Chapter-III

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3.1 COLLECTION OF SAMPLES FOR THE ISOLATION OF LACTIC ACID BACTERIA (LAB)

Four different varieties of samples (soil and dairy effluent) were collected in a sterilized container from milk processing fields of Gulbarga, Karnataka, India. All the samples were brought to the laboratory under aseptic conditions and were processed for isolation by serial dilution technique. To isolate LAB, 100 µl of aliquot from each diluted samples were inoculated on lactose agar media composed of (g/ml): lactose 0.5 %, peptone 0.5 %, beef extract 0.3 %, and agar 1.5 % adjusted at pH 6.5 ± 0.2; incubated at 37°C for 48 h and the growths of bacterial colonies were observed at every 24 h.

3.2 MORPHOLOGICAL STUDY OF LAB ISOLATES

The bacterial white colonies developed on lactose agar medium from different sources were examined for morphological characteristics such as colony characterization, gram staining, motility and sporulation as prescribed by Bergey’s manual of systematic bacteriology (Holt et al., 1994). The selected prominent white colonies of LAB were sub-cultured and maintained on deMan Ragosa Sharpe (MRS) agar at 4°C for further studies (Ragosa and Sharpe, 1960).

3.3 SCREENING OF LAB FOR LACTASE PRODUCTION

The cultivation of isolated strains and for screening of LAB producing lactase was examined by using MRS agar medium containing 1.5 % of lactose (as an additional carbon source) infused with 40 µl of X-Gal (5-bromo.4-chloro.3-indolyl-β-D-galactospyranoside); is an inert chromogenic substrate for lactase that promotes lactose utilization. Lactase hydrolyzes X-Gal into colorless galactose and 4-chloro-3-
bromindigo which forms intense blue precipitate that develop blue colony screening. Further, the screened isolates were subjected for quantitative estimation.

3.4 QUANTITATIVE ANALYSIS OF LAB FOR LACTASE PRODUCTION UNDER SUBMERGED FERMENTATION

To detect the potent lactase producer, one loopful inoculum of each primarily screened LAB isolates were inoculated in 250 ml Erlenmeyer flask containing 100 ml of modified MRS medium composed of (g/ml): Protease peptone (1 %), Beef extract (1 %), Yeast extract (0.55 %), Lactose (2 %), Tri-ammonium citrate (0.2 %), Sodium acetate (0.5 %), K$_2$HPO$_4$ (0.2 %), MgSO$_4$.7H$_2$O (0.01 %), MnSO$_4$.4H$_2$O (0.005 %) and Tween 80 (0.1 ml); adjusted at pH of 6.5 ± 0.2 and fermented for 24 h at 37°C in shaker incubator at 100 rpm. Afterwards, biomass were collected and processed for enzyme extraction.

3.5 EXTRACTION OF CRUDE ENZYME BY CELL-DISRUPTION METHOD

The cells were harvested after 24 h of incubation by centrifugation at 10000 rpm for 10 min at 4°C. The supernatant discarded wherein the cell pellet was crushed and washed with 4.5 ml of 0.1M Sodium phosphate buffer (pH 6.5) followed by vigorous vortex tube centrifuged (GYROZEN, 1580R) at 10000 rpm for 10 min at 4°C. Finally the pellet was suspended in 5 ml of 0.1M Sodium phosphate buffer (pH 6.5) containing intracellular enzyme were stored in the refrigerator for extraction by using lysozyme-EDTA treatment.

3.5.1 Stock Solution of Lysozyme-EDTA

Dissolve 0.33g of lysozyme in 10 ml of TE buffer containing 1mM EDTA and 10mM Tris-HCl, adjusted to pH 8.0.
3.5.2 Procedure

To the 100 ml of cell suspension 10 ml of lysozyme solution was added and incubated for 30 min at room temperature. It was centrifuged at 8000 rpm for 10 min and cell free supernatant (crude enzyme) was kept at 4°C until needed for enzyme assay, protein estimation and purification.

3.6 ENZYME ASSAY

Assay for the activity of lactase was performed as per the method described by Miller (1998) using lactose analog oNPG (o-nitrophenyl-β-D-galactopyranoside) as a chromogenic substrate. The reaction mixture was composed of 0.5 ml of crude enzyme and 2.5 ml of substrate solution containing 4 mg/ml of oNPG in 0.01M Sodium phosphate buffer (pH 6.4). After incubation for 15 min at 55°C, 1 ml of 1M Sodium carbonate (Na₂CO₃) was added to the mixture to stop the reaction. If excess oNPG is added, the amount of o-nitrophenol (oNP) produced is proportional to the amount of lactase and time of the reaction that produces a yellow color as shown in Figure 6. The concentration of oNP was measured at 420 nm in UV-Visible Spectrophotometer (SYSTRONICS, AU-2700) and the enzyme activity was calculated by using standard oNP calibration curve.

![Figure 6: Represents mechanism of enzyme substrate reaction (Source: http://www.wikipedia.com/onpg/substrate reaction)](image-url)
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3.6.1 Construction of Standard o-nitrophenol (oNP) Curve

3.6.1.1 Standard oNP solution: Dissolve 1.3910 mg of oNP in 100 ml of distilled water to get a final concentration of 1µmole of oNP per ml per min.

3.6.1.2 Sodium carbonate solution: 10.59 g of 1M Na₂CO₃ dissolved in 100 ml of distilled water.

3.6.1.3 Procedure

During the assay, free oNP at different dilutions level was used to construct a standard graph. The known amounts of standard oNP solution with several dilutions were prepared (Table 6, see in chapter 4). Each dilution gave a specific yellow color under the assay conditions with absorption peak at 420 nm. The absorbance value of each aliquot was plotted against oNP concentration to construct a standard calibration curve.

3.6.2 Enzyme Activity

The lactase activity was determined by measuring the amount of oNP released from oNPG substrate under assay conditions as discussed above. The unit of enzyme activity was calculated by using the following equation:

\[
\text{Enzyme Activity (IU)} = \frac{\text{Concentration of oNP released}}{\text{Reaction time} \times \text{Volume of enzyme} \times \text{Total volume}}
\]

One unit of lactase activity (IU) was defined as the amount of enzyme that liberates 1µmole of oNP per ml per min under assay conditions.
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3.7 BIOCHEMICAL AND PHYSIOLOGICAL PROPERTIES OF ISOLATE KLSA22

The potential isolate KLSA22 was scrutinized for biochemical and physiological properties as per the method prescribed in Bergey’s manual of systematic bacteriology (Holt et al., 1999). The biochemical properties such as catalase test, starch hydrolysis and nitrate reduction tests were examined. Under physiological properties, the test isolate was also examined for the utilization of different sugars viz., arabinose, glucose, lactose, maltose, sorbitol, sucrose and xylose. Further, the isolate KLSA22 was subjected to molecular identification.

3.8 IDENTIFICATION OF LACTOBACILLUS SP. KLSA22

The molecular study of the isolate signifies the systematic position of the bacterial strain confirmation. The methods of characterization briefly described as follows:

3.8.1 Isolation of Genomic DNA

The genomic DNA extraction of isolate KLSA22 was performed by the method as described by Hosek et al., (2006). The predominantly genomic DNA of the bacterial cells is surrounded by the cell membrane along with cell wall to be disrupted so that DNA is released into the extraction buffer.

3.8.1.1 Reagents requirement for the extraction buffer

3.8.1.1.1 Lysis Buffer

It is prepared by adding 10 mM tris HCl (pH 8) with 5 mM EDTA and 5 % SDS. This detergent is a necessary cofactor for the most nucleases to chilated magnesium ion.
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3.8.1.1.2 Saturated Phenol

The phenol crystals are liquefied and heated at 65°C. It is mixed with equal proportion of Lysis buffer. The phenol removes protein by denaturation and precipitation that allows the solvent to separate and resultant forms two layers in separating funnel. Thus saturated phenol formed at the lower layer collected carefully.

3.8.1.1.3 Chloroform : Isoamylalcohol

The isoamylalcohol acts as an antifoaming agent in a solution. It is prepared by mixing with chloroform in 24:1 ratio volume.

3.8.2 Protocol for DNA extraction

Take 1.5 ml of bacterial culture KLSA22; centrifuge it for 10,000 rpm for 5 min at 4°C. Discard the supernatant; suspend the pellet in 250 μl of lysis buffer. Heat the tube at 60°C for 20 min. Add equal volume of saturated phenol, centrifuge it for 10,000 rpm for 5 min at 4°C. Transfer the supernatant into fresh tube; add equal volume of chloroform and isoamylalcohol in 24:1 ratio with 100 μl of 3M sodium acetate. Centrifuge at 10,000 rpm for 10 min at 4°C. Transfer this supernatant in fresh tube and add equal volume of chilled ethanol. Incubate for 10 min at 37°C and re-centrifuge at 10,000 rpm for 10 min at 4°C. Finally, the DNA obtained in pellet was stored in TE buffer.

3.8.3 PCR Amplification

In an automated thermo-cycler extremely purified DNA was amplified by PCR using 16S forward primer (5’- AGAGTTTGATCHYGGYTYAG-3’) and 16S reverse primer (5’-ACGGCTACCTTGTTACGACTT-3’) as a template DNA. The PCR mixture (25μl) contained 16.25 μl of distilled water, 2.5 μl of TAE buffer, 4 μl of dNTP, 1 μl of each primer, 1 μl of template DNA, and 0.25 μl of Taq DNA polymerase. The PCR
cycling condition involves an initial denaturation at 94°C for 5 min (1 cycle), primer annealing at 58°C for 40 sec (35 cycles), extension at 68°C for 1 min, last denaturation at 94°C for 1 min and final extension was allowed at 68°C for 10 min. Finally, the amplified PCR product was obtained and it was subjected to electrophoresis using 1.2 % agarose gel in TAE buffer and visualized by staining with ethidium bromide. The PCR product was purified by washing with 2 % sodium acetate and 70 % of ethanol and eluted from the gel. Forward and reverse sequence reactions of PCR amplicon were carried out on the sequencer (ABI3730XL) to obtain the 16S rRNA gene sequence.

3.8.4 Evolutionary Analysis

The amplified PCR product containing 16S rRNA gene was determined for taxonomic position of the potential isolate KLSA22 by using the BLASTn program to identify the most similar sequences were downloaded from GenBank database (http://www.ncbi.nlm.nih.gov/entrez) (Altschul et al., 1990). The phylogenetic tree was constructed to illustrate the evolutionary relationship with neighbour-joining method using ClustalW algorithm in MEGA software version 4.1 (Tamura et al., 2007).

3.9 EVALUATION OF BIOPROCESS FOR THE PRODUCTION OF LACTASE

To evaluate the efficiency of substrate lactose for the production of lactase was determined through SmF by using modified MRS medium containing: Protease peptone (1 %), Beef extract (1 %), Yeast extract (0.5 %), Tri-ammonium citrate (0.2 %), Sodium acetate (0.5 %), K₂HPO₄ (0.2 %), MgSO₄·7H₂O (0.01 %), MnSO₄·4H₂O (0.005 %) with 2 % of synthetic pure lactose (2 g) or cheese whey (lactose content 2 ml) was used as a rich source of carbon substrate, at pH of 6.5 ± 0.2 adjusted, and then 0.1 ml of Tween 80 was added to 250 ml Erlenmeyer flask containing 100 ml of production medium. This medium was autoclaved at 121°C for 15 min afterward, 1 % loopful
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inoculum of *Lactobacillus sp.* KLSA22 was inoculated into the medium. The lactase production was carried by employing agitation speed at 100 rpm and as well as at static condition. The flasks were incubated at 37°C for 48 h, thereafter enzyme activity was calculated at every 24 h incubation period.

**3.10 OPTIMIZATION OF PHYSICAL PARAMETERS FOR LACTASE PRODUCTION**

The optimum physical parameters for the maximum production of lactase by *Lactobacillus sp.* KLSA22 were determined. The parameters investigated under SmF are initial pH of the fermentation medium, incubation temperature, inoculum size, agitation speed, and fermentation period. The effect of each parameter and its range was evaluated independently keeping others constant. The resultant parameter and its range which found with enhancement in enzyme production were incorporated in the next experiment while optimizing another parameter.

**3.10.1 Initial pH of Fermentation Media**

To evaluate the optimal initial pH of the fermentation media for enhanced level of lactase production by *Lactobacillus casei* KLSA22, a set of each Erlenmeyer flasks containing 100 ml of fermentation media were adjusted by using oNPG (as a lactose analog chromogenic substrate) in a series of buffers at different pH range from 3 to 9 specifically 0.1M acetic acid-sodium acetate buffer (pH 3.0 to 6.0), 0.1M sodium phosphate buffer (pH 6.5, 7.0, 7.5) and 0.1M Tris HCl buffer (pH 8.0, 9.0). The flasks were sterilized and inoculated with 1 ml of pre-inoculum and kept for incubation at 37°C for 72 h timeline. Further the activity of enzyme was measured at every 24 h.
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3.10.2 Incubation Temperature

To obtain optimum fermentation temperature for maximum production of lactase, a set of each Erlenmeyer flasks containing 100 ml pre-sterilized media adjusted with the pH of 6.5 were inoculated with 1 ml of loopful pre-inoculum. Thus inoculated flasks were kepted for incubation at 20, 25, 30, 35, 37 and 40°C respectively for 72 h timeline. Thereafter enzyme assay was assessed at every 24 h.

3.10.3 Inoculum size

For the determination of optimum size of the inoculum, each Erlenmeyer flasks containing 100 ml of pre-sterilized fermentation media with pH 6.5 were separately inoculated with different size of pre-inoculum i.e., ranged from 0.5 to 2 % and incubated at 35°C for 72 h timeline. Thereafter enzyme assay was carried out at the interval of 24 h incubation. Simultaneously, viable cell count of \( L. \text{ casei} \ KLSA22 \) strain was also observed till 48 h of incubation period at 35°C.

3.10.4 Effect of Agitation speed

To determine the control of agitation speed over the productivity of the lactase by \( L. \text{ casei} \ KLSA22 \), a set of 250 ml Erlenmeyer flasks containing 100 ml of pre-sterilized fermentation media were inoculated independently with 1 ml of pre-inoculum. Thus inoculated Erlenmeyer flasks were placed in temperature controlled shaker incubator with different agitation speed at 35°C for 48 h. Along with this, one flask was incubated with same conditions at static state. The different agitation speeds maintained in this investigation are 50, 100, 150, and 200 rpm respectively and further each flask were processed for enzyme activity at the interval of 24 h.
3.10.5 Incubation period

To find out the optimum time course needed for the maximum production of lactase by \( L. \) casei KLSA22, the 100 ml fermentation media was placed in 250 ml Erlenmeyer flask, adjusted the pH up to 6.5, sterilized and inoculated with 1 ml of pre-inoculum. Thus time course on the production of lactase was determined by incubating the production medium at different time intervals (12 to 72 h) at 35°C. Thereafter enzyme activity was measured at every 24 h.

3.11 PROCESS ECONOMIZATION FOR LACTASE PRODUCTION

In this particular study, the necessity of the supplementation of various additional nutritional factors on the production of lactase by \( L. \) casei KLSA22 was investigated. The main aim of this investigation was to increase the enzyme productivity and lowering the cost of production. Here different carbon, nitrogen, and metal ions sources were supplemented independently at different concentration with basal fermentation media and their effect towards the yield of lactase were assessed. The resultant nutrient and its concentration which enhanced the enzyme yield were incorporated in the further experiment while optimization of other sources as part of process economization.

The basal production medium composed of lactose (0.5 %), cheese whey (0.5 %), yeast extract (1 %), \( \text{K}_2\text{HPO}_4 \) (0.2 %) and \( \text{MgSO}_4.7\text{H}_2\text{O} \) (0.1 %) were being used for further process optimization and economization.

3.11.1 Carbon Sources

Since two different rich source of carbon substrate i.e., pure lactose and cheese whey were used as an essential lactose supplementation for bioprocess of lactase production (as discussed earlier), further their respective optimum concentration need to
be analyzed for lowering the cost of production. At varying concentration range from 0.5 to 2 % for both pure lactose and cheese whey were supplemented independently in each Erlenmeyer flask containing 100 ml of basal fermentation media. Thus prepared flasks were sterilized, inoculated with 1 ml of pre-inoculum and kept for incubation at 35°C for 72 h. Thereafter, enzyme assay was assessed at every 24 h.

3.11.2 Nitrogen Sources

In this study, the influence of nitrogen source supplementation on lactase production was determined, a set of each Erlenmeyer flasks containing 100 ml of fermentation medium supplemented with 0.25 to 1.5 % concentration of different nitrogen sources like beef extract, yeast extract, protease peptone and peptone, respectively and adjusted at pH 6.5. Further the flasks were sterilized, inoculated with 1 ml of pre-inoculum and kept for incubation at 35°C for 72 h. Thereafter, enzyme activity was assessed at the interval of 24 h.

3.11.3 Metal Ions

To determine the requirement of additional metal ions for the enhanced production of lactase, a set of each Erlenmeyer flasks containing with 100 ml of fermentation media along with previously optimized carbon, nitrogen sources were prepared for SmF. Thus the prepared flasks were supplemented with different metal salts at varying concentration range from 0.02 to 0.14 %. The metal salts employed in this investigation are zinc sulphate (ZnSO₄), copper sulphate (CuSO₄), magnesium sulphate (MgSO₄) and manganese sulphate (MnSO₄), adjusted at pH 6.5 and sterilized. The flasks were inoculated with 1 ml of pre-inoculum and kept for incubation at 35°C for 72 h. Thereafter, enzyme activity was assessed at the interval of 24 h.
3.12 ENHANCED PRODUCTION OF LACTASE BY RESPONSE SURFACE METHODOLOGY USING *LACTOBACILLUS CASEI* KLSA22

Response Surface Methodology (RSM) is a statistical technique for designing the experiment that is suitable for development and finding the best optimum value for the bioprocess that lead to enhance the enzyme yield.

3.12.1 Experimental Design

To explore the effect of variables on the response in the state of investigation, process parameters were optimized for the enhance production of lactase by *L. casei* KLSA22. The second-order experimental design i.e. central composite design (CCD) was applied using Design Expert software (trial version 10.0, Stat-Ease Inc., Minneapolis, USA). The levels of variables like pH (A), temperature (B), Substrate concentration (C) (Lactose + Cheese whey), and Incubation time (D) used in the experimental design as listed in Table 3. The variables from low (-1) to high (+1) were used to study the effect of independent variables on production.

<table>
<thead>
<tr>
<th>Symbols</th>
<th>Variables</th>
<th>Range</th>
<th>Code level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-1</td>
<td>0</td>
</tr>
<tr>
<td>A</td>
<td>pH</td>
<td>6.0 - 7.0</td>
<td>6.0</td>
</tr>
<tr>
<td>B</td>
<td>Temperature (°C)</td>
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<td>30</td>
</tr>
<tr>
<td>C</td>
<td>Substrate Concentration (g/L)</td>
<td>0.1 - 1.1</td>
<td>0.1</td>
</tr>
<tr>
<td>D</td>
<td>Incubation time (h)</td>
<td>43 – 53</td>
<td>43</td>
</tr>
</tbody>
</table>

-1 = low variables, 0 = previously optimized conditions and +1 = high variable
In the present investigation, thirty experiments were conducted in a set of 250 ml Erlenmeyer flasks containing 100 ml sterile production media which was prepared accordingly the design using CCD for the study of four factors each at three levels (-1, 0, +1), and other optimized nutritional parameters were kept constant. All experiments for estimating lactase production by assay method (Miller et al., 1988) were performed in triplicates and their average values obtained were taken as response. The experimental data was further analyzed by employing multiple regressions and second order polynomial fitted for predicting optimal levels was expressed by equation:

\[ Y = \beta_0 + \beta_1A + \beta_2B + \beta_3C + \beta_4D + \beta_{11}A^2 + \beta_{22}B^2 + \beta_{33}C^2 + \beta_{44}D^2 + \beta_{12}AB + \beta_{13}AC + \beta_{14}AD + \beta_{23}BC + \beta_{24}BD + \beta_{34}CD \]

Where \( Y \) stands for response variable, A, B, C, and D represents the levels of variables according to Table 1 and \( \beta_0, \beta_1, \ldots, \beta_{34} \) represent coefficient estimates with \( \beta_0 \) having the role of a scaling constant.

### 3.12.2 Model Validation

Further, the actual values (enzyme activity) of each variables of experimental design were incorporated for statistical and numerical analysis of the model using Design Expert 10.0 program analysis of variance (ANOVA) to obtain the interaction between the process variables and the response. The statistical significance of the model was analyzed by Fisher’s F-test in the same program and the quality of fit of polynomial model was expressed by the coefficient of determination (\( R^2 \)) that is associated with probability P (F) and correlation coefficient (R). During RSM implementation a mathematical model generated was validated by conducting experiment in triplicates at best predicted optimal variables of the medium. For each variable, the quadratic models
were represented as contour plots (three-dimensional) and response surface graphs generated which signifies the model validation.

3.13 PURIFICATION OF LACTASE

Purification of enzymes is very essential for detailed studies on their properties and also needed for a specified target application. In the present study, after the biomass production of lactase by *L. casei* KLSA22 through SmF under optimized conditions; cells were extracted by cell-disruption method using lysozyme-EDTA treatment (as discussed earlier see page 2). Thus obtained cell free supernatant (crude enzyme) was treated with the following three steps of purification techniques.

3.13.1 SALT PRECIPITATION METHOD

3.13.1.1 Materials required: 50 ml of measuring cylinder, ice bags, glass rod, ammonium sulphate, and 10mM Tris HCl buffer of pH 8.0.

3.13.1.2 Protocol

To the 30 ml of crude extract, 21 g of ammonium sulphate was added pinch-wise by placing the beaker under ice cold conditions with continuous stirring overnight to precipitate the protein. The precipitated protein was retrieved by centrifuging at 10,000 rpm for 10 min at 4°C. The pellet was collected and dissolved in 10 ml of 10 mM Tris HCl buffer (pH 8.0) which was later subjected to dialysis.

3.13.2 DIALYSIS

3.13.2.1 Materials required: Nitro cellulose membrane, distilled water, 2 % Sodium bicarbonate solution, beaker, glass rod and thread.

3.13.2.2 Activation of dialysis membrane

About 8 cm of dialysis bag was boiled in 100 ml of distilled water for 10 minutes to open the pores of dialysis membrane. Then it was boiled in 100 ml of 2 %
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Sodium bicarbonate solution for 10 min to remove glycerol coated on the dialysis membrane. The bag was again boiled in 100 ml of distilled water for 10 min to remove residual sodium bicarbonate.

3.13.3.6 Loading the sample

The activated dialysis membrane was gently rub to open the mouth, one end of the dialysis membrane was tied using thread and the obtained protein sample was placed inside the dialysis bag. After addition of sample, the other end of the membrane was tied with thread tightly. The dialysis bag was then suspended in a beaker containing 100 ml of 10 mM Tris HCl buffer (pH 8.0). This setup was kept in refrigerator overnight; thereafter the protein present in the bag was dissolved in 10 ml of same buffer.

3.13.2 ION-EXCHANGE CHROMATOGRAPHY

The ion-exchange chromatography (IEC) form relies on the attraction between oppositely charged particles. Ion exchange separations are carried out mainly in columns packed with an ion-exchanger. There are two types of ion exchangers namely, cation and anion exchangers. Cation exchangers possess negatively charged groups and these will attract positively charged cations. These exchangers are also called acidic ion exchange materials because their negative charges result from the ionization of acidic groups (For eg. Carboxyl methyl). Anion exchangers have positively charged groups that will attract negatively charged anions. The term basic ion exchange material is also used to describe this exchanger, as positive charges generally results from the association of the protons with basic groups (For eg. Diethylaminoethyl (DEAE)-cellulose).

3.13.3.1 Materials required: Column for chromatography, eppendorf tubes, beaker and pipette.
3.13.3.2 Reagents required: Stationary phase → DEAE cellulose

Mobile phase → 50 mM Tris HCl buffer of pH 8.8

Salt Gradient → 25 mM NaCl in 50 mM Tris HCl buffer

3.13.3.3 Preparation of Stationary Phase: Take 3 g of DEAE cellulose and make slurry in 10 ml distilled water.

3.13.3.4 Preparation of Mobile Phase: Dissolve 0.6 g of 50 mM Tris HCl Buffer (pH 8.8) in 50 ml distilled water. Make up the volume up to 100 ml by adding more distilled water.

3.13.3.5 Protocol

The column was taken and washed properly. To the column, DEAE-cellulose slurry was loaded up to 10 cm. Leave the column undisturbed for one hour to form the column matrix. To the column 10 ml of 50 mM Tris buffer (pH 8.8) was loaded. The buffer was eluted from the column. This process was repeated twice as washing with buffer provides better flow rates.

3.13.3.6 Loading the sample

10 ml of dialyzed protein sample was poured in column using a pipette. Sample was eluted from the column. Effluent was poured back in the column and re-eluted. Process was repeated thrice to ensure all the proteins present in the dialysis sample binds with DEAE Cellulose.

3.13.3.7 Applying counter ions (salt gradients): 40 ml of 25 mM NaCl in 50 mM tris pH 8.8 was applied to the column. The effluent was collected in fractions of 4 ml.

Each eluted fractions were collected from above three respective purification steps and subjected to enzyme assay and protein estimation. The activity of enzyme was assayed by the method prescribed by Miller (1998) using standard oNP curve (see
Figure 7 in chapter 4). Similarly, the concentration of purified protein was also determined by the method of Lowry et al., (1951) using standard BSA curve (see Annexure 1). Further, each purified protein sample was subjected to molecular weight determination by SDS-PAGE. Afterwards, specific activity of enzyme, purification fold, and total activity yield were calculated using the following equations:

\[
\text{Specific activity of enzyme (IU/mg)} = \frac{\text{Total enzyme activity (IU)}}{\text{Total protein (mg)}}
\]

\[
\text{Purification fold} = \frac{\text{Specific activity of enzyme in purified extract}}{\text{Specific activity of enzyme in crude extract}}
\]

\[
\text{Yield (\%)} = \frac{\text{Total activity of enzyme in purified extract}}{\text{Total activity of enzyme in crude extract}}
\]

### 3.14 MOLECULAR WEIGHT DETERMINATION OF PURIFIED PROTEIN BY SODIUM DODECYL SULPHATE POLY-ACRYLAMIDE GEL ELECTROPHORESIS

Electrophoresis technique involves the separation of proteins based on their size. When heated under denaturing conditions, proteins become unfolded and coated with the anionic detergent sodium dodecyl sulphate (SDS), acquiring net negative charges irrespective of their intrinsic electrical charges. When this sample is loaded on a gel, the negatively charged protein molecules migrate towards the positively charged electrode in an electric field. After gel electrophoresis, the size of the peptide can be estimated by comparing its migration distance with that of standard molecular weight proteins. The acrylamide gels are formed by polymerizing acrylamide with a cross-linker (bis-
acrylamide) in the presence of a catalyst tetramethylethylenediamine (TEMED) and an initiator ammoniumpersulfate (APS) with a suitable gel buffer (Tris). Solutions are normally degassed prior to polymerization. Oxygen molecules inhibit polymerization and additionally heat will be produced during polymerization. The rate at which the gels polymerize can be controlled by varying the concentrations of TEMED and APS.

In this system the gel is composed of ¾ of separating gel and ¼ of stacking gel with two buffers for making the gel. If the sample is loaded directly on the top of the gel the sharpness is lost and the protein band in the gel will be as broad as possible. The problem is overcome by polymerizing a stacking gel on the top of resolving gel. When electrophoresis starts in such a system the protein and ions migrate into the stacking gel. The proteins concentrate in very thin zone called the stack between the fast moving Tris ion and slow moving glycine ion. As a result the bands produced are very sharp and clear.

3.14.1 Requirements of Solutions for Gel preparation

**Acrylamide and Bis-acrylamide solution:** Acrylamide – 3 g, Bis-acrylamide - 0.08 g, Distilled water – 10 ml, 10 % SDS – 1 g of SDS in 10 ml of distilled water, and 10 % APS – 1 g of APS in 10 ml distilled water.

**Electrode Buffer:** 25 mM Tris HCl of pH 6.8, 200 mM glycine and 0.1 % (w/v) SDS and Distilled water -200 ml.

**Sample Buffer:** 0.2M (w/v) Tris HCl of pH 6.8, 20 % (v/v) Glycerol, 10 % (w/v) SDS, 0.05 % (w/v) bromo-phenol blue, and 2.5 % (v/v) β-mercaptoethanol

**Staining Solution:** Coomassie Brilliant Blue - 0.2 g, Methanol – 40 ml, Glacial Acetic Acid – 10 ml, and Distilled water – 100 ml.
Destaining Solution: Methanol – 40 ml, Glacial Acetic Acid – 10 ml, and Distilled water – 100 ml.

3.14.2 Procedure

The PAGE unit was assembled properly after thorough washing. The glass plates were clamped together using bulldog clips after placing the spacers between them. White petroleum jelly was applied to the bottom edges of glass plates. The end of plates were sealed using cellophane tape. According to the Table 4 stock solution were prepared. Sufficient volume of separating gel was poured and left undisturbed until it solidified. Then the stacking gel was added above the separating gel. The comb was carefully inserted into the gel and allowed the solution to solidify.

Table 4: Preparation of SDS-PAGE gel

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (mL)</th>
<th>Reagent</th>
<th>Volume (mL)</th>
</tr>
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<tr>
<td>Distilled water</td>
<td>10</td>
<td>Distilled water</td>
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</tr>
<tr>
<td>1.5 M Tris-HCl (pH 8.8)</td>
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<td>0.5 M Tris-HCl (pH 6.8)</td>
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</tr>
<tr>
<td>Acrylamide &amp; Bisacrylamide (30 % / 0.8 % w/v)</td>
<td>12</td>
<td>Acrylamide &amp; Bisacrylamide (30 % / 0.8 % w/v)</td>
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<tr>
<td>10 % SDS</td>
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<td>10 % SDS</td>
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<tr>
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</table>
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After solidification, comb was carefully removed from PAGE set up without disturbing the wells. The cellophane tapes were removed from the slides; white petroleum jelly was carefully removed with the help of blotting paper. The bulldog clips were removed. Thereafter placed the assembled glass a plate of solidified gel into the chamber was filled with electrophoresis buffer. 500 µl of crude and purified protein from each step of purification were mixed with 2 ml of sample buffer and heated independently in boiling water bath approximately for 2 min to denature the proteins. These proteins samples and along with the standard protein marker (MW 260 kDa) were loaded in the wells to find out the molecular weight independently. The power supplies of 250 V current was provided for 4 h by which the bromophenol blue dye reaches the bottom of the gel and then power supply was turned off; the gel mould was removed from the apparatus and immersed in a tray containing staining solution. The gel was stained at room temperature for 30 min followed by de-staining until the gel turns transparent and finally visualized for the blue colored protein bands.

3.15 CHARACTERIZATION OF PURIFIED LACTASE

Activity and stability of purified lactase was carried out to understand the influence of different physicochemical factors such as pH, temperature, substrate concentration ($K_m$ and $V_{max}$) by following the standard procedures as mentioned below. Activity and stability of enzyme was recorded under the influence of varied conditions.

3.15.1 Effect of pH on Activity and Stability of Lactase

The effect of pH on lactase activity was determined by assaying the enzyme activity at different pH values ranging from 3.0 to 9.0 using 0.05 M of the following buffer systems: sodium acetate (3.0, 4.0, 5.0), sodium phosphate (pH 5.5, 6.0, 6.5, 7.0, 7.5) and Tris-HCl (pH 8.0, 9.0). The relative activities were based on the ratio of the
activity obtained at certain pH to the maximum activity obtained at that range and expressed as percentage.

The pH stability of lactase was investigated in the pH range of 3.0 to 9.0 using same buffer systems as mentioned above. Enzyme solutions were mixed with the buffer solutions and incubated at 37°C for 60 min. Afterwards aliquots of the mixtures were taken to measure the residual lactase activity (%) with respect to control, under standard assay conditions.

3.15.2 Effect of Temperature on Activity and Stability of lactase

The effect of temperature on lactase activity was determined by performing the standard assay procedure at different temperatures ranging from 20 to 50°C (20, 25, 30, 35, 37, 40, 45, 50°C). The relative activities (as percentages) were expressed as the ratio of the lactase activity obtained at certain temperature to the maximum activity obtained at the given temperature range. The stability of the crude lactase was investigated by measuring the residual activity after incubating the enzyme under water bath at various temperatures ranging from 37 to 70°C for 60 min.

3.15.3 Determination of Kinetic parameters

The purified enzyme extract solution was used to observe the effect of substrate concentration on lactase activity. The kinetic properties of lactase were determined using varying concentration of the substrate (oNPG and lactose). The Michaelis constant ($K_m$) and maximum rate ($V_{max}$) values of purified lactase were investigated by performing enzyme assay at 37°C using 0.05 M sodium phosphate buffer at pH 6.5 with substrate concentrations ranging from 0.5 to 25 mM for oNPG and from 1 to 100 mM for lactose. Further, the kinetic parameters ($K_m$ and $V_{max}$) for both substrate independently calculated by Lineweaver-Burk double reciprocal plots.
3.16 IMMOBILIZATION OF LACTASE BY Ca-ALGINATE ENTRAPMENT METHOD

Immobilization of purified lactase with Ca-alginate was carried out as per the standard procedure of entrapment technique prescribed by Adinarayana et al., (2005). The purified lactase solution was mixed with sodium alginate (2 %) solution in 1:1 ratio. The stock solution of 1 ml lactase was mixed with 9 ml of sodium alginate solution (ie. the total volume of matrix and enzyme mixture to be 10 ml). The lactase–alginate mixture was extruded drop-wise into 20 ml of chilled 0.2 M CaCl$_2$ solution with gentle stirring at 4°C using hydrodynamic syringe to form beads. Further the beads were cured for about 2 h in CaCl$_2$ solution and then filtered CaCl$_2$ solution was collected for enzyme activity determination. The beads thus formed were washed 3—4 times with de-ionized water and later with 50 M Tris-HCl buffer of pH 7.0. The formed beads were recovered by filtration and thoroughly washed with distilled water. The resultant spherical beads were dried and stored in 0.1 M sodium phosphate buffer (pH 7.0) at 4°C for further studies.

3.17 CHARACTERIZATION OF FREE AND IMMOBILIZED LACTASE

3.17.1 Effect of pH and temperature on free and immobilized lactase

The activity of free and immobilized lactase was assayed in 0.05 M sodium phosphate buffer (pH 5.5 to 8.5) at 37°C. The activity at pH 6.5 was taken as control (100 %) for the calculation of remaining percent activity. In addition to effect of temperature on relative activity of free and immobilized lactase was evaluated at different temperature (20 to 70°C). The relative activity at 37°C was taken as control (100 %) for the calculation of remaining percent activity.
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3.17.2 Storage and Thermal Stability of free and immobilized lactase

The stability of free and immobilized lactase was evaluated during storage at 4°C for 4 wk (in 0.1 M Sodium phosphate buffer of pH 7.0). Thermal stability of free and immobilized lactase was also analyzed after 6 h of incubation at 55°C. The activity determined on first day was considered as control (100 %) for the calculation of remaining percent activity.

3.17.3 Reusability of Immobilized Lactase

The catalytic activity of immobilized lactase was evaluated using a repeated batch process in order to investigate the operational stability of lactase for continuous processing. Immobilized beads (0.5 g) were incubated with oNPG substrate at 55°C for 10 min and the resultant reducing oNP substance was estimated by assay method (Miller, 1988). After each batch reaction, beads were washed with 0.1 M sodium phosphate buffer pH 7.0 and stored in same buffer at 4°C. The activity determined on first day was considered as control (100 %) for the calculation of remaining percent activity after repeated uses.

3.18 APPLICATION STUDIES

The practical application of lactase is still faced with many technical problems though it has wide application in food and dairy industry. Despite the interest in probiotics as the source of lactase, the industrial applications of processes based on the enzymatic hydrolysis of lactose are limited, as the enzyme is intracellular. Therefore, there is an intense interest to explore more promising lactase with adequate properties and yields for industrial use. However, the hydrolysis of lactose using lactase supplement is one of the most promising biotechnological applications in food industries, which can be used to solve the problems of lactose-intolerant people.
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Moreover, the use of immobilization technology is of significant importance from an economic viewpoint. Immobilization has been found to be the convenient method to make reuse of cells, to obtain higher cell densities in bioreactors and easier purification of the final product. In addition, the continuous operation is more easily and efficiently controlled while using this technology. Despite of another fact, a major drawback in the use of potassium bromate and potassium iodate in the bread making sector has been banned in many countries but not in India; as these chemicals are listed as "hazardous" for public health. That is the potassium bromate – classified as a category 2B carcinogen (possibly carcinogenic to humans) and the potassium iodate could trigger thyroid disorders (http://www.cseindia.org). Therefore, the use of whole cells as a source of lactase in the baking technique is an interesting alternative, which can be further explored from the economic viewpoint. In view of this, the present work has been designed into following application studies.

1. Bread bun preparation using purified lactase
2. In-vitro study in preparation of lactose-free milk
3. In-vivo study on evaluation of lactose maldigestion in methotrexate-induced gastrointestinal mucositis wistar rats.

3.18.1 BREAD BUN PREPARATION USING PURIFIED LACTASE

100 g of wheat flour, 50 g sachet of baker’s yeast were purchased and the following protocol was carried out in SAPNA Bakery, Hagarga cross, Ring road, Kalaburagi.

3.18.1.1 Protocol

To 10 g of flour, 5 ml of yeast suspension (5g of baker’s yeast suspended in 100 ml of package drinking water). Add 0.05ml of a purified lactase suspension to above
mixture flour and mixed well with a spatula. After optimal addition, the dough's were fermented in bake furnace up to 20 min.

3.18.2 IN-VITRO STUDY IN PREPARATION OF LACTOSE-FREE MILK

Lactose hydrolysis in milk was investigated using 100 ml of toned milk (Nandini-Good life, commercial brand of KMF dairy plant, Bangaluru) was purchased and brought to the laboratory for conducting the experiment.

3.18.2.1 Protocol

Pasteurized toned milk content 4.8 % of lactose (Nandini-Good life, commercial brand of KMF dairy plant, Bangaluru) was skimmed by centrifugation at 10,000 rpm for 10 min at 4°C. This skimmed milk (100 mL) was treated with 690 mg of standard Lactase Enzyme (Nature’s Way®, USA) and the test sample 1 g of immobilized lactase in stirred batch process at 50°C for 2 h. Each sample aliquots of 4 ml were collected in separate set of test tubes at 20 min regular intervals of time for 2 h. Finally, the hydrolyzed reaction mixture was stopped by placing each sample in a boiling water bath for 5 min. The hydrolysis of lactose produced D-glucose was estimated at 540 nm by using glucose oxidase–peroxidase (GOD–POD) assay (Leary et al., 1992).

3.18.3 IN-VIVO STUDY ON EVALUATION OF LACTOSE MALDIGESTION IN METHOTREXATE-INDUCED GASTROINTESTINAL MUCOSITIS WISTAR RATS

Lactose maldigestion or intolerance is due to the deficiency of Lactase in the intestinal brush border of humans after consumption of milk or dairy products. The passage of lactose to the small intestine can lead to tissue dehydration, poor calcium absorption and generation of hydrogen, carbon dioxide gases, abdominal pain, diarrhea, bloating, flatulence, blanching, and cramps. The other adverse effect of gastric mucosa
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leads to mucositis is a severe and unbearable side effect of chemotherapy, especially in children (Moe and Holen, 2000; Sonis et al., 2004; Chen et al., 2009).

Animal experiment was approved by the chairman, IAEC (Institutional animal ethics committee) members of RxBioScienceIndia Pvt. Ltd, Hyderabad. And the experiment was conducted in the same laboratory.

3.18.3.1 Methodology

Animals: Wistar rats were maintained in cabin type isolators at standard environmental conditions. The cabin temperatures between 22 – 25°C, humidity 40-70% with 12:12 hour dark/light photoperiod were maintained. Three months old adult animals were used for the experiments (Figure 7).

![Figure 7: Maintenance of Wistar Rats in Cages](image)

3.18.3.2 Experimental Design

Three months old Wister rats were divided in to 4 groups Group-I (Normal untreated rats serves as negative control), Group-II (Mucositis untreated rats serves as positive control (MTX 60mg/kg body weight), Group-III (Mucositis rats treated with...
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Lactose + Standard lactase (0.25 to 1.00 mg/100g body weight), Group- IV (Mucositis rats treated with lactose + test sample (Isolated purified lactase 0.25 to 1.00 mg/100g body weight). In each groups six number of wistar rats were used (Figure 8 and Table 5).

Figure 8: Oral dosage of Sample

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Groups</th>
<th>Group details</th>
<th>No. of animals in each group</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Group-I</td>
<td>Normal untreated rats serves as control</td>
<td>6 No.s</td>
</tr>
<tr>
<td>2</td>
<td>Group-II</td>
<td>Mucositis untreated rats serves as positive control (MTX 60mg/kg body weight)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Group-III</td>
<td>Mucositis rats treated with lactose + Standard Lactase (0.25 to 1.00 mg/100g body weight)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Group-IV</td>
<td>Mucositis rats treated with lactose + test sample (Purified Lactase 0.25 to 1.00 mg/100g body weight)</td>
<td></td>
</tr>
</tbody>
</table>
3.18.3.3 Induction of Mucositis

Methotrexate (MTX) is an anti-metabolite which is recognized as most effective chemotherapeutic agent for leukaemias, choriocarcoma and Burkitts lymphoma. It is also called as Amethopterin which acts as folic acid antagonist. It has been recognized as a highly effective drug for the treatment of Psoriasis, Rheumatoid Arthritis. Anticancer drugs are capable of causing damage to DNA if the dose is high. Methotrexate was procured from the market (10 mg/vial as a powder form). The drug was dissolved in 0.9% sodium chloride (mg/ml concentration). The reconstituted solution is stable for 24 h at room temperature. Methotrexate was injected to animals through intra-peritoneal cavity One injection was given for every day and continue for seven days (Figure 9).

In this study we have used 10mg (60mg for kg body weight) of methotrexate for a single dose for 7 days to induce Mucositis. From third day on wards orally supplemented standard lactase (50mg) and purified lactase (50mg) for the experimental animals for twice a day for seven days.

Figure 9: Sub-cutaneous injection
3.18.3.4 Histopathology of Intestine

The animals treated and untreated with above said vehicles for 7 days were sacrificed and collected the intestine in 10 % formaldehyde solution and kept for 48 hours at room temperature to fixing the tissues (Figure 10). The tissues were dehydrated in ethanol then cleared in xylene and embedded in paraffin blocks and made 5μm thick sections using microtome. The sections were de-paraffinized in two changes of xylene for 10 minutes, rehydrated in 100 % ethanol, 90 % ethanol, and 70 % ethanol for 5 minutes each, and stained with hematoxylin and eosin as prescribed by Lukovac et al., (2008).

![Figure 10: Dissection of Wistar rat represents (A) Wistar rat Sacrifices (B) Section of Intestine](image)

3.18.3.5 Lactose Digestion and Glucose Absorption Test

After administrations of lactose, the blood samples will be obtained to quantify blood enrichment of lactose derived glucose and of glucose. The quantification of lactose-derived glucose and glucose enrichment in blood from blood spots to be performed by using Glucometer (Van Dijik et al., 2003). Absorption of lactose-derived
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glucose during the experimental period is calculated as area under the curve of glucose concentration (time 0 – 60 min), respectively.

3.18.3.6 Lactase Activity in Animal Experimental Design

Mucosa of frozen duodenal, jejunal, and ileal sections were scraped on ice to make tissue homogenates and diluted 1–10 times in distilled water as described previously (Dahlqvist, 1964; Lukovac et al., 2008). Lactase activity was assayed spectrophotometrically by the method of Dahlqvist (1964). Activity was expressed as units (one μmol/mg of lactose hydrolysed protein/hr at 37°C) per gram of total protein present in the homogenates. The protein content of the homogenate was assayed by the method of Lowry et al (1951) using std BSA protein curve (see Plate 10, Table 19 and Figure 49 in Annexure 1).