CHAPTER – III
MATERIALS AND METHODS

3.1. Materials:

3.1.1. Bacterial strains:
Different strains of LAB including *L. acidophilus*, *L. rhamnosus*, *P. acidilactici*, *L. corniformis*, *L. fermentum*, *L. brevis*, *L. plantarum* and *L. paracasei* were purchased from NCDC (National collection of Dairy Culture), National Dairy Research Institute (NDRI), Karnal, India.

3.1.2. Chemical reagents:
All the chemicals used were of highest possible quality and of analytical grade unless otherwise stated. Pro-4mβNA substrate was procured from Bachem Feinchemikalein AG, Bubendorf, Switzerland. CM-sephadex, Sephadex G-100 and Fast garnet GBC (o-aminoazotoluene diazonium sulphate) were purchased from Sigma Aldrich. Q-sepharose, Man Rogosa Sharpe (MRS) media, lysozyme, sodium dihydrogen phosphate, disodium hydrogen phosphate, sodium dodecyl sulphate (SDS), TritonX-100 (TX-100), ethanol, CoCl₂, CaCl₂, EDTA, NaCl, sodium acetate, Tris, cobaltous chloride, β-mecaptoethanol, acrylamide, N-N’methylene-bis-acrylmlde, ammonium per sulphate, BSA, sucrose, glucose, lactose, galactose and ammonium sulphate were purchased from Himedia Chemical Laboratory, Bombay. Glycerol, dimethyl sulphoxide, n-Butanol, Barium chloride, EDTA, zinc chloride, glacial acetic acid, ammonium chloride, HCl were purchased from Rankem. Coomassie brilliant blue R₂₅₀ was purchased from SRL. Bromophenol blue (BPB), Fast Garnet GBC, various synthetic substrates viz. H-Leu-Ala-βNA, H-Gly-Phe-βNA, Lys-Ala-βNA, H-Gly-Pro-Leu-βNA, Phe-Arg-βNA₂HCl, Leu-βNA, Gly-Arg-4mNAHCl, H-Asp-βNA, H-Gly-Phe-βNA, Phe-Arg-4mβNA, H-Trp-βNA, H-Gly-βNAHCl, L-Tyr-βNA, H-Ser-Tyr-βNA were from Sigma Aldrich. DTE, DTT, glutathione, N-ethylmaleimide (NEM), 5,5’-dithiobis (2-nitrobenzoic acid) (DTNB), iodoacetamide, leupeptin, bestatin, AEBSF, PMSF, 4-nitrophenyl iodoacetate, 4-chlorobenzoic acid, 1,10 phenanthroline, pepstatin were all procured from Sigma chemical Co., St. Louis, MO, USA. Marker proteins of 14.3-97.4 kDa range for molecular weight determination were obtained from Bangalore Genei, India.
3.1.3. **Instruments used:**

Laminar air flow was used for preparation of bacterial cultures. Incubator shaker from Gene™ was used for incubation of bacterial cultures. Refrigerated centrifuge of Dynamica, model number Velocity 18 R was used during experiments. Digital magnetic stirrer of Tarson spinot was used for mixing. Amicon ultrafiltration cell (Model 8200) with YM 10 membrane was used for concentration of samples. Centrifuge 5415 R of eppendorf was used during experimental steps. Dialysis membranes used were from Himedia. Amicon ultrafiltration cell (Model 8200) with YM 10 membrane was used when needed. Sonicator (UP2005, ultrasonic processor, Hielscher ultrasound technology) was employed for sonication. Spinix-vortex shaker from Tarson (cat. 3020) was used for vortexing. Eutech pH meter (Sr No. 76349) and Systronic digital pH meter (MK VI Sr. No. 5770) were used to adjust pH of solutions. All incubations were done in boiling water bath at respective temperatures. Pipetting was done with micro pipettes from Riviera and Borosil. Gel electrophoresis apparatus was purchased from Bangalore Genei. Absorbance at different wavelength was recorded at Digital double beam spectrophotometer 2202 from Systronics.

3.1.4. **Media:**

**MRS media:**

MRS media was prepared by dissolving 55.16 gm of MRS in one litre water. This was autoclaved at 121°C with 1 bar pressure for 20 min.

3.1.5. **Substrate solution:**

All substrates were dissolved in DMSO (4 mg/ml). Substrate of β-galactosidase o-nitrophenyl-β-D-galactopyranoside (ONPG) was freshly prepared in water (10 mM) because it undergoes autohydrolysis.

3.1.6. **Stopping reagent:**

One molar sodium acetate (pH 4.2) was used as stopping reagent for assay of proteolytic enzymes. For β-galactosidase, 1M Na₂CO₃ was used as stopping agent.

3.1.7. **Coupling reagent (GBC):**

Freshly prepared Fast Garnet GBC was used as coupling reagent (0.1g/100 ml) for assay of proteolytic enzymes.
3.1.8. KCN-Acetone ninhydrin solution:

KCN-Acetone ninhydrin solution was prepared by mixing 50 ml of 5% ninhydrin solution in acetone with 250 ml of KCN-acetone solution (prepared by diluting 5 ml of 0.01 M KCN in 60% ethanol to 250 ml with acetone).

3.2. Methodology:

3.2.1. Media and culture conditions:

All the bacterial strains purchased from NCDC, NDRI Karnal, were revived and maintained in MRS broth as per the protocol provided by NDRI. The strains were preserved as glycerol stocks. Glycerol stocks were prepared by mixing 0.5 ml bacterial culture with 0.5 ml of 50% glycerol and stored at -20°C for further use. The individual bacterial strain was sub cultured in MRS broth after 15-20 days to maintain the culture. Prior to experimental use, the strains were propagated twice in MRS broth at their corresponding incubation temperature.

3.2.2. Growth profile of *L. plantarum* and *P. acidilactici*:

MRS broth media was inoculated with overnight grown culture of *L. plantarum* and *P. acidilactici* and incubated at 37°C and 32°C respectively in an orbital shaker at 250 rpm. The cultures were grown till late-exponential growth phase. Growth profiles of both bacteria were obtained by recording absorbance of culture at 600 nm at regular time intervals for 2-3 days.

3.2.3. Assay for Proline iminopeptidase (PIP):

PIP activity was measured by using Pro-4-mβNA as substrate. Reaction mixture was prepared by adding 100 µl of enzyme to 880 µl of assay buffer (Tris-HCl pH 7.4) followed by incubation at 37°C for 10 min. Then reaction was started by adding Pro-4-mβNA (20 µl), 4mg/ml in DMSO and mixture was incubated at 37°C. After 20 min, the reaction was stopped by adding 1.0 ml of stopping reagent (1M Na-Acetate buffer, pH 4.2). The enzymatically released 4-methoxybetanaphthylamine (-4mβNA) was detected using a coupling reagent *i.e.* Fast Garnet GBC (1mg/ml in distilled water). The dark pink colour was extracted into 2.0 ml of n-butanol and was estimated at 520 nm. The enzyme activity was calculated in terms of nanomoles of 4mβNA released per min per ml enzyme solution by using the following formula:

\[
\text{Activity (nanomoles/min/ml)} = \frac{\text{OD}_{520} \times 10^9 \times 2.0 \times 10^{-3} \times 10}{\varepsilon \times t}
\]
\[ \varepsilon \rightarrow \] molar extinction coefficient of 4m\(\beta\)NA under assay condition.

\[ t \rightarrow \] is the reaction time in min

\[ 2.0 \times 10^{-3} \rightarrow \] is the volume of n-butanol in liters.

\[ 10 \rightarrow \] is multiplication factor for calculating enzyme activity as 0.1 ml enzyme was used for reaction

\[ 10^9 \rightarrow \] is used for converting moles into nanomoles.

One unit of enzyme activity is expressed as that amount of enzyme which releases one nanomole of 4-methoxy-\(\beta\)NA per minute from substrate under assay conditions.

### 3.2.4. Collection of biomass for PIP:

MRS broth was prepared by sterilization at 121\(^\circ\)C at 15 psi for 20 min. The broth was then inoculated with \textit{L. plantarum} and grown at 37\(^0\)C for 36 h in an orbital shaker at 250 rpm. Then the cells were harvested by centrifugation at 9000 rpm for 25 min at 4\(^\circ\)C and suspended in 50 mM sodium phosphate buffer pH 7.4 and sonicated for 1 min at 7 kHz frequency. Proline iminopeptidase (PIP) activity was screened in sonicated cell suspension.

### 3.2.5. \(\beta\)-galactosidase assay:

#### 3.2.5.1. Assay for bacterial cells:

Bacterial cell pellets were harvested, washed twice and suspended in the same volume of Z buffer (One litre contains: 16.1 g \(\text{Na}_2\text{HPO}_4\cdot7\text{H}_2\text{O}\) (0.06M), 5.5 g \(\text{NaH}_2\text{PO}_4\cdot\text{H}_2\text{O}\) (0.04M), 0.75 g \(\text{KCl}\) (0.01M), 0.246 g \(\text{MgSO}_4\cdot7\text{H}_2\text{O}\) (0.001M), 2.7 ml \(\beta\)-mercaptoethanol (0.05M), pH 7.0. Absorbance of cell suspension was measured at 600 nm against Z buffer. For each reaction mixture 0.1 ml cells were diluted to 1 ml with Z buffer. Diluted cells were permeabilized by adding 100 \(\mu\)l chloroform and 50 \(\mu\)l 0.1 % SDS. The tubes were vortexed for 30 sec and equilibrated for 5 min in water bath at 28\(^\circ\)C. Reaction was started by adding 0.2 ml of \(o\)-nitrophenyl-\(\beta\)-D-galactopyranoside (ONPG, 4mg/ml) substrate. The reaction mixture was vortexed and incubated at 28\(^\circ\)C for 10 min. The reaction was stopped by adding 0.5ml of 1M \(\text{Na}_2\text{CO}_3\) and contents were centrifuged to remove debris and chloroform. OD was recorded at 420 nm and 550 nm for each tube. Miller units for \(\beta\)-galactosidase were calculated using following formula.

\[
\text{Miller units} = \frac{1000 \times [(\text{OD}_{420} - 1.75 \times \text{OD}_{550})]}{(T \times V \times \text{OD}_{600})}
\]

Where \(\text{OD}_{420}\) and \(\text{OD}_{550}\) are read from the reaction mixture, \(\text{OD}_{600}\) is cell density in the washed cell suspension, T- reaction time (min), V- culture volume (ml) used in assay.
3.2.5.2. Assay for cell free extract:

β-galactosidase activity in *P. acidilactici* was determined according to Bhomik et al., 1990. Cell free extract (0.2 ml) was added to 1.6 ml Z buffer. The reaction was started by adding 0.2 ml 10 mM o-nitrophenyl-β-D-galactopyranoside (ONPG). Reaction mixture was incubated at 37°C for 10 min and the reaction was stopped by adding 1 ml of 1 M Na₂CO₃. The absorbance was read against a suitable blank at 420 nm. One unit of enzyme activity is defined as the amount of enzyme that liberated 1 µmole of ONP from the substrate per minute under assay conditions.

3.2.6. Collection of biomass for β-galactosidase:

*P. acidilactici* was grown in MRS medium for 24 h at 32ºC with shaking at 250 rpm. Then the cells were harvested by centrifugation at 9000 rpm for 25 min for the collection of biomass.

3.2.7. Screening of LAB for proline iminopeptidase activity:

Different strains *viz.* *L. acidophilus*, *L. rhamnosus*, *P. acidilactici*, *L. corniformis*, *L. fermentum*, *L. brevis*, *L. plantarum* and *L. paracasei* were grown in MRS media. Cells were harvested by centrifugation at 9300g at 4ºC for 25 min and resuspended in 50 mM sodium phosphate buffer pH 7.4 and sonicated for 1 min at 7 kHz frequency. Sonicated cell suspension of different LAB were assayed for proline iminopeptidase activity.

3.2.8. Extraction of PIP:

*L. plantarum* was selected for PIP studies. The bacterial strain was grown in MRS medium for 36 h at 37ºC with shaking at 250 rpm. Then cells were harvested by centrifugation at 9000 rpm for 25 min for collection of biomass. PIP was associated with the membranes of *L. plantarum*. The enzyme was extracted by method of Attri et al. (2012) with slight modifications. Washed cell pellets were harvested by centrifugation at 10,000 rpm at 4ºC for 10 min and resuspended in 50 mM sodium phosphate buffer, pH 7.4. Lysozyme (500,000 units/g wet weight) was added to cell suspension and stirred for 1 h at 37ºC and then centrifuged at 10,000 rpm at 4ºC for 25 min. The cell pellet was suspended in 50 mM sodium phosphate buffer, pH 7.4 containing 500 mM NaCl (standardized) and 1.5% Triton X-100 (sonication buffer) and subjected to sonication for 1 min at 7 KHz frequency while keeping in ice bath. This homogenate was centrifuged at 13,000 rpm for 60 min. Supernatant was collected. The
sonicate (pellet of the above step) was washed and resuspended in sonication buffer and immediately transferred to -20°C for 30 min and then homogenized at 37°C for 10 min. This step was repeated twice and the supernatant obtained after centrifugation (13,000 rpm for 30 min) was pooled with previously collected supernatant. The supernatant obtained was taken as crude enzyme solution. The above solution was dialyzed for 24 hours with 3-4 changes of the Na-Acetate Buffer pH 5.9, 0.1% Triton X-100.

3.2.9. Extraction of β-galactosidase:

*P. acidilactici* cells were incubated for 24 h and then harvested by centrifugation at 9000 rpm for 25 min. Intracellular β-galactosidase was extracted using different protocols:

3.2.9.1. **Sonication:** Cell suspension (in 50mM Na-Pohsphate buffer pH 7.0) was sonicated for 5 min in ice bath. The extract was centrifuged at 15000 g for 10 min according to Feliu et al. (1998) with slight modifications including standardization of time of sonication using 1 to 10 min of sonication period. The cell free extract was used to assay the enzyme activity.

3.2.9.2. **Lysozyme-EDTA treatment:** Lysozyme solution was prepared by dissolving 50 mg of lysozyme in 1.5 ml Tris EDTA buffer pH 8.0. Seventyfive microlitre of this was added per ml, incubated for 30 min at room temperature and then centrifuged at 15000 g for 10 min. β-galactosidase activity was checked in the supernatant.

3.2.9.3. **SDS-chloroform treatment:** One hundred microlitre of chloroform and 50 ul of 0.1% SDS were added to 10 ml of cell suspension and incubated for 30 min at room temperature while vortexing. Then it was centrifuged at 15000 g for 10 min and assayed for enzyme activity.

3.2.9.4. **Enzyme extraction by lysozyme:** Cell pellets were suspended in 5.0 ml of 0.05M Na-phosphate buffer (pH 6.8) followed by vigorous vortexing. Lysozyme (10 mg ml⁻¹) was added to it and incubated at 37°C for 15 min followed by addition of 0.5 ml of 4 M NaCl solution and again incubated at 37°C for another 50 min. It was centrifuged at 10,000xg for 15 min. Resultant supernatant was used for enzyme assay and protein determination.

3.2.10. Purification of enzymes:

After extraction, the enzymes were purified using successive chromatographic steps. Each enzyme solution was dialysed and concentrated prior to each chromatography step.
3.2.10.1. Dialysis of enzyme solution(s):

The dialysis membranes were boiled in 1% Na₂CO₃ solution for 1h and then in water. This process was repeated 2-3 times. These membranes were finally rinsed thoroughly with distilled water and stored at 4°C. The protein solution was dialyzed for 24 hours with 3-4 changes of the appropriate buffer.

3.2.10.2. Chromatographic columns:

3.2.10.2.1. CM-Sephadex C-50 (cation-exchange) column:

The cation exchanger slurry was activated by dipping 10 g of slurry in 1 litre water. It was then kept at room temperature for 48 h with intermittent extensive washing with distilled water. Then the slurry was incubated with 500 ml of 0.5 N NaOH for 1 h and then washed with distilled water till pH comes to 8.0. Then 500 ml of 0.5 N HCl was added to the slurry and left for 1 h. This step was repeated twice and as a result, gel shrunk significantly. Then the slurry was extensively washed with distilled water till pH comes to 5.0. This was equilibrated with 50 mM sodium acetate buffer (pH 5.9), containing 0.1% Triton X-100. Then the column (30 x 1.5 cm) was packed by pouring the slurry along the sides of column and washed with several volumes of equilibrating buffer (50 mM sodium acetate buffer, pH 5.9 and 0.1 % Triton X-100) at a flow rate of 1 ml/min.

3.2.10.2.2. Sephadex G-100 column:

For preparation of sephadex G-100 column, 50 g of Sephadex G-100 slurry was swollen in 500ml of 50 mM Tris-HCl buffer, pH 7.0, for 72 h. The slurry was decanted several times in order to remove the fines. It was deaerated during and after swelling then carefully poured in a glass column (30x1 cm) to avoid any air bubble. The outlet of column was closed and slurry was allowed to settle under gravity. Tris-HCl buffer 50 mM, pH 7.4 and 0.1% Triton X-100 was run through the column with flow rate of 0.5 ml/min. Finally the slurry was settled at a column length of 28 cm.

Determination of void volume and total volume of Sephadex G-100 column:

The uniformity of the packed gel was checked before separation of proteins. Void volume and total elution volume were also determined. This was done by loading the Sephadex G-100 column with 5.0 ml of 0.2% Blue Dextran-2000 and 2% Bromophenol blue and then column was run at a flow rate of 0.5 ml/min using same buffer as mobile phase. The blue dextran moved as a uniform sharp blue band through the column and revealed a symmetrical elution profile.
3.2.10.3. Concentration of protein:

Protein samples were concentrated at different stages of purification by using ultrafiltration Amicon stirred cell with YM 10 membrane. Membrane ultrafiltration is a pressure-modified, convective process that uses semipermeable membrane to separate proteins in aqueous solutions by molecular size, shape and/or charge. It allows proteins of molecular weight up to 10,000 Da to filter through, leaving behind the high molecular weight proteins. A compressed nitrogen pressure of 4 to 5 psi was used to operate the device and sample was concentrated up to few ml.

3.2.11. Estimation of Protein:

Proteins were quantified by Lowry’s method (1951) using bovine serum albumin (BSA) as standard using the following protocol.

Solution A: 2% sodium carbonate in 0.1 N NaOH.
Solution B: 1% CuSO$_4$ in water.
Solution C: 2% sodium potassium tartarate in water.
Solution D: One ml of solution B and 1 ml of solution C were mixed with 98 ml of solution A. This is prepared just before use.
Reagent E: 1N Folin’s reagent. Just before use, commercially available Folin-Ciocalteau’s reagent was diluted to 1N with water.

Standard BSA: Standard BSA (1 mg/ml) solution was prepared. Its absorbance at 280 nm was 0.667. In duplicate sets of tubes varying concentration of BSA was added and volume was made to 0.5 ml with distilled water. Then 3 ml of solution D was added. The tubes were incubated at 37°C for 15 min. Three hundred microlitres of reagent E was added to each tube and tubes were again incubated at 37°C for 30 min. The tubes were read at 670 nm and the standard curve was obtained by plotting amount of protein versus absorbance. The unknown protein samples were also assayed along with under the same conditions and values of proteins were calculated from the standard curve of BSA.

3.2.12. Electrophoresis:

Davis gel electrophoresis and in situ gel assay were used to check the homogeneity of purified enzymes.

3.2.12.1. Davis gel electrophoresis:

For Davis gel electrophoresis gels (12 %) were prepared as according to the method of Davis (1964). Glass plates were cleaned properly and rinsed with ethanol.
Gel casting assembly was set and the following components were pipetted together (Table 3.1).

Table 3.1 Components of Native-PAGE

<table>
<thead>
<tr>
<th>Components</th>
<th>Separating Gel (ml)</th>
<th>Stacking Gel (5%) (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide: bisacrylamide (30:8%)</td>
<td>4.070  4.884  6.105</td>
<td>0.75</td>
</tr>
<tr>
<td>H₂O</td>
<td>5.260  4.308  3.225</td>
<td>3.45</td>
</tr>
<tr>
<td>1.5 M Tris-HCl pH 8.8</td>
<td>3.125  3.125  3.125</td>
<td>-</td>
</tr>
<tr>
<td>0.5 M Tris-HCl pH 6.8</td>
<td>-       -       -</td>
<td>0.715</td>
</tr>
<tr>
<td>APS</td>
<td>0.190  0.190  0.190</td>
<td>0.020</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.010  0.010  0.010</td>
<td>0.004</td>
</tr>
<tr>
<td>Total</td>
<td>12.5  12.5  12.5  5.0</td>
<td></td>
</tr>
</tbody>
</table>

Gel solution (12 %) was poured in gel plates and overlaid with water-saturated isobutanol. Isobutanol was poured off after polymerization and top of the gel was carefully washed with water. Then stacking gel solution (5%) was poured in gel plates. The comb was fitted for the formation of loading wells, with precaution so that there were no air bubbles. After polymerization of stacking gel, the comb was removed and the wells were washed with the reservoir buffer. The gel was fitted in the electrophoresis chamber having reservoir buffer (25 mM Tris and 250 mM glycine, pH 8.3).

The purified sample (15 µl) was dissolved in 10 µl sample buffer (50 mM Tris.HCl, pH 6.8, 0.1% bromophenol blue and 10% glycerol) and loaded onto the gel. 10 µl of markers were also loaded and the gel was run at 50 mA at 4°C. When the marker dye reached the interphase of the separating and stacking gels, the current was increased to 100 mA. The gel was allowed to run till bromophenol blue dye reached the bottom of the gel. The gel was carefully removed from the plates and stained with staining solution (0.25% Coomassie Brilliant blue R-250 in methanol: water: acetic acid (45:45:10) for 3 h. The gel was then destained using destaining solution (methanol: water: acetic acid, 45:45:10).
Silver staining was also used for one enzyme (β-galactosidase). Protein bands were fixed by immersing in a mixture of 40% methanol and 13.5% formaldehyde for 15 min with instant shaking. Then the gel was washed twice with distilled water. After that gel was incubated with 0.1% silver nitrate solution for 20 min and then rinsed with distilled water. Then the gel was immersed in developer solution (3% sodium carbonate solution containing 0.5% formaldehyde and 0.02% Na₂S₂O₃) for 15 min or until properly stained. After that the reaction was stopped by transferring the gel in stopping solution (25% isopropanol solution containing 10% acetic acid) for 5 min. Then the gel was washed with distilled water and stored in water.

3.2.12.2. In-situ gel assay:

For in-situ gel assay, gel and samples were prepared as described in 3.2.9.1. Before loading the samples, the gel was pre-electrophoresed for 2 h. Gel was removed and cut into two halves. One half was stained with Coomassie Brilliant Blue while the other half was stained for enzyme activity. For activity staining, the gel was washed thoroughly with assay buffer followed by incubation with substrate at 37°C and then the gel was put in Fast Garnet GBC (1mg/ml) for 15 min for colour development. Gel was finally stored in water.

3.2.13. Molecular weight determination:

The molecular weight was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing and reducing conditions and also by matrix-assisted laser-desorption ionization time-of-flight (MALDI-TOF).

3.2.13.1. SDS-PAGE:

SDS-PAGE (12%) was run according to Laemmli et al. (1970). After cleaning the glass plates were rinsed with ethanol. Assembly was prepared and components of SDS-PAGE were pipetted together as given below.
Table 3.2 Components of SDS-PAGE

<table>
<thead>
<tr>
<th>Components</th>
<th>Separating Gel</th>
<th>Stacking Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(12%) (ml)</td>
<td>(15%) (ml)</td>
</tr>
<tr>
<td>Acrylamide: bisacrylamide</td>
<td>4.884</td>
<td>6.105</td>
</tr>
<tr>
<td>(30:8%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H₂O</td>
<td>4.308</td>
<td>3.225</td>
</tr>
<tr>
<td>1.5 M Tris-HCl pH 8.8</td>
<td>3.125</td>
<td>3.125</td>
</tr>
<tr>
<td>0.5 M Tris-HCl pH 6.8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10% (w/v) SDS</td>
<td>0.0625</td>
<td>0.0625</td>
</tr>
<tr>
<td>APS</td>
<td>0.19</td>
<td>0.19</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Total</td>
<td><strong>12.58</strong></td>
<td><strong>12.71</strong></td>
</tr>
</tbody>
</table>

Required components for the preparation of 12% gel solution were mixed and immediately poured in gel plates. The solution was overlaid with water-saturated isobutanol. After polymerization, isobutanol was poured off and the gel top was washed carefully with water. Stacking gel solution was prepared and poured in gel plates. The comb was fitted carefully. After polymerization of the stacking gel, the comb was removed and the wells were washed with the reservoir buffer. The gel was fitted in the electrophoresis chamber containing reservoir buffer (25 mM Tris, 250 mM glycine, 0.1% SDS, pH 8.3).

Purified protein (15 µl) was mixed with 10 µl sample buffer (50 mM Tris.HCl, pH 6.8, 2% SDS, 0.1% bromophenol blue, 2µl of β-ME and 10% glycerol) and heated at 100°C for 10 min, cooled to room temperature and then loaded on gel. Ten microlitres of the markers were also loaded and the gel was run at 50 mA. When the marker dye reached the interphase of stacking and separating gels, the current was increased to 100 mA. The gel was allowed to run till the bromophenol blue dye reached the bottom of the gel. The gel was carefully removed from the plates and stained with 0.25% Coomassie Brilliant blue R250 (methanol: water: acetic acid, 45:45:10) for 3 h following destaining with destaining solution (methanol: water: acetic acid, 45:45:10). The total length of gel and the distance to which each protein migrated was measured. The mobility and relative mobility were calculated as follows:
Mobility = \frac{\text{Distance travelled by protein X length of gel before staining}}{\text{Distance travelled by tracking dye X length of gel after staining}}

Relative mobility = \frac{\text{Mobility of a particular protein}}{\text{Mobility of the fastest moving protein}}

A curve was obtained by plotting the molecular weights of marker proteins phosphorylase B (97.2 KDa), serum albumin (66.4 KDa), ovalbumin (44.287 KDa) carbonic anhydrase (29 KDa), β-lactoglobulin (20 KDa) and lysozyme (14 KDa) along the semilogarithmic coordinate versus their respective R_m. R_m for PIP and β-galactosidase was calculated and their molecular weights were calculated from graph.

3.2.13.2. MALDI-TOF:

Mass spectrometry is used for mass measurement by producing charged molecular species in vacuum and their separation by magnetic and electric fields based on mass/charge ratio (m/z). The purified protein sample was mixed with sinapinic acid (SA) matrix in 1:1 ratio and 2µl was spotted onto the MALDI plate. Sample was air dried before analyzing on AB SCIEX 5800 MALDI TOF/TOF instrument and further analysis was done with DATA EXPLORER SOFTWARE for obtaining the intact mass.

3.2.14. Peptide mass fingerprinting:

3.2.14.1. Trypsin digestion:

Trypsin digestion was performed in gel slice. Band of purified enzyme on gel was diced into small pieces and destained up to four times at 10 min interval until the gel pieces became translucent white. Gel pieces were dehydrated using acetonitrile and Speedvac till they dried completely followed by rehydration with DTT for one hour. Gel pieces were then incubated with iodoacetamide for 45 min. The gel was incubated with ammonium bicarbonate solution for 10 min. Then the supernatant was removed and gel was dehydrated with acetonitrile for 10 min and Speedvac till complete dryness. Samples were digested overnight with MALDI grade trypsin at 37ºC and the digested sample was transferred to fresh eppendorff tubes. The gel pieces were extracted thrice with extraction buffer and the supernatant was collected each time and then Speedvac till complete dryness. The dried peptide mixture was suspended in TA buffer (30% acetonitrile containing 0.1% trifluoroacetic acid).
3.2.14.2. Fragments analysis:

The digested sample was mixed with equal volume of a matrix solution (α-cyano-4-hydroxy cinnamic acid (HCCA) (10 mg/ml) in 70% acetonitrile and 0.03% trifluoric acid). The mixture was dried at room temperature. Peptide mass spectra was obtained using MALDI-TOF/TOF mass spectrometer (Bruker Ultraflex III TOF/TOF) after calibration with peptide mixture for the m/z range of 700-4500 Da by using the peptide peak of bradykinin (757.39 Da), angiotensin-II (1046.54 Da), angiotensin-I (1296.68 Da), substance P (1347.73 Da), bombesin (1619.82 Da), ACTH fragment 1-17 (2093.08 Da), ACTH fragment 18-39 (2465.19 Da), somatostatin 28 (3147.47 Da). Sample molecules were ionized, extracted into analyzer region of mass spectrometer and were separated according to their m/z ratio. After sample detection, signals were stored in terms of relative abundance as function of m/z ratio. All mass spectra obtained in the reflector mode were calibrated using several matrix ion peaks as internal standards. The mass peak was taken as input in MASCOT using different parameters \textit{viz.} (a) fixed modifications-carbamidomethyl (C), (b) variable modifications-oxidation (M), (c) cleavage by trypsin. The generated peptide mass list was used for the online data base search to find and match the protein identity. The search “MASCOT server” (www.matrixscience.com) was used to obtain the protein identity by undertaking the Peptide Mass Fingerprinting approach. A search was performed in NCBI, MSDB and SwissProt database by following search parameter: Mass Tolerance-50 to 100 ppm, maximum number of missed cleavages was set to 1 for all samples.

3.2.14.3. Sequence comparison:

BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and Clustal W were used for Sequence alignment of tryptic fragments of purified protein and homology search. Sequences were aligned using Clustal W (http://www.ebi.ac.uk/Tools/msn/clustalw2). The complete sequence alignment was also generated using Clustal W. Sequence database was searched with BLAST which performs pairwise alignment between query sequence and target sequence in each database.

3.2.15. Physicochemical Characterization:

3.2.15.1. pH optimum and pH stability:

pH optima was determined in pH range of 4-10.5 by assaying enzymes in assay buffers of different pH \textit{viz.} 50 mM sodium acetate buffer (pH 4-5.5), sodium phosphate buffer (pH 6-6.5), Tris-HCl buffer (pH 7-8.5) and glycine NaOH buffer (pH 9.5-10.5).
To determine pH stability enzymes were incubated with buffers in pH range 4-9.5 at 37°C for 10 min and then assayed at optimum pH. Percent activity was calculated with respect to maximum activity. All the assays were performed in triplicate and mean value was reported.

3.2.15.2. Temperature optimum and temperature stability:

To determine temperature optima, β-galactosidase and PIP were assayed at different temperatures: 0°, 5°, 10°, 20°, 30°, 37°, 45°, 50°, 55°, 65° and 75°C along with a control at each temperature. Temperature stability was determined by incubating the enzymes at different temperatures (0-70°C) for 10 min and then assaying at optimum temperature. The assays were performed in triplicate and mean value was reported.

3.2.16. Kinetic Characterization:

Enzymes β-galactosidase and PIP were assayed with different concentrations of their substrates ONPG and Pro-4mβNA respectively and $K_m$ and $V_{max}$ were calculated from Michaelis Menton Plot, Line Weaver plot and Hanes Plot. Specificity constant i.e., $K_{cat}/K_m$ were also calculated.

3.2.17. Effect of enzyme inhibitors and thiol compounds:

Effect of different inhibitors, PMSF, NEM, Iodoacetic acid, 1,4-Dithioerythritol, PCMB, AEBSF, 4-Nitrophenyl iodoacetate, Puromycin, EDTA, DEPC and thiol compounds including Cysteine, Glutathione, β-ME and DTT on enzyme activity was studied by preincubating the enzymes with different chemical reagents for 10 min at 37°C. Then assays were performed at optimum temperatures. All the assays were performed in triplicate and mean value was reported.

3.2.18. Effect of metal ions:

Effect of chloride salts of different metal ions i.e., FeCl$_3$, NaCl, KCl, HgCl$_2$, ZnCl$_2$, CuCl$_2$, MnCl$_2$, LiCl, CaCl$_2$, and CoCl$_2$ on activity of purified enzymes was studied by preincubating the enzymes with different dilutions of freshly prepared metal ion solutions for 10 min. Then the assays were performed. Enzyme activity without added metal salt was used as control. All the assays were performed in triplicate and mean value was reported.

3.2.19. In-silico characterization of purified enzymes:

3.2.19.1. Comparative modeling:

LOMETS (LOcal MEta-Threading-Server) is a locally installed meta-server method for protein structure prediction. It generates predicted protein structure by
ranking and selecting models from 8 state-of-the-art threading programs. Spatial restraints are combined from the consensus of top 20 threading alignments (Nei and Kumar 2000). For a given target, 200 models are generated by 10 component servers where each server generates 20 models as sorted by their Z-scores in each algorithm. Top 10 models were finally selected on the basis of Coverage, Z value, sequence identity and confidence scores (Wu and Zhang 2007). The "Confidence Score" column indicates the confidence of the predicted template which is based on a scoring function that takes into account the Z-score of the template, the confidence of the particular server and the sequence identity between the query and the template.

The modeled protein was validated by PROCHECK (Laskoswki et al. 1993), PSVS (Eisenberg et al. 1997), ERRAT (Colovos et al. 1993), PROVE (Pontius et al, 1996) and SAVES (the Structure Analysis and Verification Server) (http://nihserver.mbi.ucla.edu). For validation, Ramachandran plot was drawn by PROCHECK and PSVS. Phylogenetic analysis was done by using Maximum likelihood method based on JTT matrix based model (Nei and Kumar 2000).

3.3. Application studies of PIP:
3.3.1. Collagen degradation:

Collagen degradation by purified PIP was studied according to Ding et al. (2014). The reaction mixture was prepared by mixing 50 mg collagen from bovine achilles tendon (Sigma), Purified PIP (10 U ml\(^{-1}\)) and/or neutral protease (NP) (50 mg, 100 U) in 50 mM Tris-HCl buffer of pH 7.5 and the final volume was made 50 ml and reaction mixture was agitated at 37°C. After 1 h, reaction was terminated by heating in water bath for boiling 20 min. The supernatant was collected by centrifugation at 10,000xg, 4°C for 10 min (Table 3.3). Hydrolytic activity of PIP for collagen was studied in combination with other enzymes also. So enzyme cocktail of DPP-II, purified from another LAB *Pediococcus acidilactici* (Dimpi 2015), neutral endoprotease and PIP was used to hydrolyse collagen. Amino acids in the supernatant were determined by ninhydrin test, TLC, and HPLC. HPLC was done from CDFD, Hyderabad.

**Table 3.3 Experimental design for enzymatic hydrolysis of collagen**

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Enzyme</th>
<th>Temperature</th>
<th>Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td></td>
<td>Collagen dissolved in 50 mM Tris HCl, pH 7.5</td>
</tr>
<tr>
<td>2</td>
<td>Neutral protease (NP)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>NP</td>
<td>37°C for 1 h</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>PIP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>NP+PIP+DPP-II</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.3.1.1. Qualitative Ninhydrin Test:

Few (2-5) drops of ninhydrin solution (0.2% in acetone) was added to 1 ml of test solution (supernatant). Then the reaction mixture was mixed thoroughly and kept in boiling water bath for 5 min for colour development. Amino acids give pink, purple or violet blue colour and imino acids like proline and hydroxyproline give a yellow colored complex.

3.3.1.2. Quantitative Determination of free amino acid:

To 2 ml of test solution, 2 ml of 0.2 M acetate buffer (pH 5.0) was added. A blank was run with 2 ml of buffer in place of test sample. One ml of KCN-acetone ninhydrin reagent was added to each tube and mixed thoroughly. The tubes were kept in boiling water bath for 20 min and then cooled under running tap water. Volume was made 10 ml with distilled water. Absorbance was recorded at 570 nm against blank. Free amino acids were quantified in reference to standard curve of glycine.

3.3.1.3. Thin layer chromatography:

Thin layer chromatography was used to visualize products of collagen degradation. Silica gel plates (5 X 20 cm) were prepared by dissolving silica gel in water. Plates were air dried and activated at 100°C for 30 min before use. Standard proline solution (1mg/ml) was prepared in 10% isopropanol and spotted on TLC plate along with sample(s) to be analysed. Plates were air dried and subjected to TLC using butanol: acetic acid: water (4:1:5) as mobile phase. After completing TLC, the plates were dried and sprayed with 0.25% ninhydrin (0.25g in acetone). The plates were then heated at 110°C for 10 min in oven for color development.

3.3.1.4. HPLC Analysis:

Sample preparation for amino acid analysis experiment: To one ml sample 4 ml methanol was added. It was vortexed, incubated at -20°C for overnight, centrifuged and supernatant was collected. Supernatant was subjected to nitrogen flow for 1 h at 60°C. Dried sample was kept in thermomixer for 1 h at 45 °C after adding coupling reagent (10 µl phenyl isothiocyanate, 70 µl methanol, 10 µl triethyl amine and 10 µl filtered MQ). To this sample 200 µl of buffer A (10 mM sodium acetate adjusted to pH 6.4 with 6% acetic acid) was added and 20 µl of this was used as injection volume.

Gradient system: Agilent TC-C18 column of dimensions 4.6×250 mm, 5-Micron was used with instrument Agilent 1100 series. Buffer B (10 mM sodium acetate and 60% acetonitrile adjusted to pH 6.4 with 6% acetic acid) was used for analysis with flow rate of 1ml/ml and absorbance was recorded at 254 nm.
3.3.2. Meat tenderization by PIP:

Chicken meat from the leg part was obtained after 1 day of slaughter from a local slaughter house and stored at -20°C. It was tempered overnight at 3-4°C and cut into small pieces (3x3x2 cm). Every piece of meat was dehydrated using sucrose after covering the chicken pieces with semipermeable membrane at 4°C for 18 h. After dehydration each piece of meat was treated with 2 volumes (w/w) of Neutral protease, Purified PIP, a mixture of Purified PIP and neutral protease (in 50mM Tris-HCl, pH 7.4), sonicated cells of *L. plantarum*, a mixture of sonicated cells and neutral protease and a mixture of neutral protease, purified PIP and purified DPP-II from *P. acidilactici*. An untreated sample (control) was dipped in deionized water after osmotic dehydration (Gerelt et al. 2000). After enzyme treatment each sample was stored for 24 h at 4°C and then subjected to microscopic analysis and SDS PAGE after preparing myofibrils. Each experiment of meat tenderization was repeated three times.

3.3.2.1. Preparation of Myofibrils:

Myofibrils were made according to Busch et al. (1972) with slight modifications. Muscles were grounded (for 15 s) and suspended in 6 volumes (w/v) of 50 mM Tris-HCl buffer (pH 7.6) containing 100 mM KCl and 5 mM EDTA by using a blender. The myofibrils were sedimented at 1000 g for 10 min and resuspended in 5 volumes of same buffer and grounded using a blender (for 10 s). The resuspended myofibrils were again sedimented at 1000 g for 10 min, and this process of resuspension sedimentation was repeated for five times. After fifth wash, the myofibrils were suspended in the same buffer and passed through a 20 mesh nylon net to remove connective tissue. The strained myofibrils were sedimented at 1000 g for 10 min and washed thrice with 1M KCl and finally suspended in 100 mM KCl and 1 mM NaN₃.

3.3.2.2. SDS PAGE analysis:

Myofibrils were solubilised in 0.01M sodium phosphate buffer (pH 7.0) containing 5% SDS and 1% 2-mercaptoethanol and boiled in boiling water for 2 min followed by centrifugation at 10,000 g for 15 min. The clear supernatant was analyzed by SDS-PAGE (7.5%) according to Laemmli (1970) with slight modifications. The electrophoresis was carried out on 7.5% polyacrylamide (bisacrylamide/acrylamide, 30:8% (w/w)) slab gel containing 10% SDS. Then the gel was removed and stained with 0.25% Coomassie Brilliant blue R250 (methanol: water: acetic acid, 45:45:10) for 3 h following destaining with destaining solution (methanol: water: acetic acid, 45:45:10).
3.3.2.3. Microscopic analysis:

Microscopic analysis of the treated samples was done. Traditional method of slide preparation for collagen was used by simply putting the collagen fibres on glass slide and covering it with cover slip. The slides were observed at 40X resolution of light microscope.

3.4. Application studies of β-galactosidase

3.4.1. Milk Lactose hydrolysis by β-galactosidase:

Milk lactose hydrolysis was studied according to Dwevedi et al. (2009) with slight modifications. Ten percent defatted milk powder was dissolved in distilled water (to avoid the turbidity of the solution for uninterrupted spectroscopic analysis). Soluble enzyme (3×10⁻¹ U) was added to 5 ml of reaction mixture was incubated at 50°C. Aliquots of 20 µl were withdrawn at regular intervals of time. Glucose oxidase peroxidase were used to estimate the released glucose content. The percentage of unhydrolyzed lactose was calculated as

\[
\% \text{ Lactose unhydrolyzed} = \frac{\text{Glucose present before } \beta \text{-galactosidase treatment}}{\text{Glucose present after } \beta \text{-galactosidase treatment}}
\]

The rate constant of lactose hydrolysis was determined from the slope of plot between log % unhydrolyzed lactose versus time using the formula:

\[
\text{Slope} = -\frac{k}{2.303} \quad \text{whereas rate constant } k = \frac{2.303}{t} \log \frac{100}{100-x}
\]

Where ‘x’ is the unhydrolyzed lactose. Therefore, time required for the hydrolysis of 50 % lactose could be calculated as \( t_{1/2} = \frac{0.693}{k} \)

3.4.2. Synthesis of galactooligosaccharides (GOS):

GOS synthesis was studied using purified β-galactosidase enzyme as well as sonicated cells of \( P. \ acidilactici \). Reaction mixture was made by mixing 30% of lactose solution in sodium phosphate buffer, pH 7.0 with 2.5 units of enzyme (both purified β-galactosidase and sonicated \( P. \ acidilactici \) cells). Then the mixture was incubated at 48°C for 4 h in orbital shaker (150 rpm). Aliquots of 100 µl were collected after 2 h, boiled at 100°C to stop the reaction, centrifuged at 5000 rpm and analysed by TLC.

Reaction conditions for maximum GOS synthesis by sonicated cells of \( P. \ acidilactici \) were optimized in a glass vial (20ml) using response surface methodology.
(RSM). Lactose (10-50% w/v) was used as substrate and sonicated \( P.\ acidilactici \) cells (600 µl corresponding to 5 units of \( \beta \)-galactosidase enzyme) were used as source of \( \beta \)-galactosidase. Reactions were carried out at pH 7.0 in orbital shaker (150 rpm). Temperature (20-60°C), enzyme unit (0.05-5 units) and time of incubation (1-24 h) of reaction were also optimized. Aliquots of 100 µl were collected at regular interval of 2 h. Reaction was stopped by boiling the samples at 100°C for 5 min and samples were then centrifuged at 5000 rpm. The samples were qualitatively analysed by TLC and quantitatively by high performance liquid chromatography (HPLC).

### 3.4.2.1. Thin layer chromatography (TLC):

TLC was carried out using silica gel plates (5×20 cm) using n-butanol:methanol:water (70:20:10 v/v/v) as a mobile phase. 1 µl of the test sample was spotted and dried on TLC plates along with standard glucose, galactose and lactose. After completing the run, plates were dried, sprayed with 35% \( H_2SO_4 \) in ethanol and dried in oven at 100°C for 5 min to visualise the compounds.

### 3.5. Lactic acid production by \( L.\ plantarum \) and \( P.\ acidilactici \) using whey and industrial wastes as substrates:

#### 3.5.1. Preparation of starter cultures:

The bacterial cultures were grown in 50 ml of MRS medium in 250 ml flask. After sterilization, the medium was inoculated with a loopful of cells from agar slant and incubated at 37°C and 150 rpm for 24 h.

#### 3.5.2. Liquid State Fermentation:

Liquid state fermentation was carried out using whey and sugarcane juice as a substrate. Whey was purchased from a dairy in Kurukshetra. Whey clarification was carried out by protein precipitation by heating the whey at 90°C for 20 min to precipitate proteins. Precipitated proteins were removed by centrifugation at 4,000 rpm for 20 min and supernatant (whey) was supplemented with yeast extract (0.5%), MnSO_4 (50 mg/l), and CaCO_3 (0.5%). This whey was sterilized at 121°C for 20 min and used as fermentation medium for the production of lactic acid using \( L.\ plantarum \) and \( P.\ acidilactici \). All the fermentation conditions were optimized using whey as substrate. Yeast extract was not added while optimizing yeast extract concentration Sugarcane juice was purchased from local market and was used as substrate in the same way as described for whey.
3.5.3. Solid State fermentation (SSF):

SSF was carried out using industrial wastes i.e., sugarcane bagasse (SB), apple pomace (AP), and grape pomace (GP) as substrates. These wastes were obtained from the juice corners of local market. Substrates for SSF were oven dried and grounded to powder form. Powdered substrates (12.5 g) were moistened with 50 ml of 4X sucrose medium (sucrose (31g/100ml), MnSO₄ (50mg/l), CaCO₃ (0.5%), CuSO₄ (40mg/l), NH₄NO₃ (1%) and (0.5%) yeast extract) and autoclaved. This was used as fermentation medium by inoculating with *P. acidilactici* and *L. plantarum*.

3.5.4. Fermentation:

Fermentation was carried out in flasks, in comparison to control in which medium composition was kept same but not inoculated with strains. Fermentation medium was inoculated and incubated. The medium was centrifuged at 3000 rpm for 30 min to pellet out bacterial cells and the supernatant was used for lactic acid estimation. In case of SSF, after incubation, the lactic acid was extracted with 25 ml of water at 40°C for 1 h and supernatant was collected after centrifugation at 3000 rpm for 30 min and used for estimation of lactic acid.

3.5.5. Lactic acid Estimation:

Lactic acid produced by fermentation was determined using titration method. 25ml of culture broth of LAB isolates was transferred into 100ml flask. One ml of phenolphthalein indicator (0.5% in 0.5% alcohol) was added to this and titrated against 0.1 N NaOH. Lactic acid concentration (g/l) was calculated according to Fortina et al. (1973) with slight modifications using the following formula:

\[
\text{Lactic acid (g/l)} = \frac{X \times N \times 90.08}{Y}
\]

X = volume (ml) of NaOH used  
N = Normality of NaOH used  
90.08 = Molecular weight of lactic acid  
Y = Volume of titrant used

3.5.6. Experimental Design and Statistical Analysis:

RSM (Response Surface Methodology) was used to analyze and validate the conditions for lactic acid production from whey using *L. plantarum* and *P. acidilactici*.
A central composite design was employed with four variables viz. temperature, pH, time and yeast extract concentration. The minimum and maximum values were set for all the variables. RSM provided 30 runs for lactic acid production. All the experiments carried out orderly and analyzed the best condition for lactic acid production. Analysis of variance (ANOVA) was applied to validate the model.

3.5.7. Optimization of different parameters using whey as substrate:

Different process parameters such as pH (5.0-6.5), temperature (32-42°C), yeast extract (0-10 gdm⁻³) and inoculum size (1-5% v/v), were varied over a range during fermentation to optimize these parameters for maximal lactic acid production.