mahler et al (Mahler, HuBSCHER et al. 1955) at the optimized pH for *P. aeruginosa Ph3, 5Y2* and *Bacillus cereus GHMS* at 30°C temperature. 3 ml of 20 mM sodium borate was added to 75 µL of 5 mM uric acid solution and 20 µL of the crude enzyme. The blank solution was taken by adding 20 µL of buffer instead of a crude enzyme. The blank and test sample were incubated at 30°C for 10 min. The decrease in absorbance of substrate UA concentration in the test samples were noted down using UV-Visible spectrophotometer (UV-1800, Shimadzu Corp., Made in Japan) at 293 nm (Nanda and Jagadeesh Babu 2014).

1 Unit (U) of enzyme activity was defined as the amount of uricase needed to convert 1µmol of UA into allantoin per minute at the optimized parameters measured by equation 1.

$$Volume\,activity\,(U/ml) = \frac{OD\,(OD_{blank} - OD_{test}) \times Vt \times df}{12.2 \times 1.0 \times t \times Vs} \quad \text{.....Eqn. 1}$$

Experimental methods

3.3. Plate Assay Method

The capability of microbes to produce extracellular uricase was primarily tested with nutrient agar plate assay qualitative method. The nutrient agar plates were prepared

with the composition (g/L) peptone 5.0, yeast extract 3.0, NaCl 3.5, agar 25.0, phenol red 0.1, and uric acid 1.5. Wells of 5 mm diameter were drilled at the center of agar plates. The 200 µL of fresh 48 h old microbial cultures were added to those made wells. Phenol red dye was added to distinguish the clear zone around the wells due to change in pH after UA degradation. The diameter of clear zones is directly related with the amount of uricase produced. The control was prepared without addition of the inducer uric acid.

3.4. Uricase production in shake flask

The primary screened uricase producers were further used to produce uricase enzyme in shake flask by inoculating 5% (v/v) of each screened microbial culture separately in a medium (g/L) composition of beef extract 1.0, yeast extract 2.0, peptone 5.0, NaCl 5.0 and uric acid 1.0 (5.94 mM), pH 7.0 and incubated for 24 h at temperature 30°C under agitation speed of 120 rpm. After incubation period, the extracellular uricase production was quantified in supernatant as a crude enzyme obtained by centrifugation at 11962 *g* at 4°C for 20 min.

3.5. Effect of uric acid concentration on uricase Production in shake flask

5 % of inoculum of *P. aeruginosa Ph3, 5Y2* and *B. cereus GHMS* were inoculated into the separate 250 mL Erlenmeyer flasks containing 50 mL aliquots of nutrient broth with 0.5, 1.0, 1.5, 2.0, and 2.5 g/L of UA as inducers for the uricase production and incubated at pH 7.0, temperature 30°C and at 120 rpm for a period of 24 hours. Finally, the effect of uric acid concentration in the production medium was studied by assaying the uricase activity.

3.6. Effect of pH on uricase production

The growth of microorganism varies with the pH of the growing medium due to their habitats. In this study, the 48 h old screened selected microorganisms were inoculated with size 5% in the above mentioned medium containing 1.5 g/L of uric acid separately and incubated for 24 h at temperature 30°C under agitation of 120 rpm. The initial pH of the medium was varied separately from 6.0 to 9.0 by using 0.5 N HCl and NaOH solution.

3.7. Effect of temperature on uricase production

The effect of temperature on the uricase production was studied using the screened microorganisms such as *B. cereus GHMS, P. aeruginosa Ph3, and P. aeruginosa 5Y2* by inoculating the 5% of culture in the above mentioned nutrient medium containing 1.5 g/L of uric acid having initial pH 7.0 and incubated for 24 h of period at 120 rpm of agitation speed at various temperatures (°C) ranging at 20, 25, 30, 35, 40, and 45 separately in triplicates (Atsbha, Haki et al. 2015). The enzyme uricase production was quantified by assaying the uricase activity (U/ml).

3.8. Effect of agitation speed (rpm) on uricase production

Since the uricase production by using *B. cereus GHMS, P. aeruginosa Ph3, and P. aeruginosa 5Y2* was an aerobic process, so the production was dependent on the agitation speed of the shaker. In this study, the screened uricase producers were separately inoculated with 5% size in the above mentioned production medium (pH 7.0) with 1.0 g/L of uric acid in triplicates and incubated for 24 h at 30°C temperature at different shaking speed (rpm) ranged by 100, 120,140, 160, 180, and 200 rpm.

After incubation period the crude extract were assayed for uricase produced during the fermentation.

3.9. Effect of pH and temperature on enzyme activity

The effects of pH and temperature on uricase activity were studied. The uricase activity of uricase isolate from *B. cereus GHMS, P. aeruginosa Ph3, and P. aeruginosa 5Y2* strains was measured in 20 mM buffer solution in the pH range of pH 6.0 - 9.5 within a temperature range of 20-45°C. To maintain pH 6-7, sodium phosphate buffer, and for pH 7.5 to 9.5, borate buffer were used (Germano, Pandey et al. 2003). The optimum parameters were selected on the basis of maximum UA degradation measured at 293 nm.

3.10. *In vitro* degradation study of UA

In another way, the *in vitro* UA degradation study was carried out by *Up-right* polarized light microscopy (PLM) (Olympus BX-63). To determine the deposition of urate crystals in synovial joints, PLM is efficiently used (Pascual and Jovani 1995; Schlesinger 2005). On this basis, a degradation study of UA was carried out by the crude uricase of *B. cereus GHMS, P. aeruginosa Ph3, and P. aeruginosa 5Y2* strains under 20X magnification with respect to blank (UA and buffer instead of crude enzyme) .

3.11. Effect of Metal Ions on Uricase activity

The effect of different metal ions on uricase activity was analyzed. The metal ions are effectors molecules that act either as activator or inhibitor of the enzyme activity. Various salts of metals of 1 mM concentrations including Ni, Zn, Mg, Co, Fe, Mn,

and Cu were dissolved in 20 mM borate buffer of optimum pH for the respective strain. The 50 µL of respective crude enzyme were pre-incubated in 3 mL of buffer containing metal ion separately for 2 h at 30°C and the uricase activity was assayed (Saeed, Yousry et al. 2004).

3.12. Production enhancement of uricase by *Bacillus cereus GHMS*

Among the screened uricase producers *B. cereus GHMS, P. aeruginosa Ph3, and P. aeruginosa 5Y2,* the *B. cereus GHMS* has produced maximum uricase under optimum conditions and hence was carried forward for further enhancement of uricase.

The uricase production was enhanced by following means:

A] Optimization of Physical parameters of SMF:

The optimized physical parameters of shake flask study including agitation speed, pH and temperature were used to determine the time duration of fermentations were studied. The 48 h old 5% *B. cereus GHMS* inoculum was inoculated in a medium (g/L) composition of beef extract 1.0, yeast extract 2.0, peptone 5.0, NaCl 5.0 and uric acid 1.0 and uricase activity (U/mL) and wet cell biomass at 550 nm was assayed at various time periods.

B] Effect of Inducers on uricase production

The uricase induction was studied using different precursors of UA including adenine and guanine (purine bases) and xanthine of purine metabolic pathway. These are structurally UA analogues and their effects on the inductions of uricase at the proper

concentrations were assayed. The effects of inducers with various concentrations ranging from 0.5, 1.0, 1.5, 2.0, 2.5 g/L were used for the induction of uricase.

C] Effect of surfactants on uricase production

The present study aimed to increase the uricase yield from *Bacillus cereus* in the optimized conditions in the presence of surfactants. Surfactants are the amphiphilic compounds that lower the surface as well as interfacial tensions by gathering at the interface of immiscible fluids and improve the solubility, mobility, bioavailability of water insoluble compounds (Silva, Cerqueira et al. 2012). These are the surface active agents that lower the surface tension between the two liquids, or between a liquid to a solid. The basic purpose of using surfactant was to increase the bacterial cell membrane permeability (Reese and Maguire 1969) for the inducer absorption and enzyme release (Singh, Van Hamme et al. 2007). The effects of various surfactants 1 % (w/v) including labolene, Tween-80, and polyvinyl alcohol (PVA), and Triton X-100 were studied for the increased production of enzyme and the control was prepared with devoid of surfactant (Matkar, Chapla et al. 2013). Labolene is an anionic detergent used commonly for cleaning lab glasswares.

D] Optimization of nutrient medium components

I] Taguchi methodology

Optimization methodology adopted comprised four phases *viz*., design of experiments (DOE), performing experiments, analysis and validation. The various steps involved in Taguchi optimization was schematically depicted in Fig. 3.1. With the help of design of experiments Taguchi methodology generates large number of experiments having reduced errors referred as orthogonal arrays (OA) (Chang, Tsai et

al. 2006; Azin, Moravej et al. 2007; Wei, Lai et al. 2007; Shaligram, Singh et al. 2008).

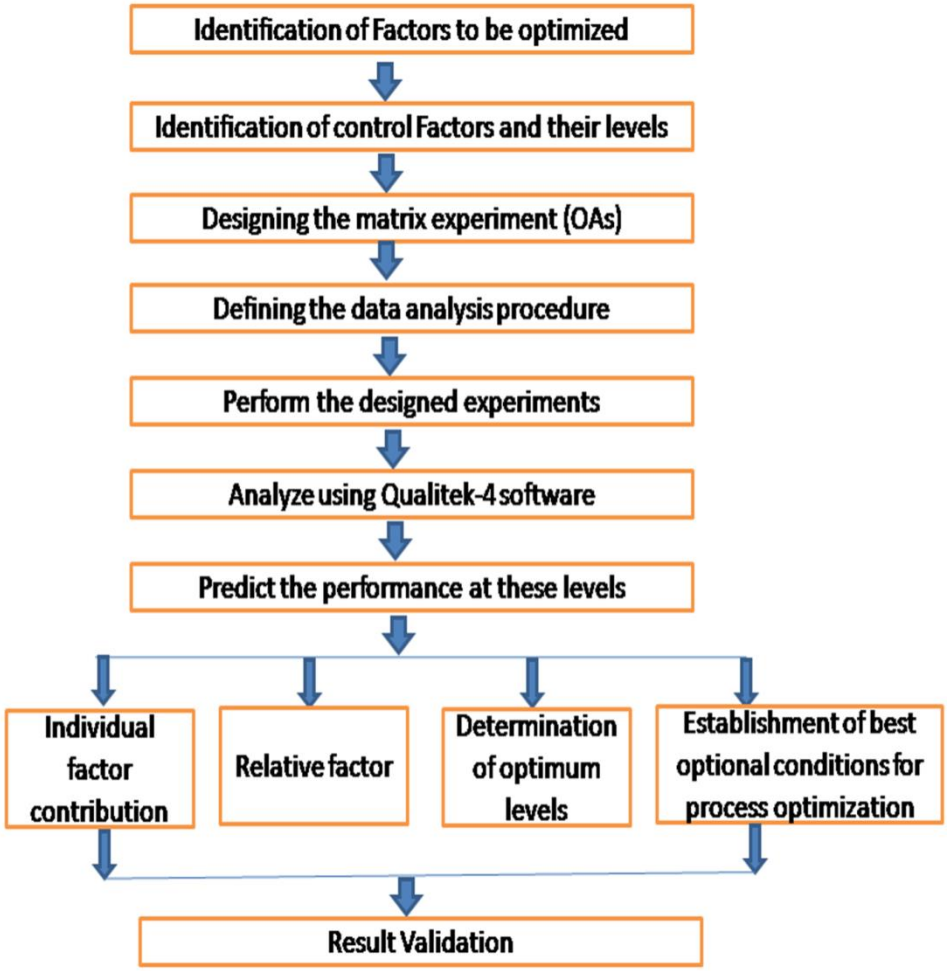

Fig. 3.1: Various steps involved in Taguchi optimization

II] Design of experiments (DOE): Phase I

The initial stage in phase I was to decide the different parameters to be optimized in the nutrient medium that has relevant effects on uricase from *Bacillus cereus GHMS* (*Bc-uricase)* production. Parameters were chosen and the ranges were further assigned based on the group consensus consisting of design engineers, scientists, and technicians with relevant experience. Based on the obtained experimental results, the six parameters possessing significant influence on the *Bc-*

uricase yield were chosen for current Taguchi DOE study to optimize the medium components. The significant influence of selected parameters included malt extract, corn steep liquor (CSL), CuSO4, uric acid, L-Glutamic acid, and KH_2PO_4 on *Bc-uricase* production was studied. In the present study, OAs of L18 (indicating 18 experimental trials) was constructed using DOE of selected parameters with each three assigned levels as represented in Table 3.6.

Table 3.6: Levels of variables used in Taguchi experimental design.

Sr. No.	Factors (%) (w/v)	Level 1	Level 2	Level 3
1	Malt extract	0.5	1	3
2	Corn steep liquor(CSL)	0.5	1	3
3	Copper sulphate	0.02	0.05	0.07
4	Uric acid	0.1	0.15	0.3
5	L-Glutamine	0.02	0.04	0.06
6	KH_2PO_4	0.02	0.04	0.06

III] Submerged fermentation experiments: Phase II

The pure culture of *Bacillus cereus* used as inoculum of size 10 % (v/v) grown for 48 h was inoculated into 50 mL medium of initial pH 7.0 of each designed trial and kept for fermentation at 30°C and 120 rpm for 24 h. Submerged fermentation experiments of designed 18 trials were performed for *Bc-uricase* production in triplicate (mean ± standard deviation of double determination). *Bc-uricase* production was measured in terms of *Bc-uricase* activity (U/mL).

IV] Analysis of experimental data: Phase III

The obtained experimental data in terms of *Bc-uricase* activity was analyzed using Qualitek-4 (Nutek Inc., MI, USA) with "bigger is better" performance quality. Analysis of variance (ANOVA) study determines the influence of individual parameters on the *Bc-uricase* production, optimum contribution, severity index (SI) of the experimental trials. In Taguchi method, the performance is measured by calculating the signal to noise ratio (S/N) for each experimental trial using equation 2.

$$SN = -10\log\sum_{n=1}^{18}(1/Y^2)/n \quad \text{.....Eqn. 2}$$

Y value represents the experimental value obtained for each trial and n is the total number of trials.

V] Validation: Phase IV

From the obtained results of *Bc-uricase* production statistically significant optimum levels of the selected parameters or process variables were determined by analysis of experimental data using analysis of variance (ANOVA), severity index (SI), and main effects of individual parameters. On the basis of optimized parameters and their assigned levels further validation can be performed experimentally.

3.13. Scale-up in 7.5 L bioreactor

a) Scale-up and validation of *Bc*-uricase production in a bioreactor

The optimized parameters were used for further validation in 7.5 L bioreactor (BioFlo/Celligen 115, New Brunswick, USA) using 5 L medium as working volume in batch cultivation mode at optimum growth conditions of 30°C and pH 7.0 by auto control of acid or base addition by pH-mV controller (Mettler-Toledo, USA) as shown in Fig. 3.2. The Dissolved oxygen (DO) at 30% saturation was maintained the speed of agitator at 300 rpm by cascading the air flow rate and monitored by DO

probe (Mettler-Toledo, USA). The reactor was sterilized in an autoclave at 121°C for 20 min. The 48 h old 10% (v/v) inoculum of *Bacillus cereus GHMS* was inoculated into the medium of the bioreactor and 200 mL samples were withdrawn at regular interval of 6 h. The relationship between the uricase production and growth of *Bacillus cereus* over the time period was described using Luedeking-Piret model as expressed by the equation 3.

$$\frac{dp}{dt} = \alpha \frac{dX}{dt} + \beta X \quad \text{.....Eqn. 3}$$

Where, P is product uricase concentration, X is cell concentration, α and β are growth and non-growth related constants.

The parameters; dry cell weight (g/L), uricase activity (U/mL), total carbohydrate (g/L) and inducer uric acid (g/L) concentration was measured in withdrawn samples. The total carbohydrate and inducer uric acid present were measured by the Phenol-sulphuric acid method (Laopaiboon, Nuanpeng et al. 2009) and standard uric acid assay respectively.

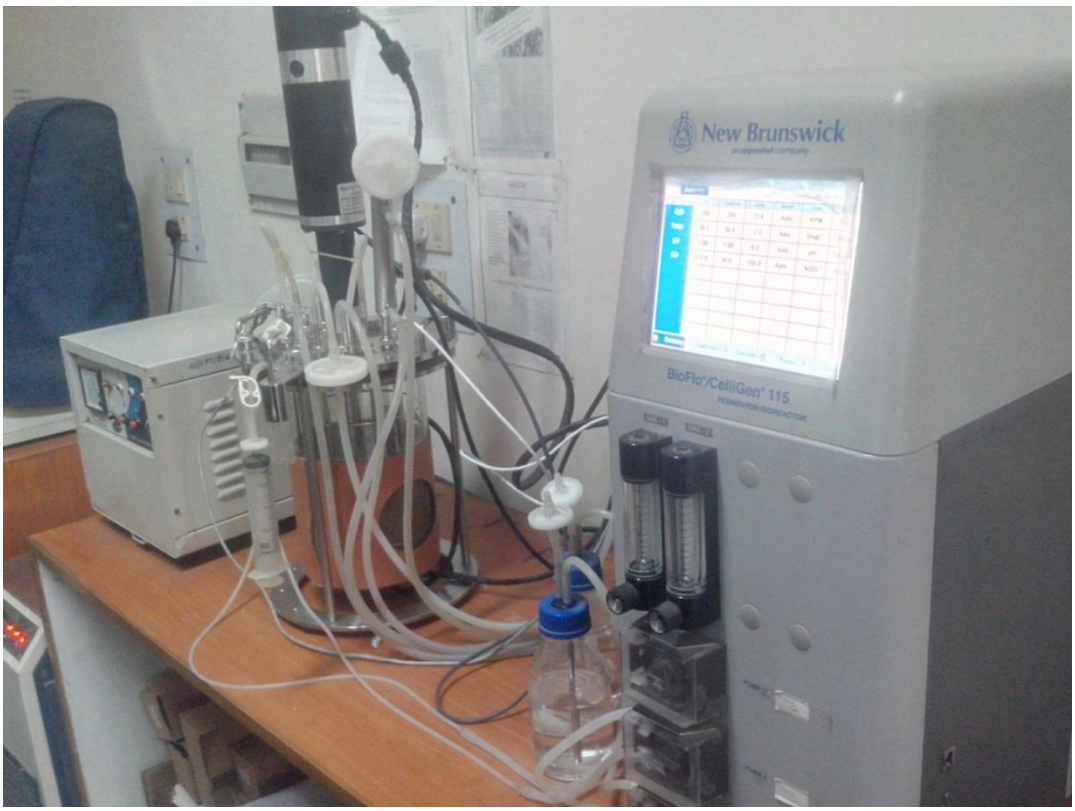

Fig. 3.2: 7.5 L fermenter (BioFlo/Celligen 115, New Brunswick, USA) Working volume (2.8-5.6 L)

3.14. Purification of uricase from *Bacillus cereus*

a) Precipitation using Ammonium sulphate

200 mL of fermentation broth was taken at every 6 h and the extracellular uricase present in the supernatant was obtained by centrifugation at 11962*g*, 4°C for 20 min. The solid ammonium sulphate starting from 30 to 90% (w/v) was added to the obtained supernatant (crude enzyme) while stirring on ice bath until the saturation was achieved. After dissolution of salt, the enzyme precipitate was collected by centrifugation at 11962*g*, 4°C for 20 min. The obtained pellet was re-suspended in 20 mM sodium borate buffer (pH 7.5). The dissolved ammonium sulphate and the low molecular weight proteins were removed by using 20 kDa cut-off dialysis membrane and dialyzing against borate buffer (20 mM) pH 7.5 at 4°C with continuous stirring

with change of buffer at 3 h intervals three times. The complete removal of salt is necessary as it may again precipitate the protein. The presence of salt was analyzed by Nessler's reagent until the dark orange colour changes to light yellow colour. The dialyzed fraction was analyzed for protein concentration and *Bc-uricase* activity. The fraction with highest *Bc-uricase* activity was further purified on the basis of charge and size.

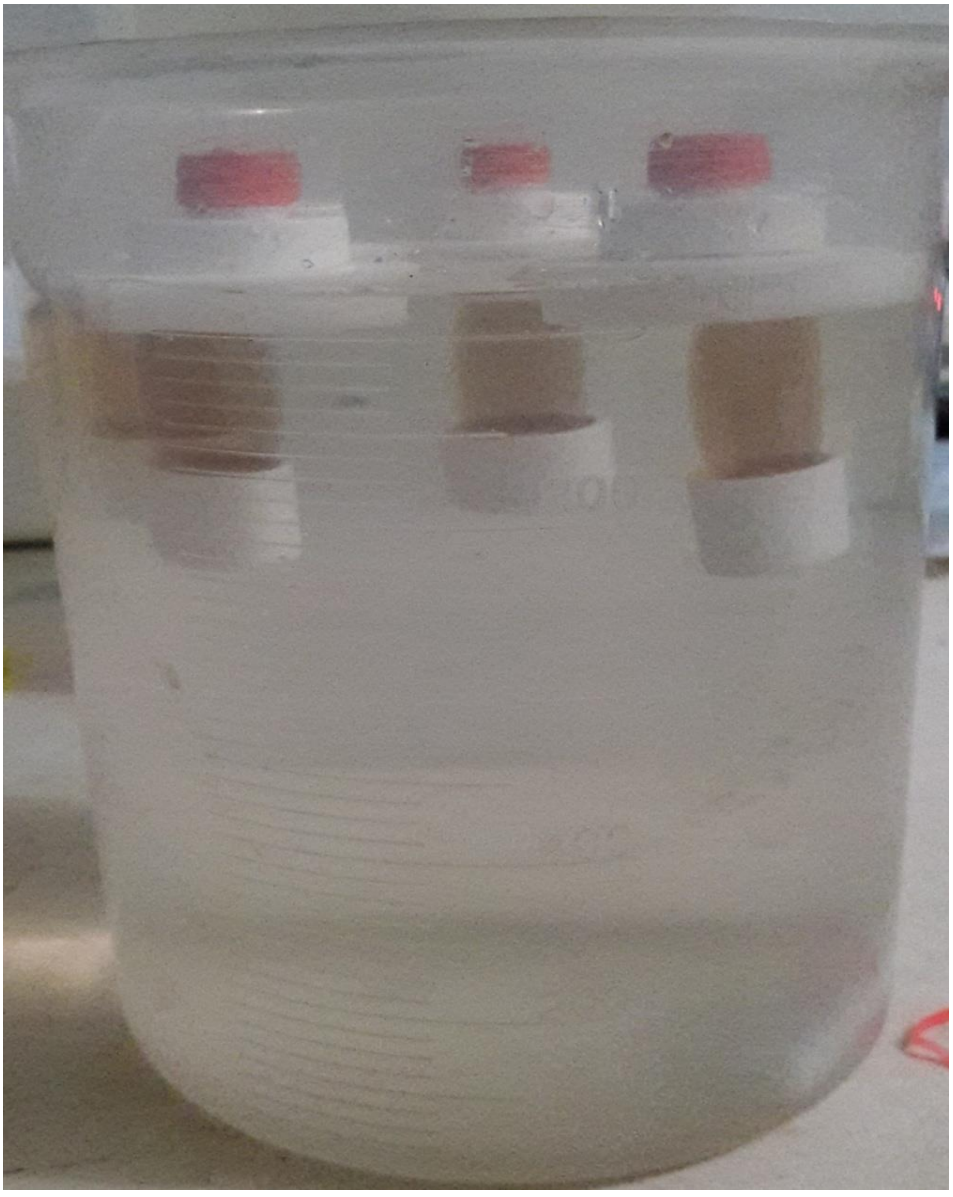

Fig. 3.3: Dialysis using 20 kDa cut-off membranes at 4^0C

b) Determination of isoelectric point

The isoelectric point (pI) of above dialyzed enzyme sample was determined by pH- precipitation profile. The pI of the dialyzed fraction with maximum *Bc-uricase* activity were determined as per the protocol described earlier (El-Sayed 2011). The aliquots of 0.164 mg/ml protein were incubated at various pH values ranging from 2.5 to 9.0 using different buffers with pH including glycine-HCL (2.5-3.5), sodium acetate (4.0-6.0), Tris-acetate (6.0-7.0), borate (7.5-9.0) buffers for 24 h at 4°C followed by centrifugation at 11962*g* for 20 min at 4°C. The amount of precipitated protein was quantified by Biuret assay. The isoelectric point was delineated as the pH at which the maximum amount of protein gets precipitated.

c) Ion-Exchange Chromatography (IEC)

Based on the pI, the *Bc-uricase* was found to be anionic in nature. For further purification the dialyzed sample (14 mL) was concentrated by centricon tube to 2 mL and 1.5 mL was loaded onto DEAE-sepharose column (GE Healthcare, 1.5 cm ×10 cm) which was pre-equilibrated with 20 mM sodium borate buffer, pH 7.5. The washing of column was thoroughly carried out using the same buffer. *Bc-uricase* was then eluted with the linear gradient of NaCl from 0 M to 1.5 M NaCl dissolved in the same buffer. The flow rate and pressure was kept constant throughout the experiment at 0.8 mL/min and 0.3 MPa, respectively and 1.0 mL fractions were collected. IEC was performed by coupling with the Fast Protein Liquid Chromatography (FPLC) system (AKTA Prime, GE Healthcare, USA). Each fraction was analyzed by SDS-PAGE, to check the purity of enzyme (Jianguo, Gaoxiang et al. 1994).

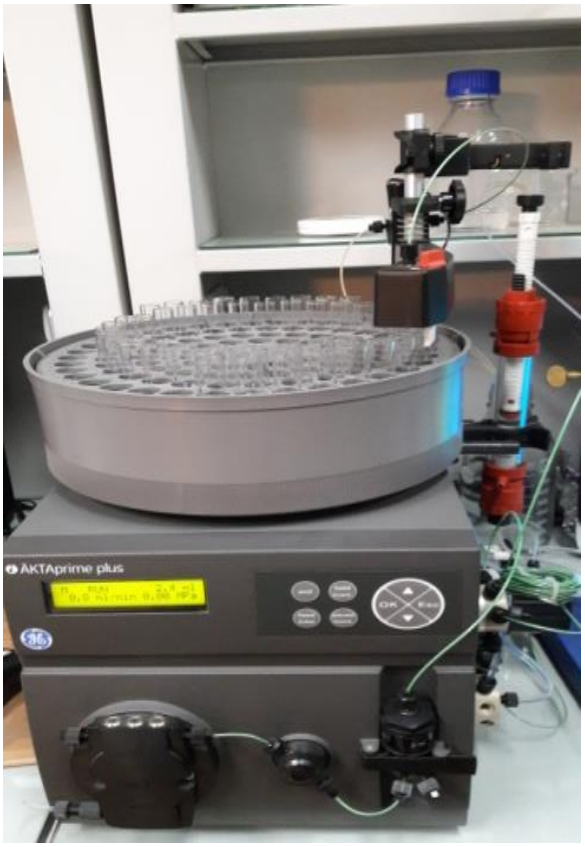

Fig. 3.4: Anion-exchange chromatography using DEAE-Sepharose column

d) Size-Exclusion Chromatography (SEC)

The fractions(from 20 to 26) obtained from DEAE-Sepharose column purification having *Bc-uricase* activity (7 mL) were pooled and concentrated by centricon tube up to 2 mL and then subjected to further purification by SEC using Superdex 75 column (HiLoad 16/60, GE Healthcare Life Sciences). Superdex-75 offers increased resolution of proteins in shorter time. SEC was performed by coupling with the Fast Protein Liquid Chromatography (FPLC) system (AKTA Prime, GE Healthcare).SEC was performed at a flow rate of 0.8 mL/min using 20 mM borate buffer (pH 7.5), 0.3 MPa pressure and 2 mL fractions were collected. Each fraction was analyzed to check the purity of enzyme by sodium dodecyl sulphate-

polyacrylamide gel electrophoresis (SDS-PAGE) on 12% (w/v) polyacrylamide gels, using method reported earlier (He 2011).

Fig. 3.5: Size-exclusion chromatography using Superdex-75pg column

3.15. Molecular characterization of *Bc-uricase*

a) In-gel trypsin digestion

The purified *Bc*-uricase band on SDS-PAGE was cut followed by in-gel trypsin digestion protocol (Shevchenko, Tomas et al. 2006). The purified uricase band was further characterized by excising the gel band with fresh laser blade and transferred it to the sterile fresh Eppendorf microcentrifuge tube. Further the Coomassie stained gel band was destained using the destaining solution (methanol: water: acetic acid in 45:45:10). The destained gel piece was rinsed with 25 mM Ammonium Bicarbonate (ABC) solution. Then the gel piece was dehydrated using 50 µL solution (2:1 mix of

Acetonitrile (ACN): 50 mM of ABC) for a period of five minute. After dehydration, the gel slice was rehydrated 50 µL of 20 mM dithiothreitol for 60 min by incubating at 60^0C in order to reduce the disulfide linkage if any present in the protein followed by alkylating the thiol group of cysteines with the help of iodoacetamide. The alkylation prevents the further formation of disulfide linkages between the cysteines of peptides. The alkylated peptides was further allowed to dehydrate and digested with 20 µL of trypsin protease (20 µg per vial in 1 mL of 25 mM of ABC) and incubated for overnight at 37^0C. Finally the peptide was extracted using 25-50 µL extraction solution (60% Acetonitrile in 1% trifluoroacetate) solvent-solvent extraction method and then the pooled extracted peptide was dried by centrifugal evaporation followed by adding 0.5 µL on the MALDI plate with mixing 0.5 µL of α-cyano-4-hydroxycinnamic acid matrix for further characterization of peptide by MALDI-TOF MS.

b) MALDI-TOF MS analysis

The purified *Bc-uricase* band on SDS-PAGE was cut followed by in-gel trypsin digestion protocol(Shevchenko, Tomas et al. 2006) for mass spectrometric characterization of protein using MALDI TOF/TOF (5800system, Sciex). In-gel trypsin digested *Bc-uricase* sample was mixed with the alpha-cyano-4-hydroxycinnamic acid matrix and subjected to MALDI-TOF analysis. The monoisotopic mass values were assigned to singly charged peptide fingerprints using Biotools software (Brucker, Daltonics). These fingerprints were then searched in non-redundant protein sequence database of NCBI using the following parameters such as, trypsin as digestive enzyme, maximum cleavage site missed up to one, carbamidomethylation as a fixed modification for cysteines, oxidation as variable

modifications for methionine, peptide mass tolerance of ±100 ppm and fragment Mass Tolerance of ± 0.8 Da in MASCOT software (Matrix Science). The Mascot score p< 0.05 was used for the positive identification of *Bc*-uricase.

c) Phylogenetic analysis of *Bc-uricase*

The deduced *Bc-uricase* sequence was searched against UniProtKB/Swiss-Prot database to identify and retrieve the homologous biochemical characterized sequences. The analysis involved 29 amino acid sequences. The sequences were aligned by using Clustal W program and phylogenetic tree was constructed using MEGA6 (Tamura, Stecher et al. 2013). The evolutionary history was inferred by Neighbor-Joining method(Saitou and Nei 1987) using default parameters. Stability of phylogenetic tree was tested by 2000 Bootstrap rearrangements. The branches corresponding to the partitions reproduced in less than 50% bootstrap replicates were collapsed. All positions containing gaps and missing data were eliminated. There were a total of 253 positions in the final dataset.

d) Secondary structure analysis of *Bc*-uricase

The secondary structure of *Bc-uricase*was predicted with the help of PSIPRED v3.3 method (http://bioinf.cs.ucl.ac.uk/psipred/). Circular dichroism (CD) was also performed to determine the percentage of α-helices and β-strands. The Circular dichroism spectroscopic analysis was performed using 0.19 mg/mL concentration of SEC purified *Bc-uricase* having specific activity of 87 U/mg in 20 mM sodium borate buffer (pH 8.0) in the Far UV range (190-250nm). The CD spectrum was recorded on a spectro-polarimeter (J-815Jasco Corporation, Tokyo) at 25°C. The resulted CD spectrum of protein was expressed by difference in molar extinction coefficients (Δε, deciliter mol^{-1} cm^{-1}) as a function of wavelength (Kelly et al, 2005). The composition

of secondary structure was predicted using web based K2D3 server(Louis-Jeune, Andrade-Navarro et al. 2012).

e) Structure modeling, energy minimization and validation of *Bc-uricase*

The amino acid sequence of *Bc-uricase* deduced from MALDI-TOF analysis was used for template based tertiary structure prediction using Raptor X server (Källberg, Wang et al. 2012). The modeled structure of *Bc-uricase* was refined by energy minimization using the ModRefinerserver (Xu and Zhang 2011). The refined structure was assessed by SAVES server (https://services.mbi.ucla.edu/SAVES/). The homologous structure of *Bc-uricase* was searched using DALI web server(Holm and Rosenstrom 2010).

3.16. Physico-chemical properties of the purified uricase

The properties of the purified uricase based on partial purification, charge and size basis has been studied.

a) Effect of pH on uricase activity and stability

Since all the enzymes are very specific to the pH of the reaction mixture for the optimum activity. The pH of the reaction mixture was adjusted with different proportions of buffers such as sodium acetate (4.0 – 6.0), Tris-acetate (6.0 – 7.0) and borate (7.5 – 10.0) of each 20 mM strength and the uricase activity was measured. The stability of uricase in buffers were determined by incubating the enzyme at different pH of buffers for 1 h at 30°C and uricase assay was performed in terms of measuring the residual activity at the optimum pH (Ghorbel, Sellami-Kamoun et al. 2003). Prior to incubating the uricase at various pH, the uricase activity was measured at the optimum pH which was considered as 100 % activity.

b) Determination of optimum temperature and stability

Since most of the enzymes are protein in nature, so their structures and functions are highly dependent on the temperature of the catalytic environment. The optimum temperature for uricase activity was determined by incubating the reaction mixture at various temperatures (^{0}C) such as 20, 25, 30, 35, 40, and 45 for the period of 10 min (Yoon, Choi et al. 1996; Immanuel, Dhanusha et al. 2006). The thermostability of the uricase was determined by incubating 50 µL of the uricase enzyme in 20 mM borate buffer of pH 8.0 for 30 min. at various temperatures such as 20, 25, 30, 35, 40, 45, 50, 55, and 60^{0}C and the activity were assayed.

3.17. *In vivo* applications of uricase in hyperuricemia

To regulate the concentration of uric acid in serum and joints of the hyperuricemic animal by enzyme therapy, the purified uricase from *B. cereus* was studied thoroughly. The treatment of gout and hyperuricemia by the administration of purified uricase which acts on the uric acid in serum oxidatively and lowers down the concentration of uric acid. The *in vivo* efficacy of the purified uricase was studied in male Swiss albino mice weighing 25-30 gm. Before initiating the experimental study, the mice were allowed to adopt for 15 days in the laboratory environment to sustain the experimental procedure (Whishaw, Metz et al. 2001; Wahlsten, Metten et al. 2003).

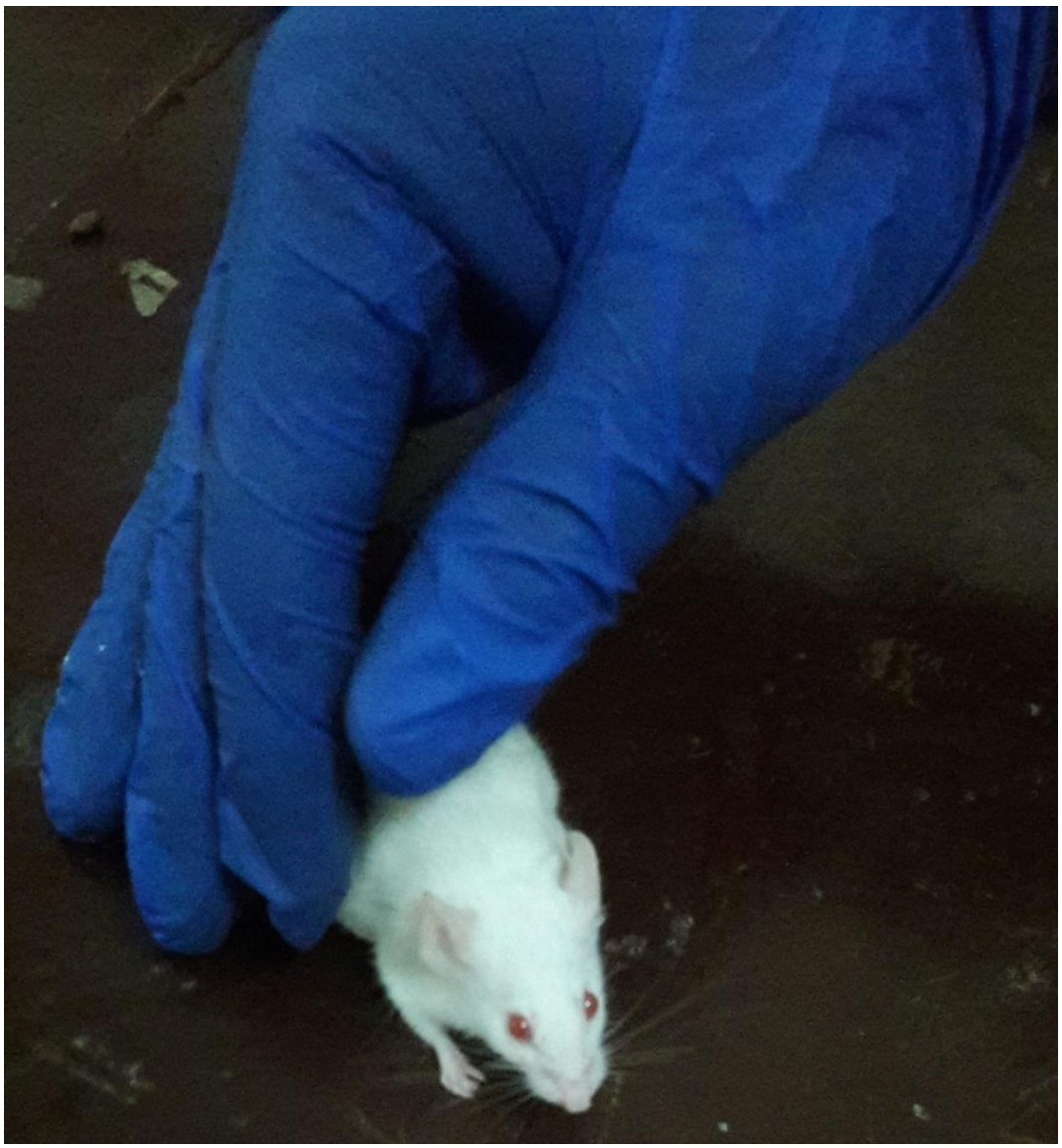

Fig. 3.6: Adaptation of male Swiss albino mice in the laboratory animal house.

a) Hyperuricemia: Uricase deficient mice model

To study the therapeutic efficacy of purified uricase having 87 U/mg of specific activity, the uricase deficient mice model were selected. The activity and stability of uricase present in mice was inhibited by intraperitoneally (IP) administration of uricase inhibitor i.e. potassium oxonate (300 mg/kg) into the mice (Wang, Wang et al. 2010; Shi, Wang et al. 2012). The potassium oxonate inhibits the uricase of mice by

means of competitive inhibition mechanism (Fridovich 1965; Zhao, Zhu et al. 2006). This indeed increased the uric acid concentration in the serum of mice known as hyperuricemia mice (Jin, Yang et al. 2012). After intraperitoneally administration of potassium oxonate inhibitor, the uric acid concentration was regularly measured at specific time (h) 1, 3, 6, 12, 24, and 48 h by collecting blood samples from retro-orbital vein of mice. The effect of potassium oxonate treated mice was significantly compared with that of the control mice and the uric acid concentration was measured in serum spectrophotometric method (Kaikkonen, Porkkala-Sarataho et al. 2002).

Fig. 3.7: Intraperitoneal rout of administration of potassium oxonate and uricase

b) *In vivo* study of purified uricase

The therapeutic efficacy of the purified uricase was studied in the uricase deficient hyperuricemic mice. After 1 h of administration of potassium oxonate the uric acid concentration was increased significantly and then 5 µg dosage of uricase with activity of 87 U/mg was administered by injecting intraperitoneally. Further uric acid concentration in the serum was measured at the interval of time 1, 3, 6, 12, 24, and 48 h from the collected blood samples and significant therapeutic activity of the uricase was determined. The therapeutic efficacy of the uricase was determined by decrease in the uric acid concentration in the serum at significant level which was compared with the oxonate treated hyperuricemic mice. All the experiments were performed using three groups containing four mice (n=4) in each group.

c) Immunogenicity study of uricase

In order to replace the existing outrageous chemotherapy for regulating the uric acid concentration in the patients suffering from hyperuricemia and gout, the uricase protein based therapy should be non-immunogenic in nature with good significant biocompatibility with the human body. So the immune responsive nature of the purified *B. cereus* uricase was studied using laboratory acquainted Swiss albino mice. The intraperitoneal administration of 5 µg dose of uricase was injected on 0 day and the total IgG present in serum were measured (Smith, Lloyd et al. 1979; Parekh, Dwek et al. 1985; Caliceti and Veronese 2003). On day 15th, the same 5 µg dose of uricase as a booster dose was injected via intraperitoneal rout in the same group of mice and the total IgG were measured again on day 21st. The increase in concentration of total IgG on 21st day after the immunization with one booster dose on 15th day

ensures the immunogenic nature of protein (Tsuji, Hirose et al. 1985; Babu, Pattnaik et al. 2008).