Chapter 3.
Materials and Methods

3.1 Culture Media and Their Components

The selection of appropriate culture media is essential for the isolation and screening of fungal strains. In this study, potato dextrose agar (PDA) and Czapek Dox agar were utilized as growth media, both obtained from Himedia (Mumbai, India). These media were supplemented with L-methionine as a substrate to facilitate enzymatic activity and phenol red as a pH indicator to monitor pH variations (Agrawal *et al*., 2018; Awan *et al*., 2017). All the ingredients for the growth media were bought from Himedia, a company in Mumbai, India, to make sure the experiments were consistent and reliable. The culture media used in this study are listed below table.

3.1.1 Potato dextrose agar (PDA)- The components per liter are as follow:

Potato infusion	- 4g
Dextrose	- 20g
Agar-Agar	- 20g
Distilled water	- 1000 mL
pH	- 7.0

3.1.2 C'zapex dox agar - The components per liter are as follow:

Glucose	- 2.00g
L-Methionine	- 5.00g
Dipotassium phosphate	- 1.52g
Potassium chloride	- 0.52g
Magnesium sulphate	- 0.52g
Ferrous sulphate	- 0.01g
Agar-agar	- 20g
Distilled water	- 1000 mL

pH - 7.0

Phenol red - 0.009% (Awan et al., 2017)

3.1.3 Optimized Media for L-methionase production - The components per liter are as follow:

Yeast Extract - 1.11 g/L

Glucose - 6 g/L

Di potassium phosphate - 0.24 g/L

Magnesium sulphate - 0.12 g/L

Potassium chloride - 0.12 g/L

Potassium nitrate - 0.72 g/L

pH - 7.00

Temperature - 30 ℃

Incubation time - 7 days

3.2 Materials

A comprehensive range of chemicals, reagents, and commercial kits were employed throughout this study for various experimental procedures, including enzymatic assays, protein quantification, fungal identification, enzyme production, purification, biochemical characterization, and *in vitro* anticancer evaluations of L-methionase.

3.2.1 Reagents for Enzymatic Assays and Protein Quantification

To conduct the quantitative estimation of L-methionase activity, L-methionine was procured from Himedia (Mumbai, India). Tris-HCl buffer was obtained from Merck, and Nessler's reagent used for ammonia detection was supplied by Sigma-Aldrich. Protein concentration was estimated using the Folin-Lowry method (Lowry *et al*., 1951). This was accomplished using sodium hydroxide, sodium carbonate, sodium potassium tartrate, and copper sulfate pentahydrate (Merck) (SRL, Surat, India). The Folin-Ciocalteu reagent, required for color development, was purchased as a ready-to-use solution from Sigma-Aldrich, while bovine serum albumin (BSA), employed as the protein standard, was acquired from Thermo Fisher Scientific.

3.2.2 Materials for Fungal Identification and Enzyme Production

For the morphological identification of fungal isolates, lactophenol cotton blue stain was used, sourced from Sigma-Aldrich (Ghosh & Maiti, 2016). L-methionase was produced using Czapek Dox broth that had been enriched with L-methionine as a supplement. Various carbon sources, including glucose, maltose, fructose, and lactose, were incorporated to optimize enzyme yield. Nitrogen sources like peptone, yeast extract, ammonium sulfate, and potassium nitrate were also included. All these components were procured from Himedia (Mumbai, India).

3.2.3 Chemicals for Enzyme Purification

The purification of L-methionase involved both precipitation and chromatographic techniques. Acetone used for protein precipitation was sourced from RANKEM. Buffer systems including potassium phosphate buffer, Tris-HCl buffer, acetate buffer, and sodium chloride were procured from Himedia (Mumbai, India). For chromatographic purification, DEAE-cellulose was used for ion-exchange chromatography, while Sephadex G-100 and Sephadex G-75 were employed for size-exclusion chromatography. These chromatographic media were from Sigma-Aldrich (Sharma & Singh, 2021).

3.2.4 Reagents for SDS-PAGE Analysis

To determine the molecular weight of the purified L-methionase enzyme, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed. The required chemicals acrylamide, bis-acrylamide, SDS, glycine, Tris base, ammonium persulfate, TEMED, bromophenol blue, Coomassie Brilliant Blue, β-mercaptoethanol, glycerol, agarose, methanol, and acetic acid were all obtained from Himedia (Mumbai, India) (Laemmli, 1970). Molecular weight protein markers were also sourced from the same supplier.

3.2.5 Buffers for Biochemical Characterization

For the characterization of L-methionase under varying pH conditions, several buffer systems were prepared in-house. These included acetate buffer (pH 4–5), phosphate buffer (pH 6–7), Tris buffer (pH 8-10), potassium phosphate buffer, and glycine-sodium hydroxide buffer (pH 9–10) prepared according to Sambrook and Russell (2001).

3.2.6 Cell Lines Reagents and Laboratory Consumables for *In Vitro* Anticancer Activity

To investigate the *in vitro* anticancer effects of L-methionase, two human cancer cell lines, namely colon (HT-29), and breast (MDA-MB-231), were obtained from the American Type Culture Collection (ATCC). The MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), used for assessing cell viability, was purchased from Thermo Fisher Scientific. Dimethyl sulfoxide (DMSO) and phosphate-buffered saline (PBS), critical for reagent preparation and washing steps, were sourced from Himedia (Mumbai, India) (Mosmann, 1983). Cell culture media, including Dulbecco's Modified Eagle Medium (DMEM) and RPMI-1640, along with streptomycin antibiotic and trypan blue stain, were procured from Sigma-Aldrich. Additional experimental materials, such as 5 mL and 10 mL serological pipettes, a 200 µL multichannel pipette, 35 mm and 100 mm Petri dishes for cell adherence, T-flasks, 96-well plates, and bovine serum albumin, were also obtained from Himedia to facilitate cell culture and assay procedures.

3.2.7 Molecular Biology Reagents

This study involved molecular analysis focused on the isolation and sequencing of the 18S rRNA gene. Only essential reagents and materials were used throughout the process. Genomic DNA was extracted using a commercial DNA isolation kit, and the 18S rRNA gene was amplified using a standard PCR master mix. Custom primers specific to the 18S rRNA gene were synthesized by Sigma-Aldrich (St. Louis, MO, USA). The resulting PCR products were then purified and sent for sequencing through a commercial service provider.

3.3 Instrumentation, Glassware, Plasticware

3.3.1 Instruments

A wide array of laboratory instruments was utilized to facilitate the various experimental workflows, including culture preparation, enzyme assays, protein analysis, electrophoresis, molecular biology techniques, and anticancer assays. The following instruments were employed:

- **Sterilization and Aseptic Handling:** Autoclave (Equitron, India) Horizontal Laminar Air Flow Cabinet (Whitely, United Kingdom), and, Biosafety Cabinet (Equitron, India)
- **Molecular Biology and Visualization:** Thermocycler (USA, Scientific), UV Transilluminator System (Genei, India), and Gel Documentation System (Bio red, USA).

- **Cultivation and Mixing Equipment:** Refrigerated Orbital Shaker (Genei, India), Mini Shaker (IKA, Germany), Ice Machine (Icematic F80C), Lyophilizer (Heto Pvt. Ltd., UK), Ultrasonicator (Lark Innovative Pvt. Ltd., India), Water Bath (Equitron, India) Hot Air Oven (Meta lab, Mumbai, India) and Microwave Oven (LG, India).
- **Centrifugation and Separation:** Microcentrifuge (Tarson, India) and Refrigerated Centrifuge (REMI Laboratory Instruments, India).
- **Spectroscopic and Analytical Tools:** UV-Visible Spectrophotometer (Labindia, India), and pH Meter (Genei, India).
- **Electrophoresis and Blotting Systems:** Vertical and Horizontal Gel Electrophoresis Units (Bio red, USA), SDS-PAGE unit (Bio red, USA), and Gel Rocker (Genei, India).
- **Incubation and Microscopy:** B.O.D. Incubator and CO_2 Incubator (Equitron, India), Bacteriological Incubator (ASIND, India) and Inverted Microscope (Olympus, USA).
- **Quantification and Measurement Tools:** Microplate Reader (Thermo Scientific, USA), Weighing Balance (Shimadzu, Japan), Magnetic Stirrer (REMI Laboratory Instruments, India), Vortex (Genei, India), Voltage Power Pack System (Bio red, USA), and Auto Micropipettes 20 µL, 50 µL, 200 µL, and 1000 µL (DLAB, China)
- **Cold Storage:** -80°C Deep Freezer (Sanyo, Japan), and 4°C Laboratory Refrigerator (LG, India).
- **Water Purification System:** Milli-Q Water System (Millipore Pvt. Ltd.).

These instruments supported the full spectrum of experimental procedures, ranging from microbial culturing to advanced molecular analysis.

3.3.2 Glassware

- High-quality borosilicate glassware was employed consistently across all experimental procedures to maintain reliability and accuracy.
- All glassware was sourced from Borosil Pvt. Ltd. (India), ensuring standardized quality across all components.
- The experimental setup utilized a variety of glassware including:
    - Conical flasks and measuring cylinders in capacities of 100 mL, 250 mL, 500 mL, and 1000 mL for solution preparation and incubation tasks.

- Beakers with the same volume range were used for mixing reagents and general sample handling.
- Test tubes with capacities of 10 mL, 20 mL, and 30 mL served for small-scale reactions and storage needs.

- Additional glassware used included:
  - Scotch Duran bottles and Borosil male reagent bottles (100 mL to 1000 mL) for chemical and media storage.
- For microbiological procedures:
- All glassware was subjected to rigorous cleaning and sterilization protocols before use to ensure aseptic conditions and eliminate contamination risks.

3.3.3 Plasticware

- Laboratory-grade plasticware was essential for maintaining sterility and ensuring the reliability of experimental procedures.
- All plastic consumables were either pre-sterilized or autoclaved prior to use, based on the particular requirements of the experiment.
- Microcentrifuge tubes with volumes of 0.2 mL, 0.5 mL, 1.5 mL, and 2.0 mL were used for centrifugation and short-term sample storage.
- Micropipette tips suitable for volume ranges of 0.2–10 µL, 10–200 µL, and 200–1000 µL were used with corresponding precision micropipettes (10 µL, 200 µL, and 1000 µL).
- All pipetting tools procured from (BRAND Scientific), ensuring consistency and accuracy in liquid handling.
- Additional plasticware, including Oakridge tubes, centrifuge tubes, plastic beakers, thistle funnels, autoclave bags, and lab trays, was obtained from Tarson (India).
- These plastic items supported a variety of laboratory tasks such as sample preparation, chemical handling, and sterilization.
- Strict sterilization protocols were followed for all plastic materials to preserve aseptic conditions and minimize contamination risks.

3.4 Methods

3.1 Geographical Locations of Soil Sampling Sites in Gujarat, India

3.4.1 Sample Collection

Soil samples are typically collected from environments likely to fungi with enzymatic potential, such as agriculture fields, compost-rich soils, or decaying organic matter. Using sterile tools, soil is collected at a depth of 5-10 cm, avoiding surface contamination, and store in sterile container for further processing.

Table 3.1. Source of Fungal isolates

Sample Name	Location	Date of Collection
Marine soil	Porbandar	7 February 2022
Marine soil	Dwarka	30 March 2022
Marine soil	Dandi	4 April 2022
Marine soil	Mandavi	13 May 2022
Aji River soil	Rajkot	15 June 2022
Nyari River soil	Rajkot	16 June 2022
Machhu River soil	Morbi	25 August 2022
Cotton Field soil	Morbi	30 August 2022
Bhagatsingh Garden soil	Rajkot	12 September 2022
Saradar Garden soil	Morbi	10 September 2022

3.4.2 Isolation of Fungi

One gram of soil sample was suspended into test tubes containing sterile distilled water. After allowing the suspended matter to settle for a few minutes, the clear supernatant was carefully decanted, and serial dilutions were subsequently prepared in the range of 10^{-1} to 10^{-6}. Subsequently, 0.1 ml of suspension from different dilution tubes was aseptically spread on to Potato Dextrose Agar (PDA) containing, potato infusion (4g/L), Dextrose (20g/L) and Agar-Agar (20g/L). The medium's pH was brought to 7.0 by adding 1N Sodium Hydroxide solution. Antibacterial agents like penicillin (70 µg/mL) and cephalosporine (75 µg/mL) were incorporated in the medium to control bacterial growth contamination. The plates were incubated at 28℃ for 5 to 6 days. Fungal colonies that developed were isolated and transferred onto potato dextrose agar for purification. The purified cultures were then stored at 4℃ for further uses (Kichu *et al.,* 2019).

3.4.3 Screening of fungal isolates for L-Methionase production

To detect L-methionase production, phenol red (0.009%) was added to the modified Czapek-Dox agar as a pH indicator before pouring the plates, and the medium was adjusted to a final pH of 7.0. Fungal mycelia were collected using a sterile needle and point-inoculated onto the prepared agar plates. The plates were incubated at 28°C for 5 to 7 days. After incubation, L-methionase activity was indicated by the appearance of a yellow halo around the fungal colonies. Isolates showing this color change were selected for further quantitative analysis.

Quantitative screening of L-methionase production was carried out using the agar well diffusion method (Rapid Plate Assay). Selected fungal isolates were cultured in modified Czapek-Dox broth and incubated at 28°C for 6 to 7 days. After the incubation period, cultures were centrifuged at 6000 rpm for 30 minutes to obtain cell-free supernatants, which were further filtered through standard filter paper. Then, 100 µL of each filtrate was loaded into wells in agar plates containing the same modified medium. The plates were incubated at 28°C for 24 to 48 hours to allow diffusion.

L-methionase activity was indicated by the development of a yellow zone around the well against a red background. The diameter of this zone (in mm) was used to quantify enzyme activity (Awan *et al*., 2017).

3.4.4 L-Methionase Enzyme Assay

The amount of ammonia produced from L-methionine was used to detect the production of L-methionase. The optimal reaction system includes 1 ml of 1% methionine in 0.5 M potassium phosphate buffer (PH 7.0) and 1 ml of raw enzyme. The reaction system was incubated at 30℃ for 1 hr. The enzymatic activity was blocked by adding 0.1 ml of 1.5 M trichloroacetic acid. The mixture was centrifuged at 5,000 rpm for 5 min to eliminate the precipitated proteins. 0.1 ml of supernatant was added to 3.7 ml of distilled water and the liberated ammonia was detected by using 0.2 ml Nessler reagent, and the developed color compound was measured at 480 nm using spectrophotometer. Enzyme and substrate blanks were used as control. One unit of L-Methionase was measured as the amount of enzyme that liberates ammonia at 1μmol/min under standard examination conditions (El-Sayed *et al*., 2015).

3.4.5 Protein Estimation

The total protein content of the fungal culture filtrates was estimated using the Folin–Lowry method. This colorimetric assay is based on the reaction of protein molecules with the Folin–Ciocalteu reagent under alkaline conditions, resulting in a blue-colored complex. The intensity of the color, which correlates with protein concentration, is measured spectrophotometrically.

In this method, an aliquot of the sample was mixed with an alkaline copper sulfate reagent (prepared by mixing sodium carbonate, copper sulfate, and potassium sodium tartrate). The mixture was incubated at room temperature for 10 minutes. Afterward, the Folin–Ciocalteu phenol reagent (previously diluted 1:1 with distilled water) was added, followed by incubation for 30 minutes in the dark at room temperature. The absorbance of the resulting blue color was measured at 660 nm using a spectrophotometer. Bovine Serum Albumin (BSA) was used to generate the standard calibration curve. Protein concentrations of the samples were calculated from the standard curve and expressed in mg/mL.

This method is widely used due to its sensitivity and reliability for estimating protein concentrations in biological samples (Lowry *et al.,* 1951).

3.4.6 Morphological Identification of L-Methionase Producing Fungi

To identify L-methionase-producing fungi at the genus or species level, morphological characterization was performed by observing both macroscopic and microscopic features. Fungal strains isolated from environmental sources such as marine and agricultural soils were cultured on potato dextrose agar (PDA) and incubated at 25–30 °C to promote growth. Colonies were examined for physical traits including size, shape, color, texture, and edge morphology, which provided preliminary identification clues. Microscopic analysis, using lactophenol cotton blue (LPCB) staining, allowed observation of key structures such as septate or aseptate hyphae and conidial arrangements. Septate hyphae indicated *Ascomycota or Basidiomycota,* while aseptate hyphae suggested *Zygomycota.* LPCB was particularly effective for highlighting fungal features, as phenol kills the cells, lactic acid preserve's structure, and cotton blue stains the chitin-rich cell walls, enabling clear visualization under a microscope (Ghosh & Maiti, 2016).

The Lactophenol Cotton Blue (LPCB) staining technique is a widely used method for the microscopic examination and identification of fungal structures. This staining solution combines four main components: phenol, lactic acid, glycerol, and cotton blue dye. Phenol acts as a fungicidal agent, effectively killing the fungal cells and preventing further growth during observation. Lactic acid serves to preserve the morphological features of the fungal elements by maintaining the structural integrity of the cell walls. Glycerol provides a semi-permanent mounting medium, preventing the sample from drying out, while cotton blue binds specifically to chitin in the fungal cell wall, enhancing visibility under the microscope.

To perform the staining, a small portion of the fungal colony is placed on a clean microscope slide using a sterile needle or loop. A drop of LPCB stain is added, and the fungal material is gently teased apart to spread it evenly. A coverslip is then carefully placed over the sample, avoiding air bubbles. The slide is observed under a light microscope, typically starting at low magnification to locate the fungal structures, followed by higher magnifications for detailed examination of hyphae, conidia, and spore arrangements.

This method provides clear contrast and is particularly effective for identifying morphological characteristics critical for classifying fungi into appropriate genera or species.

3.4.7 Molecular Identification of L-Methionase Producing Fungi

The molecular identification of L-methionase producing fungi involves a systematic approach including isolation, enzymatic screening, DNA extraction, amplification, sequencing, and phylogenetic analysis. Initially, fungal strains were isolated from various environmental samples, such a soil, using potato dextrose agar (PDA) under sterile condition. Pure fungal colonies were obtained by repeated sub-culturing and maintain on slant at 4 ℃ for further analysis. The selected isolates underwent preliminary qualitative screening for L-methionase production using colorimetric assays to detect enzyme specific product, such as ammonia. Quantitative screening was subsequently performed to measure enzymatic activity through spectrophotometric analysis, focusing on the release of α-ketobutyrate, which provides a reliable indicator of L-methionase activity.

Molecular identification of selected fungal isolate has been carried out in collaboration with SLS Research Pvt. Ltd. Surat, India. Using the DNeasy® Fungi Mini Kit (), genomic DNA was obtained from the fungal isolate and streaked on a potato dextrose agar plate as per according to the manufacturer's instructions. The quantity and purity of the extracted DNA were evaluated using a spectrophotometer. 20 ng of the eluted DNA was subsequently used for the amplification of ribosomal ITS regions by using the forward primer ITS1-F(5'-CTACCTGATCCGAGGTCAAC-3') and reverse primer ITS4-R (5'-AGGTGGACCCAGAGGGCCCTCA-3') in a Bio-Rad Thermal cycler. The 25µl PCR reaction mixture contain 2.5 µl of 10× PCR buffer, 1.5 µl $MgCl_2$ (50 mM), 50 µl dNTPs (10 mM), 1.0 µl of each primer, 0.9 µl of Taq DNA polymerase (10 U/µl) and 19.6 µl molecular biology water (White *et al*., 1990).

Amplification was achieved with the following PCR condition; initial denaturation at 94°C for 5 min, 33 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30s, extension at 72°C for 2 min each, finishing with 5 minutes of final extension at 72°C. The PCR amplicon was purified with PCR Clean-up Kit

Sequencing reaction was carried out with sequencing 27F (TACGTCCCTGCCCTTTGTAC) using BigDye Terminator v3.1 cycle sequencing kit (Thermo Fisher Scientific, USA) on ABI 3730XL Genetic Analyzer (Applied Biosystems, Foster City, CA). Similarity searches for the nucleotide sequences were done by BLAST

(http://www.ncbi.nlm.nih.gov/blast) against GenBank database to make out the identification of the isolate.

The 18s rRNA gene sequence of the strain MF13 was used as a search for homologous sequence in the nucleotide sequence database by running BLAST program. The high scoring similar to 18s rRNA gene sequence were identified from the result and retrieved from GenBank database. The identified sequence was aligned using MEGA 6.0 software. Phylogenetic trees were inferred using the Neighbor-Joining bootstrap analysis with help of MEGA 6.0 software.

3.5 Optimization of L-Methionase Enzyme

3.5.1 One-Factor-at-a-Time (OFAT)

The One Factor at a Time (OFAT) method is a systematic approach used to optimize conditions for enzymatic activity or production by varying one parameter at a time while keeping all other factors constant. For L-methionase, this method involves investigating the effect of individual variables such as pH, temperature, substrate concentration, incubation time, inoculum size, carbon sources, and nitrogen sources on the L-methionase enzyme activity or production. Each factor is tested over a defined range, and the enzyme activity is measured under standard assay conditions, such as spectrophotometric detection of ammonia production (Swain & Ray, 2007).

Fungal mycelia are cultured by inoculating them into Czapek-Dox broth, enriched with L-methionine as primary substrate. The experiment was designed using the One-Factor-At-a-Time (OFAT) approach, where individual factors were systematically varied within specific predetermined ranges to observe their effects. The inoculated cultures were then incubated under controlled conditions as outlined in the experimental protocol. The fungal interaction with various experimental conditions was thoroughly evaluated by measuring the protein concentration and enzyme activity in the samples after incubation.

3.5.1.1 Effect of pH on L-Methionase Production

The growth medium's pH is a crucial environmental component that affects microorganisms' metabolic processes, especially the production of enzymes. To evaluate the effect of pH on L-methionase production, experiments were conducted using the fungal isolate MF13 under controlled laboratory conditions.

The isolate was cultured in Czapek-Dox broth, and the pH of the medium was systematically adjusted across a range from 6.0 to 10.0 to cover mildly acidic to alkaline conditions. This range was selected to determine the optimal pH for maximum enzyme production and activity. All Cultures were kept in an incubator adjusted at a constant 28°C, which had been identified as suitable for fungal growth, and were agitated continuously using an orbital shaker set at 120 rpm to ensure proper aeration and uniform distribution of nutrients.

The incubation period was standardized to seven days to allow sufficient time for enzyme induction and accumulation. After the incubation phase, the culture supernatants have been collected and analyzed for L-methionase activity using the Nessler's reagent assay, which quantifies ammonia released during the enzymatic breakdown of methionine. Simultaneously, the overall amount of protein in the samples was estimated using the Folin-Lowry method to facilitate normalization of enzyme activity relative to protein concentration.

3.5.1.2 Effect of Temperature on L-methionase Production

Temperature is one of the most influential environmental factors affecting microbial physiology and enzyme biosynthesis. To determine the effect of temperature on the production of L-methionase, a set of experiments was conducted using selected fungal isolates. The isolates were cultured in a nutrient medium and incubated at varying temperature conditions to evaluate the enzyme's activity profile across different thermal environments. Four incubation temperatures were chosen for the study: 25°C, 30°C, 35°C, and 40°C. These temperature points were chosen to encompass the typical mesophilic range, thereby enabling the identification of both optimal and suboptimal conditions for enzyme production. The experimental design aimed to establish the temperature at which L-methionase activity reaches its peak, in addition to observe any potential decline in activity at higher or lower temperatures.

To ensure that temperature was the only variable influencing the results, all other parameters were kept constant. In particular, the pH of the culture medium was adjusted and maintained at 7.0 throughout the incubation period using a suitable buffering system. This pH was selected based on preliminary studies indicating its neutrality and suitability for the growth of fungi and enzyme activity. Each culture was incubated for a fixed duration of seven days. This incubation period was selected to allow adequate time for fungal growth and for the synthesis and accumulation of the target enzyme. After the completion of the incubation, the culture broth was filtered, and the enzyme activity was measured.

L-methionase activity was quantitatively measured using the Nessler's reagent method. This assay is depending on the detection of ammonia released during the enzymatic cleavage of L-methionine, serving as a direct indicator of enzyme functionality. In parallel, the overall amount of protein in the culture filtrates was also determined using the Folin-Lowry method, which facilitated the normalization of enzyme activity relative to protein concentration.

3.5.1.3 Effect of Substrate Concentration on L-Methionase Production

To evaluate the impact of substrate concentration on the production of L-methionase, different concentrations of L-methionine were incorporated into the culture medium. The fungal isolate MF13 was inoculated into Czapek-Dox broth supplemented with L-methionine at varying concentrations of 0.2%, 0.3%, 0.4%, and 0.5%. The pH of the medium was maintained at 7.0, and the cultures were incubated for seven days at a constant temperature of 28°C under shaking conditions at 120 rpm to ensure uniform aeration and optimal growth.

To measure L-methionase activity, the culture filtrates were collected after incubation and put into enzymatic assays. Enzyme activity was assessed using Nessler's reagent, which detects the release of ammonia as a byproduct of L-methionase activity. Concurrently, protein concentration was measured using the Folin-Lowry method, providing insights into the relationship between substrate concentration and total protein expression associated with enzyme production. This investigation enabled a comparative assessment of how increasing L-methionine concentrations influence L-methionase synthesis, supporting in the identification of the optimal substrate level for maximal enzyme yield under the specified culture conditions.

3.5.1.4 Effect of Inoculum Size on L-Methionase Production

Inoculum size is a critical parameter in fermentation processes, as it directly influences microbial growth dynamics, nutrient utilization, and metabolite synthesis, including enzyme production. To investigate the effect of inoculum concentration on L-methionase production, the fungal isolate MF13 was cultured in Czapek-Dox broth under standardized laboratory conditions.

The study was designed to evaluate the enzyme production at varying inoculum volumes, ranging from 1% to 3% (v/v). This range was selected to determine the optimal biomass concentration required for efficient enzyme synthesis without causing nutrient depletion or growth inhibition. Each inoculum concentration was aseptically introduced into the culture medium, and all flasks were incubated at 28°C for a period of seven days.

Continuous agitation at 120 rpm was maintained using an orbital shaker to ensure homogeneous mixing and proper oxygen transfer throughout the incubation period.

Following incubation, the culture filtrates were harvested and subjected to biochemical analyses. L-methionase activity was measured using the Nessler's reagent method, which measures the release of ammonia as an indicator of enzymatic conversion of methionine. Concurrently, protein content in the culture supernatants was estimated by the Folin-Lowry method to allow for normalization and comparative assessment of enzyme productivity.

3.5.1.5 Effect of Incubation Time on L-Methionase Production

The duration of incubation plays a crucial role in microbial growth and metabolite production, influencing both enzyme synthesis and secretion efficiency. To evaluate the effect of incubation time on L-methionase production, the fungal isolate MF13 was cultivated in Czapek-Dox broth under standardized conditions, and enzyme activity was monitored across a range of incubation periods.

The study investigated enzyme production over time intervals spanning from 4 to 8 days. This range was selected to capture both the early phase of enzyme expression and the point at which maximum production occurs before potential decline due to nutrient exhaustion or accumulation of metabolic by-products. The cultures were incubated at 28°C while being constantly stirred at 120 rpm to ensure proper aeration as well as uniform growth conditions.

At the end of each designated incubation period, culture samples were harvested and analyzed for L-methionase activity. The enzyme assay was carried out using the Nessler's reagent method, which detects ammonia released as a by-product of methionine degradation, serving as a reliable indicator of enzymatic activity. Simultaneously, the total protein concentration in each sample was established using the Folin-Lowry method to normalize enzyme activity and facilitate comparative evaluation across different time points.

3.5.1.6 Effect of Organic and Inorganic Nitrogen Sources on L-Methionase Production

Nitrogen is an essential nutrient that significantly influences microbial metabolism, growth rate, and enzyme synthesis. The characteristic of the nitrogen source whether organic or inorganic can have a profound effect on the regulation and yield of enzyme production. To investigate this effect, the fungal isolate MF13 was cultured in Czapek-Dox broth

supplemented with different nitrogen sources to assess their influence on L-methionase production.

The experimental setup included two categories of nitrogen sources. Organic nitrogen sources comprised peptone and yeast extract, while ammonium sulfate and potassium nitrate represented the inorganic nitrogen sources. Each nitrogen compound was incorporated into the culture medium at equivalent nitrogen concentrations to ensure uniform nutrient availability. All cultures were incubated at 28°C for seven days under shaking conditions at 120 rpm to maintain consistent aeration and nutrient dispersion. Following incubation, the culture filtrates were collected and subjected to biochemical analysis. L-methionase activity was determined using the Nessler's reagent method, which measures the release of ammonia as a result of enzymatic breakdown of L-methionine. In addition, the total protein concentration in the culture supernatant was estimated using the Folin-Lowry method to normalize enzyme activity and allow for comparative evaluation across treatments.

3.5.1.7 Effect of Carbon Source on L-Methionase Production

Carbon sources serve as the primary energy supply for microbial growth and metabolism and play a crucial role in regulating the biosynthesis of enzymes. The type and availability of carbon compounds in the culture medium can significantly influence the production of secondary metabolites, including L-methionase. In this study, the effect of different carbon sources on the production of L-methionase by the fungal isolate MF13 was systematically evaluated.

To determine the optimal carbon source for enzyme induction, Czapek-Dox broth was supplemented individually with glucose, maltose, fructose, and lactose at equivalent concentrations. These carbohydrates were selected to represent both monosaccharides and disaccharides, providing a broad spectrum for analyzing metabolic preference. The fungal cultures were kept at 28°C for seven days with constant agitation at 120 rpm to ensure uniform growth conditions and effective nutrient utilization.

At the ending of the incubation period, culture supernatants were harvested and analyzed for enzyme activity using the Nessler's reagent method, which quantifies ammonia released during the enzymatic degradation of L-methionine. Simultaneously, protein concentration in the samples was measured using the Folin-Lowry method to normalize enzyme activity relative to total protein content.

This investigation enabled a comparative assessment of different carbon sources on L-methionase production, contributing to the identification of the most appropriate carbon substrate for maximizing enzyme yield. The findings are instrumental in optimizing media formulations for future applications in industrial-scale enzyme production.

3.6 Optimization and Statistical Modeling of L-Methionase Production

To enhance L-methionase production from the selected fungal isolate *Aspergillus fumigatus* MF13, optimization and statistical modeling techniques were employed. Initially, the most suitable culture medium identified through preliminary screening was employed as the foundation for further optimization. Two statistical approaches were applied in this study: the Plackett-Burman Design (PBD) for identifying significant factors and Response Surface Methodology (RSM) for optimizing these variables. All data analysis and experimental design, were performed using Design Expert Version 13 software.

3.6.1 Plackett-Burman Design (PBD)

To determine the most influential components of the culture medium affecting L-methionase production, the Plackett-Burman design (PBD) was employed as an initial screening tool. This statistical method is highly efficient for evaluating the effects of multiple variables with a relatively small number of experimental trials, making it ideal for preliminary optimization studies (Kumar & Tiwari, 2017; Singh & Chaturvedi, 2019).

In this experiment, a total of eight independent variables were selected for evaluation. Each variable was assessed at two levels: a low level (−) and a high level (+), as outlined in (Table 2). The parameters included pH, temperature, incubation time, and the concentrations of glucose, yeast extract, potassium nitrate, potassium chloride, dipotassium phosphate, and magnesium sulfate. These factors were chosen based on their known influence on fungal metabolism and enzyme biosynthesis.

All fermentation test was conducted using 250 mL Erlenmeyer flasks containing the culture media formulated according to the Plackett-Burman matrix. The fungal isolate *Aspergillus fumigatus* MF13 was inoculated into each flask, and the cultures were kept in an incubator under the specified conditions for enzyme production.

Upon completion of the incubation period, the enzyme activity was assessed and expressed (U/mL). Each experimental run was carried out three times to ensure statistical accuracy, and the average enzyme activity was considered as the response variable.

The general form of the linear regression model applied in this design is represented as:

$$\mathbf{Y = \beta_0 + \Sigma \beta_i X_i \ (i = 1...k)}$$

Where:

- **Y** represents the response function (L-methionase activity),
- β_0 is the intercept of the model,
- β_i denotes the linear coefficients of the respective variables,
- X_i represents the levels of the independent variables.

This model focuses exclusively on the main effects of each factor, without accounting for interactions between variables. The primary objective of this approach was to determine the most important components affecting enzyme yield, which would then be subjected to further optimization in subsequent experiments.

In the present investigation, eleven variables including one dummy variable were incorporated into a total of twelve experimental runs based on the PBD matrix. The inclusion of the dummy variable enabled the estimation of experimental error and helped validate the model's robustness in identifying statistically significant factors influencing L-methionase production.

This design facilitated the identification of key parameters significantly influencing L-methionase production, thereby narrowing down the variables for further optimization.

Table 3.2 Experimental Variables at Two Levels for L-Methionase Production Using Plackett-Burman Design

Factors	Low Level (−)	High Level (+)
pH	7.5	8.5
Temperature (°C)	28	32
Incubation Time (days)	7	9
Glucose (g/L)	20	40
Yeast Extract (g/L)	2.4	5
Potassium Nitrate (g/L)	2.4	5
Potassium Chloride (g/L)	0.4	0.7
Dipotassium Phosphate (g/L)	0.4	2
Magnesium Sulfate (g/L)	0.4	2

3.6.2 Optimization of Selected Parameters Using Response Surface Methodology (RSM)

Following the screening phase, significant variables identified through the Plackett-Burman design were subjected to further refinement using Central Composite Design (CCD) under Response Surface Methodology (Kumar & Tiwari, 2017; Singh & Chaturvedi, 2019). This approach enabled the development of a mathematical model to predict and optimize enzyme production by examining the interactions between critical variables.

The CCD consisted of 20 experimental runs, including six replicates at the center point to assure the accuracy and reproducibility of the model. All experiments were conducted in triplicate to enhance statistical reliability. The response variable (R1) was described as the average L-methionase activity (U/mL) obtained under each experimental condition.

The analysis of variance (ANOVA), was used to examine the obtained data, and response surface plots were generated to visualize the effects and interactions of the selected factors. The optimal levels of each variable were predicted based on the model.

3.6.3 Model Validation

To confirm the accuracy and predictive capability of the developed statistical model, validation experiments were performed under the optimized conditions suggested by the RSM model. The actual enzyme activity obtained from these confirmatory trials was compared to the predicted values to evaluate the reliability and precision of the model. The close agreement

between experimental and predicted responses validated the effectiveness of the statistical approach used in optimizing L-methionase production.

3.7 Purification of L-Methionase Enzyme

3.7.1 Partial Purification by Aceton Precipitation Method

Partial purification of the extracellular L-methionase enzyme was carried out using the cold acetone precipitation method, a commonly employed technique for concentrating and partially purifying proteins from culture supernatants. Following the fermentation process, the culture broth was subjected to centrifugation to separate the mycelial biomass, and the resulting cell-free supernatant containing the extracellular enzyme was collected for further processing. Cold acetone precipitation has been demonstrated as an effective method for isolating and purifying enzymes such as L-methioninase from microbial sources, yielding high specific activity and maintaining enzyme integrity (Kotramada *et al*., 2020).

To precipitate the protein, pre-chilled acetone was added gradually to the supernatant in a 1:2 ratio (supernatant to acetone, v/v) under continuous stirring at 4°C. The mixture was kept at -20°C for 12–16 hours to allow efficient precipitation of protein content. After incubation, the precipitated proteins were recovered by centrifugation at 10,000 rpm for 20 minutes at 4°C. The resulting pellet was carefully collected and resuspended in suitable buffer for further examination (Sharma & Singh, 2021).

3.7.2 Purification of L-Methionase Using Size-Exclusion Chromatography

Size-exclusion chromatography (SEC), also referred to as gel filtration chromatography, was employed as a key step in the purification of L-methionase to achieve enhanced purity and to separate the enzyme from other lower and higher molecular weight impurities (Sharma & Singh, 2021). The purification procedure was performed using the **ÄKTA Start FPLC system (Cytiva)** in combination with a manually packed **HiTrap** column containing Sephadex G-75 resin. The separation process was conducted using potassium phosphate buffer (50 mM, pH 7.5) as the phase of mobility.

3.7.2.1 Column Packing Procedure

In this study, the HiTrap column was used to manually packed with Sephadex G-75, a cross-linked dextran-based gel filtration medium suitable for proteins in the molecular weight

range of 3–75 kDa. The resin was first pre-swollen in distilled water as per the manufacturer's instructions and allowed to equilibrate in 50 mM potassium phosphate buffer (pH 7.5) to match the running buffer conditions.

The slurry was carefully poured into the column using a funnel to avoid the introduction of air bubbles. The resin was allowed to settle by gravity, and a low-pressure flow (approximately 0.5 mL/min) was applied to compress the resin gently and achieve a uniform packing. After stabilization of the bed, the column was flushed with 5–10 column volumes (CV) of buffer to remove fines and ensure equilibrium. The final bed height was marked, and the system was tested for stability and reproducibility of flow before sample loading.

3.7.2.2 Chromatographic Separation

Prior to initiating the purification run, the packed column was equilibrated with two column volumes of 50 mM potassium phosphate buffer (pH 7.5) at a flow rate of 0.5 mL/min. The enzyme sample, which had been previously obtained via acetone precipitation and resuspended in the same buffer, was clarified by centrifugation and filtration through a 0.22 µm membrane filter to eliminate any particulate matter.

The clarified sample was injected into the column through the sample loop of the ÄKTA Start instrument. The chromatographic separation was performed at 1.0 mL/min, and protein elution was continuously monitored by recording absorbance at 280 nm. The eluted fractions were collected automatically in 1.0 mL volumes using the system's fraction collector.

Each collected fraction was assayed for L-methionase activity using the Nessler's reagent method and for total protein content using the Folin-Lowry assay. Fractions exhibiting high specific activity were combine and kept at 4°C for further characterization.

3.7.2.3 Column Regeneration and Storage

After the completion of each purification run, the column was regenerated to ensure uniformity and longevity of the resin. The regeneration process involved sequential washing of the column with:

1. Three column volumes of deionized water to remove residual buffer salts.
2. Three column volumes of 0.5 M NaCl to eliminate any loosely bound proteins and ionic contaminants.

3. An optional wash with 0.1 M NaOH for deep cleaning, followed by neutralization with buffer (performed occasionally, based on column usage).

For long-term storage, the column was flushed with 20% ethanol prepared in deionized water to prevent microbial growth. The column was sealed with tight-fitting caps and stored upright at 4°C until the next use.

3.7.3 FPLC Method Run Parameters

The purification of partially processed L-methionase from *Aspergillus fumigatus* MF13 was performed using a size-exclusion chromatography approach integrated into an ÄKTA Start FPLC system. A HiTrap column packed with Sephadex G-75 resin, known for its efficient separation of proteins in the 3–75 kDa range, was employed for the purification step. The total column volume was maintained at 20 mL, with careful pre-equilibration in 50 mM potassium phosphate buffer (pH 7.5) to ensure a stable chromatographic environment.

The chromatography was conducted under isocratic conditions, and all operational parameters were strictly maintained for reproducibility and resolution accuracy. The following table summarizes the run configuration used during the purification process:

Following system equilibration, the enzyme sample, previously concentrated via acetone precipitation and resuspended in buffer, was filtered and loaded into the injection loop. The flow rate was held constant at 0.5 mL/min throughout the elution phase. UV absorbance at 280 nm was used to continuously monitor protein elution.

Table 3.3 FPLC Operational Parameters for Size-Exclusion Chromatography of L-Methionase

Parameter	Set Value	Operational Range
Column Volume (CV)	20.00 mL	0.100 – 999999.0
Flow Rate	0.5 mL/min	Fixed
Equilibration Volume	0.20 CV	0.00 – 999999.0
Sample Injection Volume (Loop Volume)	1.00 mL	0.00 – 999999.0
Isocratic Elution Concentration (Buffer B)	100.0%	0.0 – 100.0
Fraction Collection Volume	1.0 mL	0.5 – 15.0
Total Isocratic Elution Volume	1.80 CV	0.00 – 999999.0

Fractions were collected automatically at 1.0 mL intervals and each was assessed for both total protein content and L-methionase activity. The fractions showing the highest specific enzymatic activity were pooled for subsequent biochemical and cytotoxic analysis.

3.7.4 SDS-PAGE Analysis for find molecular weight of L-Methionase Enzyme

After the purification of L-methionase using size-exclusion chromatography on the ÄKTA Start FPLC system with a HiTrap Sephadex G-75 column, the eluted fractions were analyzed by SDS-PAGE to determine the molecular weight of the enzyme (Laemmli, 1970; Nayak & Naik, 2016).

3.7.4.1 Preparation of Gel

A 12% resolving gel and a 5% stacking gel were prepared using standard protocols. The resolving gel was poured first between two slabs of glass and allowed to polymerize. Once solidified, the stacking gel was poured on top of the resolving gel, and a comb was inserted to create wells for sample loading.

The components of the gel included:

- Acrylamide/Bis-acrylamide solution (30%)
- Tris buffer (pH 8.8 for resolving gel, pH 6.8 for stacking gel)
- SDS (10%)
- Ammonium persulfate (APS) and TEMED (0.005 mL) as polymerization initiators

3.7.4.2 Sample Preparation and Loading

Purified enzyme fractions were mixed with SDS sample buffer (containing β-mercaptoethanol, glycerol, bromophenol blue, and SDS) in a 1:1 ratio and heated at 95°C for 5 minutes to denature the protein and ensure complete interaction with SDS.

A prestained molecular weight protein marker (Himedia) ranging from low to high molecular weights (up to 245 kDa) was used as a reference standard. Equal volumes of the denatured protein samples and marker were loaded into the wells of the gel.

3.7.4.3 Electrophoresis Conditions

Electrophoresis was performed in Tris-glycine-SDS running buffer (pH 8.5) at a constant voltage of 100–120 V until the tracking dye reached the bottom of the gel. The electrophoresis unit was connected to a power supply and maintained under constant conditions throughout the run.

3.7.4.4 Gel Staining and Visualization

Following electrophoresis, the gel was removed and stained with Coomassie Brilliant Blue R-250 solution for 2–3 hours to visualize the protein bands. Excess stain was removed by destaining the gel in a solution containing methanol, acetic acid, and distilled water until a clear background was achieved.

The stained gel was photographed, and the molecular weight of the L-methionase enzyme was estimated by comparing the migration distance of the enzyme band with that of the known molecular weight standards. This SDS-PAGE analysis confirmed the presence, purity, and the estimated molecular weight of the L-methionase enzyme following its purification by size-exclusion chromatography.

3.7.4.5 Silver Staining of SDS-PAGE Gel

To achieve high-sensitivity visualization of protein bands following SDS-PAGE, the gel was subjected to silver staining, a technique capable of detecting nanogram levels of protein. This method offers superior sensitivity compared to conventional Coomassie staining and is particularly useful for detecting low-abundance proteins after purification.

3.7.4.6 Silver Staining Procedure

The following protocol was used for silver staining of SDS-PAGE gels (Nayak, B. S., & Naik, D. G. (2016).

1. **Fixation**:
   After electrophoresis, the gel was immersed in a fixing solution consisting of 50% (v/v) methanol and 10% (v/v) acetic acid in distilled water. The gel was incubated for or overnight at room temperature with gentle agitation to immobilize proteins and remove interfering substances such as SDS.

2. **Washing**:

   The gel was washed three times with distilled water for 10 minutes each to remove residual fixative and prepare the gel for sensitization.

3. **Sensitization**:

   The gel was treated with a 0.02% sodium thiosulfate solution for 1 minute, followed by a brief rinse in distilled water (2–3 quick washes) to enhance silver binding during the next step.

4. **Silver Nitrate Staining**:

   The gel was incubated in a 0.1% silver nitrate solution, freshly prepared and kept in the dark for half an hour at room temperature with gentle shaking. During this step, silver ions bind selectively to protein bands.

5. **Washing**:

   After staining, the gel was cleaned twice with distilled water for 1 minute each to remove unbound silver ions.

6. **Development**:

   The gel was developed using a freshly prepared solution of 2% sodium carbonate containing 0.04% formaldehyde (added just before use). Protein bands began to appear within 1–5 minutes. The development was closely monitored, and the process was stopped once distinct bands emerged.

7. **Stopping the Reaction**:

   To halt further development and stabilize the staining, the gel was immersed in a 5% acetic acid solution for 10–15 minutes.

8. **Documentation**:

   The stained gel was then rinsed with distilled water and stored in a sealed plastic bag or covered container. Images were captured using a gel documentation system for further analysis and record-keeping.

3.8 Biochemical Characterization L-Methionase Enzyme

3.8.1 Determination of optimal pH on L-Methionase Enzyme Activity

The influence of the pH on the catalytic activity of purified L-methionase was evaluated using three distinct buffer systems: sodium citrate (pH 4.0–5.5), potassium phosphate (pH 6.0–7.5), and Tris-HCl (pH 8.0–10.0), each prepared at a final concentration of 50 mM. All buffer solutions were freshly prepared and adjusted to the desired pH using 1 M NaOH or 1 M HCl to ensure accuracy and consistency. Enzyme assays were conducted in triplicate, incorporating the purified enzyme, L-methionine as the substrate, and the respective buffer. The reaction mixtures were incubated at the previously established optimal temperature, and enzyme activity It was developed utilizing the Nessler's reagent method.

Absorbance was recorded at 450 nm with a UV-visible spectrophotometer, and activity was expressed in units per milliliter (U/mL). The pH value that yielded the highest activity was recognized as the optimum pH for L-methionase function (El-Sayed *et al*., 2015).

3.8.1.2 pH Stability Assay of L-Methionase

The pH stability of the purified L-methionase enzyme was evaluated by incubating 50 µL of the enzyme with 50 µL of 50 mM potassium phosphate buffer (pH 7.5) at 37°C for 15, 30, 45, and 60 minutes in the absence of substrate. This pre-incubation step was designed to assess the enzyme's structural integrity under neutral pH conditions over time.

After each incubation period, the residual enzymatic Activity had been evaluated using the Nessler's reagent method, with L-methionine serving as the substrate. Ammonia released during the reaction was quantified by recording absorbance at 450 nm. The results were expressed as residual activity relative to a non-incubated control, providing insight into the enzyme's stability under physiological pH conditions.

3.8.2 Determination of optimal Temperature on L-Methionase Enzyme Activity

The optimal temperature for L-methionase activity was assessed by incubating the purified enzyme in 50 mM potassium phosphate buffer (pH 7.5) at various temperatures: 20°C, 30°C, 40°C, 50°C, 60°C, and 70°C, each for a duration of 30 minutes. Following thermal equilibration, the enzymatic reaction was initiated by including L-methionine as the substrate. Enzyme activity It was determined using the Nessler's reagent method, which detects ammonia released during catalysis. Measurements of absorbance were carried out at 450 nm using a UV-

visible spectrophotometer, and the activity was calculated in units per milliliter (U/mL). The temperature at which the highest activity was recorded was designated as the optimum temperature, offering valuable insight into the thermal behavior and potential application range of the enzyme (El-Sayed *et al*., 2015).

3.8.2.1 Temperature Stability Assay of L-Methionase

The thermal stability of purified L-methionase was evaluated by produce the enzyme in 50 mM potassium phosphate buffer (pH 7.5) at 20°C, 30°C, 40°C, 50°C, 60°C, and 70°C for 15, 30, 45, and 60 minutes. Equal volumes of enzyme and buffer were mixed and pre-incubated in temperature-controlled water baths without the addition of substrate to assess the enzyme's structural stability under heat stress. Following each time interval, samples were rapidly cooled on ice to halt any further thermal denaturation.

Residual enzyme activity It was developed utilizing the Nessler's reagent assay, employing L-methionine as the substrate. The amount of ammonia released during the reaction was quantified by measuring absorbance at 450 nm using a UV-visible spectrophotometer. Enzyme activity was expressed relative to a control sample maintained at 4°C, enabling a comparative analysis of stability across the range of tested temperatures and incubation periods.

3.8.3 Determination of Metal Ions on L-Methionase Enzyme Activity

The impact of selected metal ions and chemical agents on L-methionase activity was evaluated to identify potential activators or inhibitors. Stock solutions of Na^{+}, K^{+}, Mg^{2+}, Ca^{2+}, Zn^{2+}, Fe^{2+}, Mn^{2+}, Cu^{2+}, EDTA, SDS, and β-mercaptoethanol were prepared at 50 mM and diluted to final concentrations of 5 mM and 1 mM in the assay (El-Sayed *et al*., 2015).

Each test was conducted in a reaction mixture containing purified L-methionase, L-methionine substrate, and 50 mM potassium phosphate buffer (pH 7.5). Additives were introduced before starting the reaction, which was incubated under optimal conditions.

Enzyme activity was assessed using the Nessler's reagent method, with absorbance read at 450 nm. Results were expressed as a percentage of the activity observed in a control reaction without additives, allowing comparison of the effects on enzymatic function.

3.9 Kinetic Study of L-Methionase

The kinetic parameters of purified L-methionase were determined using varying concentrations of L-methionine substrate: 1 mM, 5 mM, 10 mM, 25 mM, and 50 mM. Each reaction mixture contained 700 µL of 50 mM potassium phosphate buffer (pH 7.5) and 100 µL of purified enzyme. The mixtures were incubated under optimal temperature and time conditions (El-Sayed *et al*., 2015).

Enzyme activity was assessed using the Nessler's reagent method, with ammonia release quantified at 450 nm. A Lineweaver–Burk plot was constructed by plotting the reciprocal of substrate concentration (1/[S]) against the reciprocal of enzyme velocity (1/V). From the linear regression, Km and Vmax values were calculated to describe the enzyme's affinity for its substrate and its maximum reaction rate.

3.10 *In Vitro* Cytotoxic Assay for Anticancer Activity of Purified L-Methionase

3.10.1 In Vitro Anticancer Assay Using MTT Assay

The cytotoxic potential of purified L-methionase was evaluated against HT-29 (human colorectal adenocarcinoma) and MDA-MB-231 (human breast adenocarcinoma) using the MTT assay. The assay quantifies cell viability by measuring the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to purple formazan crystals by mitochondrial dehydrogenase enzymes in metabolically active cells. The following sections detail the procedures for cell thawing, passaging, seeding, L-methionase treatment, MTT assay, and data analysis.

3.10.2 Cell Thawing

1. Culture media DMEM for HT-29 and MDA-MB-231 each supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (100 U/mL penicillin, 100 µg/mL streptomycin) were pre-warmed to 37°C in a water bath for 15–20 minutes.
2. Sterile T-25 culture flasks were labeled with the cell line name (HT-29 and MDA-MB-231,), passage number, and thawing date to ensure accurate tracking.

3. Cryovials containing frozen cells were retrieved from liquid nitrogen storage (-196°C) and immediately placed in a 37°C water bath. Vials were gently swirled for 1–2 minutes until only a small ice crystal remained, minimizing exposure to DMSO cryoprotectant.
4. In a Class II biosafety cabinet, cryovials were disinfected with 70% ethanol. The cell suspension was gently transferred to a 15 mL conical tube containing 5 mL of pre-warmed culture medium to dilute the cryoprotectant and reduce cell stress.
5. Cells were centrifuged at 300 × g for 5 minutes at room temperature (22–25°C) to pellet the cells. The supernatant was carefully aspirated using a sterile pipette, ensuring the cell pellet remained undisturbed.
6. The pellet was resuspended in 5 mL of fresh culture medium by gentle pipetting to avoid cell clumping. The suspension was transferred to a T-25 flask containing an additional 5 mL of pre-warmed medium (total volume: 10 mL).
7. Flasks were incubated at 37°C in a humidified incubator with 5% CO_2. Cell adherence and morphology were monitored after 4–6 hours using an inverted microscope (10 × magnification) to confirm cell health.
8. After 24 hours, the medium was replaced with 10 mL of fresh pre-warmed medium to remove residual DMSO and non-adherent cells. Flasks were returned to the incubator and monitored daily for growth and confluency.

3.10.3 Cell Passaging

1. Cells were passaged when they reached 70–80% confluency, typically every 2–3 days, as determined by microscopic observation. Culture medium, PBS (pH 7.4), and 0.25% trypsin-EDTA were pre-warmed to 37°C.
2. In a biosafety cabinet, the culture medium was aspirated from the flask using a sterile pipette. The cell monolayer was gently washed with 5 mL of sterile PBS to remove residual FBS, which could inhibit trypsin activity.
3. Added 1–2 mL of 0.25% trypsin-EDTA to the flask, ensuring even coverage of the cell monolayer. Flasks were incubated at 37°C for 3–5 minutes, with periodic microscopic checks to confirm cell detachment. Gentle tapping of the flask was used if needed to facilitate detachment.

4. Trypsin was neutralized by adding 5 mL of culture medium containing 10% FBS. The cell suspension was transferred to a 15 mL conical tube and centrifuged at 300 × g for 5 minutes at room temperature.
5. The supernatant was aspirated, and the cell pellet was resuspended in 5–10 mL of fresh culture medium by gentle pipetting to ensure a single-cell suspension.
6. A 20 µL aliquot of the suspension was mixed with 20 µL of 0.4% trypan blue, and viable cells were counted using a Neubauer hemocytometer under a microscope (10× magnification). Cell viability was typically >95%.
7. For subculturing, cells were seeded into new T-25 or T-75 flasks at a density of 1–2 × 10^4 cells/cm^2 (e.g., ~2 × 10^5 cells for a T-25 flask) in 10–20 mL of fresh medium. Flasks were incubated at 37°C in 5% CO_2, with medium replaced every 24 hours.
8. Cells were passaged every 2–3 days or upon reaching 70–80% confluency to maintain exponential growth and prevent over-confluence.

3.10.4 Cell Seeding for MTT Assay

1. Cells at 70–80% confluency were harvested. After counting, the cell suspension was diluted to 5 × 10^4 cells/mL in the respective culture medium using a sterile pipette.
2. Sterile 96-well flat-bottom tissue culture plates were labeled with the cell line, date, and experimental conditions. Using a multichannel pipette, 100 µL of cell suspension (5,000 cells/well) was dispensed into the inner 60 wells (rows B–G, columns 2–11) to ensure consistent cell distribution.
3. The outer wells (rows A and H, columns 1 and 12) were filled with 100 µL of culture medium to serve as blank controls and minimize evaporation-induced edge effects.
4. Plates were gently tapped to ensure even cell distribution and incubated at 37°C in a 5% CO_2 humidified incubator for 24 hours to allow cell adhesion and stabilization before treatment.

3.10.5 L-Methionase Treatment

1. Stock solutions of purified L-methionase were prepared in sterile PBS and stored at 4°C. Serial dilutions were made in the respective culture medium to achieve a

concentration range (25, 50, 100, 200, 400 µg/mL) based on preliminary dose-response studies.

2. After 24 hours of cell adhesion, the medium in each well was aspirated using a multichannel pipette. Each well was replaced with 100 µL of fresh medium containing the designated L-methionase concentration.
3. Control wells received 100 µL of medium without L-methionase to serve as the untreated control group. Positive controls (Doxorubicin 0.5 µg/mL) were included in separate wells if required by the experimental design.
4. Plates were incubated at 37°C in a 5% CO_2 humidified incubator for 24–48 hours, depending on the cell line and experimental objectives, to allow L-methionase to exert its cytotoxic effects.

3.10.6 MTT Assay

1. A stock solution of MTT (5 mg/mL in PBS) was prepared, filter-sterilized (0.22 µm), and stored at 4°C in the dark. After the treatment period, 20 µL of MTT solution was added to each well using a multichannel pipette, resulting in a final MTT concentration of approximately 0.83 mg/mL.
2. Plates were incubated at 37°C in a 5% CO_2 incubator for 3–4 hours to allow metabolically active cells to reduce MTT to purple formazan crystals. The incubation time was optimized for each cell line to ensure sufficient crystal formation without over-incubation.
3. The medium was carefully aspirated from each well using a multichannel pipette to avoid disturbing the formazan crystals. Then, 100 µL of molecular biology-grade DMSO was added to each well to dissolve the crystals.
4. Plates were placed on an orbital shaker for 5–10 minutes at room temperature to ensure complete dissolution of formazan crystals, achieving a uniform purple color.
5. Absorbance was measured at 570 nm using a microplate reader. Background absorbance from blank wells (medium only) was subtracted from all readings to correct for non-specific absorbance**.**

3.10.7 Data Analysis

1. Cell viability was calculated as a percentage relative to untreated control cells using the following equation:

   Cell Viability (%) = (Absorbance $_{treated}$ - Absorbance $_{Blank}$) / (Absorbance $_{control}$ - Absorbance $_{Blank)}$ x 100

2. The half-maximal inhibitory concentration (IC_{50}), defined as the L-methionase concentration required to reduce cell viability by 50%, was determined by plotting percent cell viability against the logarithm of L-methionase concentrations. A four-parameter logistic dose-response curve was fitted using GraphPad Prism (version 9.0).

3. Experiments were conducted in triplicate wells, with at least three independent biological replicates to ensure reproducibility. Data were reported as mean ± standard deviation (SD).

4. Statistical significance of differences in cell viability between treated and control groups was assessed using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test, with a significance threshold of $p < 0.05$.

3.11 Statistics

All experimental data presented in the form of figures and tables are expressed as mean ± standard deviation (SD). Statistical analyses were conducted using SigmaPlot (version 12; Systat Software Inc., Richmond, CA, USA), with one-way analysis of variance (ANOVA) employed where applicable to determine statistically significant differences among experimental groups. A p-value of less than 0.05 was considered indicative of statistical significance. Enzyme optimization studies were carried out using Design-Expert software (version 13; Stat-Ease Inc., Minneapolis, MN, USA). Figures were created using BioRender, and graphical representations of data were generated using GraphPad Prism.