Chapter 5.
Summary and Conclusion

This study focused on harnessing the enzymatic potential of soil-derived fungi from diverse ecological niches in Gujarat, India, to produce L-methionase, an enzyme with significant promise for cancer therapy due to its ability to degrade methionine, a critical nutrient for certain tumor cells. By targeting methionine-dependent cancers, L-methionase offers a novel therapeutic approach to selectively inhibit tumor growth. The research explored fungal biodiversity across marine, riverine, and terrestrial environments to identify strains with robust L-methionase production capabilities, optimizing their yield and evaluating their therapeutic efficacy.

A total of 50 fungal isolates were obtained from soil samples collected from various sites, including coastal regions (Porbandar, Dwarka, Dandi, Mandavi), riverine systems (AbuiltinAji, Nyari, Machhu Rivers), and agricultural lands (cotton fields, gardens). Coastal and agricultural soils exhibited the highest fungal diversity, likely due to favorable conditions such as high moisture, organic matter, and nutrient availability. The isolation process involved serial dilution and plating on Potato Dextrose Agar (PDA), followed by morphological identification using lactophenol cotton blue staining to examine hyphal structures and spore arrangements. Screening for L-methionase production was conducted in two phases: qualitative screening on modified Czapek-Dox agar with L-methionine and phenol red, where yellow halos indicated enzyme activity, and quantitative assessment using the agar well diffusion method and Nessler's reagent to measure ammonia release. Among the isolates, Aspergillus fumigatus MF13 emerged as the top performer, producing a 35 mm hydrolysis zone after 48 hours and a specific activity of 1.48 U/mg, indicating exceptional enzymatic potential.

Molecular identification of MF13 was achieved through ITS region sequencing of ribosomal DNA, revealing a 99.82% similarity to Aspergillus fumigatus, confirmed by phylogenetic analysis and registered under GenBank accession number OQ690549. This precise identification underscored the reliability of ITS-based methods for fungal classification, aligning with prior studies on enzyme-producing fungi.

To maximize L-methionase production, optimization was conducted using a One-Factor-at-a-Time (OFAT) approach, followed by statistical designs, including Plackett-Burman Design (PBD) and Central Composite Design (CCD). The OFAT experiments identified optimal conditions at pH 8.0,

30°C, 0.2% L-methionine, 3% inoculum size, and 7 days of incubation, with glucose and potassium nitrate as preferred carbon and nitrogen sources, respectively. The PBD screened nine variables, pinpointing temperature, yeast extract, and dipotassium phosphate as the most influential factors, with statistical significance confirmed by ANOVA ($p < 0.05$). The CCD further refined these parameters, achieving a maximum enzyme yield of 2.57 U/mL/min at 30°C, 2.4 g/L yeast extract, and 1.2 g/L dipotassium phosphate. The resulting quadratic model, with an F-value of 146.6 and $p < 0.0001$, demonstrated high accuracy, revealing synergistic interactions among the variables, particularly between temperature and yeast extract.

Enzyme purification involved a two-step process: cold acetone precipitation to concentrate the protein and remove impurities, followed by size-exclusion chromatography using a Sephadex G-75 column. This yielded a 10.5-fold purification, reducing protein content from 12.0 mg/mL to 2.36 mg/mL while increasing specific activity from 4.0 U/mg to 40.0 U/mg. SDS-PAGE analysis, using both silver and Coomassie Brilliant Blue staining, confirmed the enzyme's purity with a single band at approximately 45 kDa, consistent with reported molecular weights for fungal L-methionases (43–50 kDa).

Biochemical characterization revealed optimal enzyme activity at pH 7.5 and 30°C, with over 80% stability after 60 minutes at pH 7.5 and near 100% retention at 30°C. The enzyme was sensitive to metal ions, with Na^+ and Mn^{2+} enhancing activity at 1 mM, while Fe^{2+} and EDTA were inhibitory, confirming its metalloenzyme nature. Kinetic studies showed a Km of 0.674 mM and Vmax of 0.871 U/mL, indicating high substrate affinity and moderate catalytic efficiency, competitive with other microbial L-methionases.

The therapeutic potential of purified L-methionase was assessed via MTT assays on HT-29 (colon cancer) and MDA-MB-231 (triple-negative breast cancer) cell lines. HT-29 cells exhibited high sensitivity, with cell viability dropping to 25% at 400 µg/mL and an IC_{50} of approximately 175 µg/mL, accompanied by morphological changes indicative of apoptosis. In contrast, MDA-MB-231 cells were more resistant, retaining 55% viability at 400 µg/mL with an IC_{50} of ~390 µg/mL, suggesting variable methionine dependency. These results highlight L-methionase's selective cytotoxicity against methionine-dependent cancers, with potential challenges for less sensitive cell lines like MDA-MB-231.

Conclusion

This study underscores the value of fungal biodiversity in Gujarat's diverse soil ecosystems as a rich source of L-methionase-producing strains, with Aspergillus fumigatus MF13 demonstrating exceptional enzymatic potential. The rigorous optimization process, combining OFAT, PBD, and CCD, significantly enhanced enzyme yield, achieving a maximum of 2.57 U/mL/min under finely tuned conditions. The two-step purification protocol yielded a highly pure enzyme with a specific activity of 40.0 U/mg and a molecular weight of ~45 kDa, exhibiting optimal performance at pH 7.5 and 30°C, robust stability, and high substrate affinity (Km = 0.674 mM). These characteristics position L-methionase as a promising candidate for industrial and therapeutic applications.

The in vitro anticancer assays revealed a strong dose-dependent cytotoxic effect, particularly against HT-29 colon cancer cells ($IC_{50} \approx 175$ µg/mL), with morphological evidence of apoptosis, affirming its potential as a targeted therapy for methionine-dependent tumors. However, the limited efficacy against MDA-MB-231 cells ($IC_{50} \approx 390$ µg/mL) suggests inherent resistance, possibly due to alternative metabolic pathways or upregulated methionine synthase activity. This variability underscores the need for combination therapies or enzyme modifications to enhance efficacy across diverse cancer types.

The findings contribute significantly to the field of enzyme-based biotherapeutics, highlighting the scalability and therapeutic promise of fungal-derived L-methionase. Future research should focus on overcoming resistance in less sensitive cancers, exploring synergistic therapies, and advancing toward preclinical and clinical trials to validate L-methionase's role in cancer treatment. Additionally, integrating advanced computational models, such as artificial neural networks, could further optimize production, paving the way for cost-effective, large-scale enzyme manufacturing for therapeutic applications.