CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

Chemicals for media preparation and general chemicals were procured from various sources such as Hi-Media (Mumbai), SRL (Chennai), Merck (Mumbai) and Qualigens Fine Chemicals (Mumbai) and were of analytical grade. Enzyme substrates and inhibitors were procured from Sigma Aldrich (St.Louis, USA.) Ion-exchange chromatography Micro-Prep® DEAE sorbent and low molecular weight protein markers were purchased from Bio–Rad Laboratories. 30 kDa Amicon® Ultra filter were purchased from Merck (Mumbai). All the reagents were prepared using deionised (Millipore) or glass distilled water. The consumables like filters, membranes, were purchased from the following companies and stored according to the manufacturer’s recommendation.

2.1.1 Culture Media

Nutrient broth (g/l)

- Peptone : 2.0
- NaCl : 5.0
- Yeast extract : 2.0
- Beef extract : 1.0
Basal medium (g/l)

\[
\begin{align*}
\text{K}_2\text{HPO}_4 & : 3.0 \\
\text{NaCl} & : 0.5 \\
\text{MgSO}_4 \cdot 7\text{H}_2\text{O} & : 0.5 \\
\text{CaCl}_2 & : 0.3
\end{align*}
\]

2.2 ISOLATION AND SCREENING

The microorganism used in this study was isolated from tamarind seed (\textit{Tamarindus indica}). The samples were dissolved in sterile water and serial dilution was performed and many pure cultures were screened and plated on substrate agar plates such as gelatin/olive oil/tributyrin agar plates. (Nutrient agar media containing 0.5 \% w/v substrates) for screening protease and lipase/esterase producing organisms. The plates were incubated at 37 °C for 24-48 h. A clear zone of hydrolysis around the colonies on substrate agar plates indicated the presence of protease and esterase. (For protease the plates were flooded with 0.1 \% solution of Coomassie blue R250). No activity was found for lipase. The stock culture was maintained as a glycerol stock (50 \% v/v glycerol) at -20 °C. This was taken for further studies.

2.3 IDENTIFICATION OF MICROORGANISM

The taxonomic characteristics of this strain were examined according to \textit{Bergey’s Manual of Determinative Bacteriology} (Holt et al. 1994) and 16S rDNA gene sequencing (Weisburg et al. 1991). The sequence homology was matched with Genbank database using BLAST. Alignment of 16S rDNA sequences were carried out using CLUSTALW program at the European Bioinformatics Institute server (Thompson et al. 1994). The neighbour-joining phylogenetic tree was constructed
with PHYLIP program. Bootstrap analysis was made by method of Felsenstein (1985). The isolate was identified as *Lysinibacillus fusiformis* AU01.

### 2.4 EFFECT OF CULTURE CONDITIONS ON GROWTH

Optimal conditions for growth were studied using nutrient broth media containing (g/l): Peptone 2.0, NaCl 5.0, Yeast extract 2.0, and Beef extract 1.0, at temperature ranges of 25-40 °C, pH ranges between 6.0-10.0, with increments of one unit. The culture media was inoculated with 1 % v/v of an exponentially growing pre-culture prepared in the same medium (nutrient broth) for 60 h on a rotary shaker at 150 rpm. The samples were withdrawn at 2 h interval. The optical Density was measured at 610 nm.

### 2.5 ANALYTICAL METHODS

#### 2.5.1 Estimation of Biomass

For estimation of dry cell weight, cells were collected by centrifugation of samples at 10,000 x g for 10 min at 4 °C. The collected cells were washed twice with distilled water and allowed to dry at room temperature under vacuum until a constant mass was attained. The growth was monitored by measuring the optical density at 610 nm and the cell free supernatant was analysed for protease and esterase activity. The obtained optical density values were converted to dry cell wt g/l using calibration curve.

#### 2.5.2 Preparation of crude enzyme

Extracellular crude enzyme solution was obtained by centrifugation of the culture broth at 10,000 x g for 10 min at 4 °C and assayed for
extracellular protease and esterase activity. To extract intracellular proteins, the cell pellet was washed twice with double distilled H₂O. Then the cell pellet were dissolved in 20 mM Tris-Cl pH 8.0 and sonicated for 4 cycles of 45 seconds (600 joules) at 60% amplitude on a ice bath (VIBRA CELL TM Ultrasonicator). The cell lysate was centrifuged at 12,000 x g for 10 min at 4 °C. The cell debris was discarded and the supernatant was analysed for protease and esterase activity.

2.5.3 Protease and esterase assay

Protease activity was measured according to the method described by Sarath et al (1989) with some modifications. The reaction mixture was composed of 250 µl of azocasein (5 mg/ml) in 50 mM Tris-Cl (pH 9.0) and an equal volume of the diluted enzyme. After 30 min incubation at 40 °C the reaction was terminated with 1.2 ml of 10%, w/v trichloroacetic acid (TCA). The mixture was kept at ice for 10 min. The precipitate was removed by centrifugation at 12,500 x g for 5 min. After adding 150 µl of supernatant to equal volume of 1 N NaOH, the absorbance at 450 nm was recorded with a Bio-Rad (Model 680) titre plate reader. A blank was prepared by the same procedure, TCA being added at zero time. One unit of protease activity was defined as the amount of enzyme required to produce an increase in absorbance of 0.1 at 450 nm.

The hydrolytic activity of esterase was measured using 4-nitrophenyl valerate as substrate. A reaction mixture containing 25 mM Tris-Cl pH 8.0, 100 µl enzyme and the reaction started by the addition of 10 µl of 4-nitrophenol valerate (50 mM dissolved in 2-propanol). The reaction was quenched by the addition of 500 µl ice cold acetone. The activity was
determined by measuring the absorbance of the liberated 4-nitrophenol against an enzyme-free blank at 405 nm. 1 U of enzyme activity was defined as the amount of enzyme needed to release 1 µg of 4-nitrophenol per min under standard assay conditions.

2.5.4  **Protein Estimation**

Protein concentration was measured by the method of Bradford (1976) using bovine serum albumin (BSA) as the standard. The assay mixture consisted of 160 µl of protein sample and 40 µl of dye, then was incubated in microtitre plate at room temperature for 5 min. Absorbance was read at 595 nm. Protein concentration in the sample was calculated by comparing with known concentration of standard graph (Appendix- II).

2.6  **OPTIMIZATION OF PROTEASE AND ESTERASE PRODUCTION**

2.6.1  **One-Variable-at-a-Time Strategy**

One-Variable-at-a-time strategy was performed for initial screening of the most significant carbon and nitrogen sources required to enhance protease and esterase production. The production of protease and esterase by AU01 was carried out in basal medium containing (g/L): ( glucose, 2; peptone, 2;NaCL 0.5; K$_2$HPO$_4$, 0.5; CaCl$_2$, 0.5 and MgSO$_4$, 0.3) with different combinations of carbon sources (glucose, fructose, maltose, sucrose, starch, trisodium citrate, glycerol and citric acid) and nitrogen (peptone, yeast extract, malt extract, beef extract, casein acid hydrolysate, skimmed milk, gelatin, ammonium sulphate, ammonium phosphate, ammonium chloride and sodium carbonate) sources (1% w/v) and metal
chlorides (CaCl₂, MgCl₂, MgSO₄, ZnCl₂, MnCl₂ and KCl) incubation time (12, 24, 36, 48 and 60), temperature (25, 30, 37 and 45 °C), pH (6, 7, 8,9) and inoculum size (0.5, 1.0, 2.0, and 4.0 %) agitation(100,200,400 rpm) were tested by one-factor-at-a time strategy. CaCl₂ and MgSO₄ were autoclaved separately and added to the medium.

Glucose and peptone was replaced by various carbon and nitrogen sources respectively. All the experiments were performed in a shake flask at 37°C for 60 hours at 150 rpm in an orbital shaker and carried out as duplicates.

2.6.2 Plackett-Burman Design

For the selection of significant variables, a set of parameters was selected based on preliminary results. A total of eleven parameters were included for selection, with each variable represented at two levels. 9 variables and 2 dummy variables were selected for Plackett-Burman (PB) design. All the variables were tested at two levels and were designated as +1 for high and -1 for low values respectively (Table 2.1). Variable such as trisodium citrate, yeast extract, CaCl₂, MgSO₄, pH, inoculum size, Temperature, Incubation time and Agitation were optimized with the Plackett-Burman method.
Table 2.1 Coded Vs Actual value of variables for Plackett-Burman design

<table>
<thead>
<tr>
<th>Variables</th>
<th>Coded values</th>
<th>-1</th>
<th>+1</th>
</tr>
</thead>
<tbody>
<tr>
<td>X_1 - Trisodium citrate, g/l</td>
<td>1.0</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td>X_2 - Yeast extract, g/l</td>
<td>1.0</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td>X_3 - CaCl_2, g/l</td>
<td>0.1</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>X_4 - MgSO_4, g/l</td>
<td>0.1</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>X_5 - pH</td>
<td>7.0</td>
<td>9.0</td>
<td></td>
</tr>
<tr>
<td>X_6 - Inoculum size, % (v/v)</td>
<td>1.0</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>X_7 - Temperature, °C</td>
<td>30</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>X_8 - Incubation time, h</td>
<td>24</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>X_9 - Agitation, rpm</td>
<td>100</td>
<td>200</td>
<td></td>
</tr>
</tbody>
</table>
Table 2.2  Coded Vs Actual value of variables for RSM analysis

<table>
<thead>
<tr>
<th>Variables</th>
<th>Coded values</th>
<th>-2</th>
<th>-1</th>
<th>0</th>
<th>+1</th>
<th>+2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trisodium citrate, g/l (A)</td>
<td>0.50</td>
<td>2.00</td>
<td>3.50</td>
<td>5.00</td>
<td>6.50</td>
<td></td>
</tr>
<tr>
<td>Yeast extract, g/l (B)</td>
<td>2.0</td>
<td>2.75</td>
<td>3.50</td>
<td>4.25</td>
<td>5.00</td>
<td></td>
</tr>
<tr>
<td>pH (C)</td>
<td>4.50</td>
<td>6.00</td>
<td>7.50</td>
<td>9.00</td>
<td>10.50</td>
<td></td>
</tr>
<tr>
<td>Inoculum size, % v/v (D)</td>
<td>0.10`</td>
<td>2.00</td>
<td>4.00</td>
<td>6.00</td>
<td>8.00</td>
<td></td>
</tr>
</tbody>
</table>

Thirty experimental runs with 5 duplicate runs at centre point were designed, responses in terms of protease and esterase activity were determined for each experiment. All parameters were taken at a central coded value considered as zero. The minimum and maximum ranges of parameters were investigated and the full experimental plan with respect to their values in actual and coded form. The response values (Y) in each trial were the average of the triplicates. The ranges of these variables were decided according to the ranges determined by the one-at-a-time method. Other variables were set at their optimum level.

2.6.4  Statistical Analysis and Modelling

The data on enzyme production were subjected to analysis of variance (ANOVA). The mathematical relationship of the independent variable and the response enzyme production was calculated by the quadratic regression equation.

2.7  ENZYME PRODUCTION IN A BIOREACTOR

In order to validate the predicted value, the experiments were carried out in triplicates using optimized media and culture conditions in
shake flasks. The final scale-up and verification of the statistical model for enzyme production was carried out in a 3L fermentor. For bioreactor studies batch experiments were conducted in 3L bioreactor (2L working volume, New Brunswick BioFlo-115) with agitation speed of 300 rpm, with constant sparging for aeration rate at 1 vvm. Initial pH of the medium was set at 8.0, and was operated under controlled pH conditions.

2.8 PRODUCTION AND PURIFICATION OF PROTEASE

For enzyme production, 100 ml of inoculum was transferred into a 3 l Bioreactor containing 2 l ml of optimized media. The fermentation was carried out at the above mentioned conditions for 16 hours.

Proteins are usually soluble in water solutions because they have hydrophilic amino acids on their surfaces that attract water molecules and interact with them. This solubility is a function of the ionic strength and pH of the solution. Proteins are characterized by their isoelectric points at which the charges of their amino acid side groups balance each other. Ammonium sulphate is the most common salt used for this purpose because of it’s good solubility in cold buffers. Ammonium sulphate fractionation is commonly used in research laboratories as a first step in protein purification because it provides some crude purification of proteins away from non-proteins and also separates some proteins. It also yields precipitated protein slurry that is usually very stable.

Ammonium sulphate (40-70%) was added to the cell free supernatant (250 ml) with gentle stirring. The precipitated proteins were collected by centrifugation at 12,500 x g for 15 min at 4 °C. The precipitate was dissolved in minimal volume of buffer A containing 20 mM Tris-Cl
buffer (pH 8.0) and dialysed against the same buffer at 4 °C. The buffer was replaced twice during the process and this was assayed for protease activity.

2.8.1 Anion-Exchange Chromatography

The most popular method for the purification of proteins and other charged molecules is ion exchange chromatography. In cation exchange chromatography positively charged molecules are attracted to a negatively charged solid support. Conversely, in anion exchange chromatography, negatively charged molecules are attracted to a positively charged solid support.

Ammonium sulphate precipitated crude enzyme was used for further purification of protease. Three millilitre of Micro-Prep® DEAE sorbent was filled into a PD-10 column without air bubbles. The slurry was allowed to settle for few minutes until a clear supernatant layer was visible at the top of the column. The column material was equilibrated with 20 ml buffer A (20 mM of Tris-Cl, pH 8.0) and wash with the same buffer to remove unbound proteins. The bound proteins were eluted stepwise by increasing the concentration gradient of NaCl (1-500 mM NaCl dissolved in Tris-Cl pH 8.0). Fractions of 3 ml were collected and assayed for protease activity.

2.8.2 Membrane Filtration

Membranes are polymer films with specific pore ratings. It concentrates a protein solution using selective permeable membranes. The function of the membrane is to let the water and small molecules pass through while retaining the protein. The solution is forced against the membrane by mechanical pump, gas pressure, or centrifugation.
The ion-exchange chromatography fractions that showed protease activity were pooled and concentrated through a membrane filter (Amicon Ultra 30K Millipore) by centrifugation at 5000 x g for 10 min at 4°C. The retentate was stored at -20°C for further studies.

2.9 RP-HPLC ANALYSIS OF PURIFIED PROTEASE

The purity of the enzyme was confirmed by RP-HPLC analysis using Agilent 1200 HPLC system. The purified protease was applied on to C-18 column (Zorbax C-18, 4.6 X 250 mm i.d., 5 µm particle size, Agilent technologies). For elution, solvent A consisting of 0.1% (v/v) Trifluoroacetic acid (TFA) in water and solvent B consisting of 0.1% (v/v) TFA in acetonitrile were used. After loading, the column was washed with 2% solvent B for 2 min, to elute any unbound protein. The bound proteins were eluted from column using a 30 min linear gradient from 2-100% (v/v) of acetonitrile containing 0.1% trifluoroacetic acid at a constant flow rate of 1 ml/min. Column temperature was maintained at 25 °C and column eluent was monitored at 280 nm.

2.10 SDS POLYACRYLAMIDE GEL ELECTROPHORESIS

Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed according to Laemmli (1970). The purified protease was run through SDS-PAGE using a 4% stacking and 12%(v/v) separating gel respectively.

- **Monomer solution**: 29.2% acrylamide and 0.8% bis-acrylamide
- **Separating gel buffer**: 1.5 M Tris-HCl pH 8.8
- **Stacking gel buffer**: 1.0 M Tris-HCl pH 6.8
- **Ammonium per sulphate**: 100 mg/ml
- **SDS**: 10% stock solution
### Separating gel (12%) - 10 ml
- 30% acrylamide-bisacrylamide : 2.80 ml
- 1.5 M Tris-HCl pH 8.8 : 2.50 ml
- 10% SDS : 0.10 ml
- APS : 0.10 ml
- TEMED : 0.008 ml
- Distilled water : 4.50 ml

### Stacking gel (4%)-5 ml
- 30% acrylamide-bisacrylamide : 0.70 ml
- 1 M Tris-HCl (pH 6.8) : 0.31 ml
- 10% APS : 0.05 ml
- TEMED : 0.005 ml
- Distilled water : 3.90 ml

### Sample loading buffer (5X)
- 1.0 M Tris-HCl pH 6.8 : 0.21 ml
- Bromophenol blue : 0.05% (w/v)
- SDS : 100 mg
- Glycerol : 0.50 ml
- 2-mercaptoethanol : 0.26 ml
Running buffer (pH 8.3)

Glycine : 0.192 M  
Tris : 0.025 M  
SDS : 0.1% (w/v)

An equal amount of ammonium sulfate precipitated and ion exchange purified samples were loaded. For reducing SDS-PAGE, samples were boiled for 5 min with sample loading dye containing disulphide bond reducing agent (β-mercaptoethanol) before electrophoresis. For non reducing condition sample was not boiled and β-mercaptoethanol was not included in the sample buffer. Molecular mass of the enzyme was determined by comparing with pre-stained protein molecular weight standards (Bio-rad). The gel was pre-run for 10 minutes. Electrophoresis was carried out at 60 V, till the dye front reached bottom of the gel.

Following electrophoresis, the gel was transferred to a clean glass or plastic container. 50 ml of 0.25% coomassie brilliant blue R-250, 50% methanol, and 10% acetic acid were added and incubated for 4 h to overnight at room temperature with shaking. The gel was destained by successive incubations in 15% methanol and 5% acetic acid in water.

2.10.1 Zymogram Analysis

For activity staining, gel was loaded in duplicate with equal amount of purified protein under non-denaturing condition. After electrophoresis, the gel was rinsed with 50 mM Tris-Cl buffer pH 8.0 for 10min. Then the gel was transferred to the substrate solution (containing 0.5 % w/v, gelatin in 50 mM Tris-Cl buffer pH 8.0) for 30 min with gentle rocking to allow the substrate to diffuse into the gel. The gel was transferred to clean petridish and incubated at 40°C for 45 min for the digestion of the gelatin by the active
protease. Then the gel was stained using Coomassie R250, destained and the clear halo around blue background was visualized showing the presence of protease activity.

2.11 IMMOBILIZATION OF PROTEASE

The purified protease was immobilized on Celite (545) by the method described by (Liu et al. 2009) with some modification. Briefly, one gram of dry celite powder was mixed with 1 % (v/v) 3-aminopropyltriethoxysilane in 50 ml acetone, stirred at 50 °C for 30 min and dried at 110 °C for 10 h. Aminopropyl celite beads were washed with water twice and suspended in 10 mM phosphate buffer solution (pH 7.5). The resulting beads were activated by adding 10 ml of 2.5 % glutaraldehyde solution in phosphate buffer pH 7.5, stirred at 25 °C for 2 h. The activated celite beads were washed with water and dried at 60 °C for 2h. The activated celite bead (500 mg) was mixed with 10 ml of purified protease (100 U/ml) and the mixture was constantly shaken at 25 °C for 12 h. The immobilized protease was recovered by centrifugation at 5000 xg for 10 min at 4 °C. The amount of protease immobilized on celite was estimated by detecting the protein concentration in the reaction solution before and after immobilization. To determine the storage stability of protease, the free and immobilized enzyme was stored at 4 °C and 25 °C for 15 days. Aliquots were withdrawn every day and residual activity was measured under standard assay conditions. To determine the operational stability of immobilized enzyme, 100 mg (800 U) immobilized protease was assayed under standard assay conditions, enzyme was recovered by centrifugation at 5000 x g for 10 min at 4 °C. Then the process was repeated 10 times to test enzyme reusability.
2.12 BIOCHEMICAL CHARACTERISATION OF AU01 PROTEASE (FREE and IMMOBILIZED ENZYME)

2.12.1 Effect of pH on Activity and Stability

To the effect of pH on protease activity was determined by incubating the purified enzyme between pH 3 to 12 at 40 °C. The effect of pH on protease stability was examined by incubating the mixture in 50 mM of respective buffers for 4 h at 25 °C. Aliquots were withdrawn at regular time intervals to test the residual activity. The following buffers used are citric acid (pH3-4), sodium acetate (pH 4-5.5), sodium phosphate (pH 6-7.5), Tris-Cl (pH 8-9), glycine-NaOH (pH 9-11) and sodium phosphate (pH 11-12).

2.12.2 Effect of Temperature on Activity and Stability

The effect of temperature on protease activity was studied from 20 °C to 70 °C, with an interval of 5 °C. The enzyme mixture was incubated at different temperature for 30 minutes. Stability studies were performed by incubating the enzyme mixture at respective temperature for 3 hour, samples were withdrawn at regular intervals (15 min) and the residual activity was measured under standard assay conditions.

The optimum temperature of the enzyme was determined by estimating the activity of protease in the temperature ranging from 20 °C to 70 °C (with the interval of 10 °C) for 10 min at pH 8.0. Activation energy (E_a) of the enzyme is calculated according to Arrhenius relationship:

\[ E = A \exp\left(-\frac{E_a}{RT}\right) \]  \hspace{1cm} (2.1)

\[ \ln E = \ln A + \left(-\frac{E_a}{R}\right)\left(\frac{1}{T}\right) \]  \hspace{1cm} (2.2)
Where, \( E \) - enzyme activity (U/mg); \( A \) - Arrhenius constant; \( E_a \) – Energy of activation; \( R \) – universal gas constant (8.314 J mol\(^{-1}\) K\(^{-1}\)); \( T \) – absolute temperature.

\( E_a \) was calculated from slope \((-E_a/R)\) of the Arrhenius plot \( \ln(E) \) vs \( 1/T \).

### 2.12.3 Thermal Inactivation Kinetics

Thermal inactivation of the enzyme was determined by incubating purified protease (free and immobilized enzyme) at different temperature ranging from 30 to 60 °C (with the interval of 5 °C) for 3 h. Samples were withdrawn at regular intervals (15 min) and residual activity of the enzyme was calculated at standard assay conditions. Thermal inactivation kinetics of the enzyme can be described by first-order kinetic model. Under isothermal conditions, the deactivation of enzyme as the function of treatment time can be described as follows:

\[
E = E_0 \exp(-k_{\text{d}}t) \tag{2.3}
\]

\[
\ln \left( \frac{E}{E_0} \right) = -k_{\text{d}}t \tag{2.4}
\]

Where,

\( E \) – Enzyme activity at time \( t \); \( E_0 \) – Initial enzyme activity;
\( k_{\text{d}} \) – Thermal deactivation constant; \( t \) – reaction time

\( k_{\text{d}} \) was calculated from slope of the plot \( \ln(E/E_0) \) vs time.

Thermal deactivation energy (\( E_{\text{d}} \)) of the free and immobilized enzyme is calculated according to Arrhenius relationship:

\[
k_{\text{d}} = A \exp\left(\frac{E_{\text{d}}}{RT}\right) \tag{2.5}
\]
\[ \ln k_d = \ln A + \left( \frac{E_d}{R} \right) \left( \frac{1}{T} \right) \]  \hspace{1cm} (2.6)

\( E_d \) was calculated from slope (-\(E_d/R\)) of the Arrhenius plot \( \ln(k_d) \) vs 1/T.

Half life of the enzyme was calculated using following relationship:

\[ t_{1/2} = \frac{2.303}{k_d} \]  \hspace{1cm} (2.7)

Change in the activation enthalpy (\(\Delta H\)), activation free energy (\(\Delta G\)) and activation entropy (\(\Delta S\)) for thermal inactivation for free and immobilized enzyme were calculated according to following equations (Ozturk et al 2012):

\[ \Delta H = E_d - RT \]  \hspace{1cm} (2.8)

\[ \Delta G = -RT \ln \left( \frac{k_d h}{k_b T} \right) \]  \hspace{1cm} (2.9)

\[ \Delta S = \frac{(\Delta H - \Delta G)}{T} \]  \hspace{1cm} (2.10)

Where, \( k_b \) — Boltz constant; \( k_d \) — Thermal deactivation constant

### 2.13 EFFECT OF ORGANIC SOLVENTS

To determine the organic solvent stability of the purified enzyme, 3 ml of crude protease was incubated with 1 ml of organic solvent at 25°C with a constant shaking for 24 hours. Samples were withdrawn every day (24 h) and the residual activities were estimated at standard assay conditions. The solvents used are glycerol, water, DMSO, methanol, ethanol, acetonitrile, acetone, 2propanol, DCM, benzene, toluene, xylene, n-hexane, n-pentene, n-heptane, n-nonane, n-octane, n-undecane.
2.14 EFFECT OF METAL IONS, INHIBITORS AND REDUCING AGENTS ON PROTEASE ACTIVITY

The effect of metal ions on purified protease was determined by pre-incubating the purified enzyme with 2, 5 and 10 mM (final concentration) of chloride salts of the metal ions (Ba\(^{2+}\), Ca\(^{2+}\), Cd\(^{2+}\), Fe\(^{2+}\), Mg\(^{2+}\), Mn\(^{2+}\), K\(^{+}\), Na\(^{+}\), Zn\(^{2+}\)), at 25 °C for 30 min. Relative activity was measured under standard assay conditions. To determine the effect of inhibitors on enzyme activity, the purified enzyme was pre-incubated with different concentration of individual inhibitors (EDTA, 1, 10 phenanthroline, PMSF) for 30 min at 25 °C. All the inhibitors were tested at different concentrations of 2, 5 and 10 mM. The residual activity was compared with control (incubated without inhibitors).

Effect of reducing agents on AU01 protease stability was tested by incubating aliquots of purified enzyme with (DTT, 2-mercaptoethanol) agents for 30 min at 25 °C. The residual activity was measured under standard assay conditions and compared with control incubated without additives.

2.15 DETERMINATION OF KINETIC PARAMETERS AND SUBSTRATE SPECIFICITY

Substrate specificity analysis of protease was determined using gelatin, wheat gluten, casein and azocasein as substrates. The reaction mixture containing varying concentrations of substrates in 50 mM Tris-Cl pH 8.0 and 10 U/ml of purified enzyme was incubated for 30 min at 40 °C. The reaction was stopped by adding equal volume of 10 % (w/v) TCA and kept in ice for 15 min. After 15 min, centrifugation was done for 15 min at 12,500 x g. The absorbance of the supernatant was read at 280 nm. The specificity and rate of the enzymatic reaction rate was measured with
different substrates at varying concentrations ranging from 0.02 mg/ml to 3 mg/ml by Michaelis-Menten enzyme kinetics, under standard conditions. The Michaelis constant ($K_m$) and the maximum reaction velocity ($V_{max}$) were estimated by the Lineweaver-Burke plot.

2.16 DETERMINATION OF AUTOLYTIC ACTIVITY

To determine autolytic activity of the protease, the purified enzyme was incubated for 4 h at 40 °C. Then after incubation the samples were analysed using RP-HPLC analysis. Auto hydrolysis can be monitored by comparing chromatogram of pure protein before and after incubation under above mentioned conditions.

2.17 REUSABILITY AND STORAGE STABILITY OF IMMOBILIZED PROTEASE

To determine the storage stability of AU01 protease, the free and immobilized protease enzymes were stored at 4 °C and 25 °C for 15 days. Aliquots were withdrawn every day and residual activity was measured using azocasein as substrate. Activity of enzyme before incubation at respective temperature was taken as 100 %. To determine the operational stability of immobilized enzyme, 50 U of immobilized protease was assayed under standard assay conditions; enzyme was recovered by centrifugation at 5,000 xg for 10 min at 4 °C. Then the process was repeated 10 times to test enzyme reusability.
2.18 MALDI-TOF-MS OF PROTEASE

The protein bands were excised from Coomassie-stained SDS-PAGE gel and subjected to trypsin digestion. After digestion the peptides were eluted from the gel using a solvent mix containing 50% acetonitrile and 5% trifluoro acetic acid. Mass spectrometric analysis of the samples was carried out by mixing protein with the MALDI matrix (α-cyano-4-hydroxycinnaminic acid). MALDI-TOF spectra were acquired using BRUKER MALDI-TOF mass spectrometer. The mass spectrum was analysed using MASCOT program by searching against the NCBI nr database to identify matching peptides. Partial amino acid sequence of AU01 protease was identified and the homologous protein sequences were identified using BLASTp tool. The closely related proteins were obtained from BLASTp analysis. The predicted protein sequence was aligned with closely related Lysinibacillus sp., protease sequences using ClustalW. Motif searches were made using the PROSITE database (http://prosite.expasy.org).

2.19 APPLICATION OF AU01 PROTEASE IN MONOLAYER CELL CULTURE

Trypsin, a protease of animal origin, is widely employed in animal cell culture. This application deals with the possibility of replacing trypsin by the alkaline protease from Lysinibacillus fusiformis AU01 in the animal tissue culture for the separation of cells.

The MCF7 cancer cell line was grown and maintained in growth media consisting of high-glucose Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum in 6 well cell culture plates. After 24 hours of cell growth, the growth media was removed
aseptically. The adherent monolayer cells remain on the bottom surface of the plate. The plates were observed under microscope and then the plates were flooded with 2 ml of PBS containing 50 U of protease and incubated for 3 min at room temperature (37 °C). A batch of 5 ml fresh media was added to the plate. Then cells were flushed out and transferred into fresh tubes. The cells were centrifuged at 2000 x g for 5 min, re-suspended in 2 ml of fresh growth medium and used for sub-culture. For control experiments, cell detachment was monitored under microscope using PBS alone and also by standard trypsinisation procedure.