Chapter 2.
Review of Literature

Cancer continues to be among the leading causes of death and morbidity globally and is the second most common cause of death in wealthy nations, after cardiovascular illnesses (Saini et al., 2020). Normal cells can undergo malignant transformation due to genetic alterations, including mutations in DNA repair genes, tumor suppressor genes, oncogenes, and genes involved in cell growth and differentiation. In 2012, 14 million new cases of cancer and 8 million deaths from cancer occurred worldwide. One million cancer diagnoses and 700,000 cancer-related fatalities were reported in India alone by 2014 (Siegel *et al*., 2022).

The rise in the incidence of cancer can be attributed to several factors, including an aging population, unhealthy lifestyles, tobacco use, poor diet, and improvements in diagnostic facilities. According to the World Health Organization (WHO), there were 8.8 million cancer-related deaths globally in 2015, and this number is projected to rise to 23 million new cancer cases by 2023 (Eloranta *et al*., 2021).

Both external factors (e.g., radiation, smoking, pollution, infectious organisms) and internal factors (e.g., genetic mutations, immune conditions, hormonal imbalances) contribute to cancer development. Smoking habits, population increase, and aging populations are the main causes of the worldwide cancer burden. Tobacco, a severe carcinogen, is strongly connected with cancers of the esophagus, lungs, hypopharynx, mouth, and tongue (Jain *et al*., 2021; Slob *et al*., 2020).

A higher risk of lung, colon, bladder, breast, leukemia, multiple myeloma, non-Hodgkin lymphoma (NHL), and prostate cancer has also linked to exposure to agricultural pesticides, including herbicides, insecticides, and fungicides like alachlor, carbaryl, metolachlor, pe ndimethalin, permethrin, and trifluralin (Melanda *et al*., 2022; Miousse, 2023).

Paternal and maternal pesticide exposure during pregnancy has also been associated with childhood leukemia, brain tumors, and other cancers (Schmidt *et al*., 2021; *Gajjar et al*., 2018; Karalexi *et al*., 2021). Pesticide exposure promotes the formation of reactive oxygen species (ROS) in tissues, leading to significant DNA and protein damage, thereby contributing to mutagenesis (Shah *et al*., 2020).

There is an urgent need for therapeutic enzymes with anticancer applications, such as L-asparaginase, cystathionine-γ-lyase, L-methionase, L-glutaminase, and L-arginase. These enzymes function by depriving cancer cells and pathogenic organisms of essential amino acids (Sugathan *et al*., 2017). Microorganisms offer an economical and efficient source for enzyme production, as they can be cultivated in inexpensive media and produce enzymes within a short time frame. Various screening methods are available to identify microbial isolates capable of producing specific enzymes.

2.1 Methionine Dependency in Cancer

Methionine dependency is a distinctive metabolic feature observed in various malignant cell lines, setting them apart from normal healthy cells. The survival and multiplying of many cancer cells are completely dependent on exogenous methionine, compared to normal cells that are capable of using homocysteine for their methionine requirements. (Kaiser, 2020).

This metabolic vulnerability has significant therapeutic implications. Methionine plays a vital role in cellular methylation reactions, including DNA, RNA, and protein methylation, which are critical for maintaining malignant transformation and rapid tumor growth. Depriving cancer cells of methionine can selectively hinder their growth and viability while sparing normal cells.

Experiments conducted in cell culture systems (*in vitro*) have demonstrated that culturing methionine-dependent cancer cells in methionine-free media, especially when combined with antimitotic agents, leads to a targeted arrest of cancer cells during the cell cycle's late S/G2 phase. This selective arrest prevents tumor cells from progressing to mitosis, thereby inhibiting their proliferation (Hoffman & Yano, 2019). Importantly, normal cells do not exhibit the same level of sensitivity, emphasizing the selectivity of methionine depletion strategies.

Animal model studies (*in vivo*) have further validated these findings. Dietary restriction of methionine in methionine-dependent tumor-bearing animals resulted in a significant extension of lifespan and a notable reduction in metastasis rates. However, complete tumor growth arrest was not achieved solely through dietary methionine deprivation. Residual serum methionine levels, maintained even under dietary restriction, may continue to support minimal tumor activity, highlighting the potential need for combination strategies (Hoffman & Yano, 2019).

2.2 L-Methionase

According to Suganya *et al*. (2017a), L-methionase is a carbon-sulfur lyase enzyme that is a member of the lyase family. This enzyme, which is dependent on pyridoxal phosphate (PLP), has great therapeutic promise, especially for the treatment of cancer (Inagaki, n.d.). L-methionase also participate in therapeutically reducing elevated homocysteine (Hcy) levels, which are recognized as a potential risk factor for cardiovascular diseases, Alzheimer's disease, and coronary artery disease (Kaiser, 2020).

PLP reduces the energy required for the conversion of amino acids into a zwitterionic carbanion, enabling the apo-enzyme to catalyze substrate bond cleavage and product formation (Richard & Amyes, 2004). PLP-dependent enzymes combine the lysine residue's ε-amino group at the active site to generate an internal aldimine, which acts as a coenzyme to raise the acidity of the catalytic site in its normal state (Boeri *et al*., 2011).

The presence of L-methionine in the culture media usually causes the cytosolic enzyme L-methionase to be synthesized (Kahraman *et al*., 2011). It comprises four identical subunits, each with a molecular weight between 41 and 45 kDa, resulting in a total molecular weight ranging from 149 to 173 kDa (M. H. Selim, Karm Eldin, *et al*., 2015). Nevertheless, it was discovered that the two separate subunits of L-methionase that were extracted and purified from Pseudomonas putida weighed 40 kDa and 48 kDa, respectively (Hoffman, 2015).

Humans require dietary intake of L-methionine to facilitate the biosynthesis of L-cysteine via the trans-sulfuration pathway (B. Sharma *et al*., 2014). On the other hand, microbes can use the de novo cysteine biosynthesis route to create sulfur-containing amino acids from inorganic sulfate. For instance, *E. coli* and plants can synthesize L-methionase through either the forward trans-sulfuration pathway using cysteine or via the de novo cysteine biosynthesis pathway utilizing inorganic sulfate (Cavuoto & Fenech, 2012).

According to the MetaCyc database, different organisms have unique pathways for the production of L-methionase. In *E. coli* K-12, L-methionase is synthesized via the L-methionine biosynthesis-I pathway, which includes the conversion of homoserine through trans-sulfuration (Zhang *et al*., 2022). *Arabidopsis thaliana* follows the L-methionine biosynthesis-II pathway, which involves synthesis from homoserine-II.

Other organisms like *Leptospira meyeri*, *Saccharomyces cerevisiae*, and *Corynebacterium glutamicum* utilize the L-methionine biosynthesis-III pathway, involving homoserine conversion and sulfhydrylation. Additionally, A belongs to the L-methionine salvage-I pathway, which is necessary for Rattus norvegicus and Homo sapiens, but the L-methionine salvage-II pathway is followed by *Bacillus subtilis, Klebsiella oxytoca*, *Klebsiella pneumoniae, Lupinus luteus,* and *Oryza sativa.*

Several bacteria including *B. subtilis*, *C. glutamicum*, *L. meyeri*, *Pseudomonas aeruginosa*, *Pseudomonas putida*, and A different super pathway of L-methionine synthesis via sulfhydrylation is linked to S. cerevisiae. In the meanwhile, *A. thaliana, L. luteus, O. sativa, Plantago* major, and *Solanum lycopersicum* perform the Yang cycle, which is also referred to as the MTA cycle (Caspi *et al*., 2014).

Furthermore, *Trichomonas vaginalis* and *Entamoeba histolytica* possess parts of a de novo sulfide biosynthesis pathway and an L-methionine catabolic pathway for cysteine formation (Jeelani *et al*., 2017). These metabolic differences between humans and parasites offer promising opportunities for the expansion of antiparasitic agents in the future.

2.3 Other Anti-Cancer Enzymes

2.3.1 L-Asparaginase with Mechanism

L-asparaginase, also known as L-asparagine amidohydrolase, catalyzes the hydrolysis of L-asparagine into aspartic acid and ammonia. It was the first enzyme extensively studied for its anti-leukemic properties (Van Trimpont *et al*., 2022). Beyond its clinical applications, L-asparaginase significantly reduces acrylamide formation, a potent carcinogen produced during the Maillard reaction in food processing (Muneer *et al.,* 2020). It is widely utilized in clinical trials for tumor therapy, food technology, and pharmaceutical production, often in combination with chemotherapy. There are several known bacterial strains that produce L-asparaginase, such as *Pseudomonas aeruginosa* (Kuwabara *et al*., 2015), *Bacillus subtilis* (Ameen *et al*., 2020), *Escherichia coli* (Medawar *et al*., 2020), *Serratia marcescens* (Kumar, 2014), Erwinia carotovora (Warangkar & Khobragade, 2010), and *Escherichia coli* (Medawar *et al*., 2020). Several fungal species such as *Aspergillus tamarii*, *Aspergillus niger*, and *Aspergillus terreus* have also been reported as significant producers of this enzyme (Sanjotha & Manawadi, 2017). Moreover, L-asparaginase production has been documented in various fungi, yeasts, and algae.

2.3.2 L-Glutaminase with Mechanism

L-glutaminase is another critical enzyme with potent anticancer and antiretroviral properties. It decreases the availability of glutamine, a necessary food for rapidly developing tumor cells, by catalyzing its breakdown into glutamic acid and ammonia. (Unissa *et al*., 2014). Additionally, it inhibits HIV reverse transcriptase activity. The therapeutic value of glutaminase lies in its ability to starve glutamine-dependent cancer cells (Yulianti *et al*., 7 C.E.). Clinically, it has been employed to treat of acute lymphocytic leukemia, as well as colon and liver cancers. In the food industry, glutaminase enhances flavor and aroma by increasing glutamic acid levels in fermented products (Barzkar *et al*., 2021). It plays a vital role in soy sauce fermentation, with salt-tolerant strains like *Stenotrophomonas maltophilia* being utilized extensively (Binod *et al*., 2017). Glutaminase activity exhibit an across variety of biological domains, including plants, animals, and microorganisms such as *Escherichia coli*, *Pseudomonas*, *Acinetobacter*, and *Bacillus* species (Karim & Thalij, 2016).

2.3.3 L-Arginase with Mechanism

The breakdown of L-arginine to L-ornithine and urea is catalyzed by L-arginase, an essential enzyme in the urea cycle (Zolfaghar *et al*., 2019). It was first identified in bacteri *Bacillus subtilis* (Ameen *et al*., 2020), *Escherichia coli* (Medawar *et al*., 2020), *Serratia marcescens* (Kumar, 2014), Erwinia carotovora (Warangkar & Khobragade, 2010), and *Escherichia coli* (Medawar *et al*., 2020). Several fungal species such as *Aspergillus tamarii*, *Aspergillus niger*, and *Aspergillus terreus* have also been reported as significant producers of this enzyme (Sanjotha & Manawadi, 2017). Moreover, L-asparaginase production has been documented in various fungi, yeasts, and algae.

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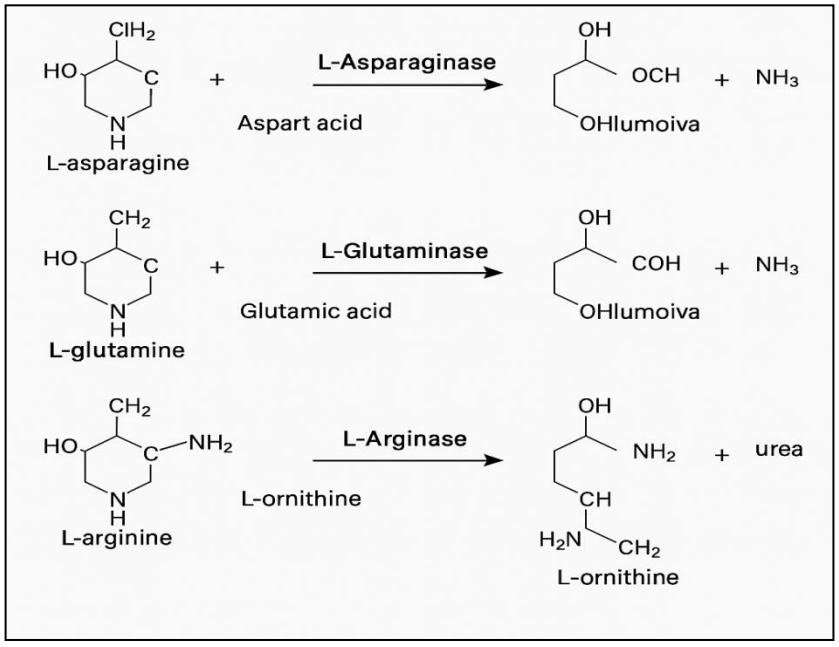

Figure 2.1 Hydrolytic Reactions Catalyzed by Anti-cancer Enzymes (El-Sayed et al., 2021)

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2.4 L-Methionase Mechanism of Action

By cleaving the α-γ bond of L-methionine, L-methionase catalyzes the direct conversion of L-methionine into α-ketobutyrate, ammonia, and methanethiol (With et al., 2016; B. Sharma et al., 2014) (Figure 2.2).

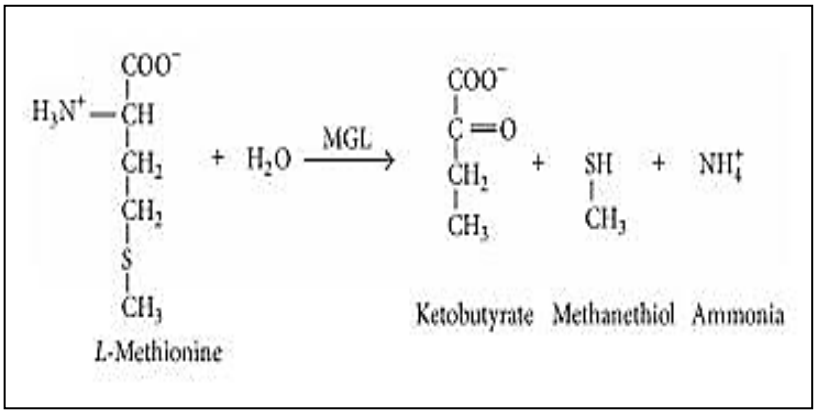

Figure 2.2 Formulation of the reaction for the L-methionase and PLP-catalyzed L-methionine (Yao *et al*., 2020)

The enzymatic activity of L-methionase proceeds through the following sequential steps:

1. **Development of External Aldimine**: The amine group of L-methionine attacks the internal aldimine (Schiff's base) structure of the enzyme-PLP complex. This leads to the formation of an external aldimine and the release of the lysine residue from the active site.

2. **Deprotonation at the α-Position**: The enzyme then abstracts a hydrogen atom from the α-position of the methionine molecule, leading to the development of a ketoimine intermediate.

3. **Formation of Quinonoid Intermediate**: A quinonoid intermediate is generated as the thiol group (−SH) is released, following the donation of a hydrogen atom to the β-position of the tyrosine hydroxyl group in the active site.

4. **Hydrolytic Cleavage**: The imine bond is subsequently attacked by a water molecule, resulting in the release of α-ketobutyrate as a product.

5. **Internal Aldimine Formation:** When the lysine residue of L-methionase reattacks the PLP complex, a new internal aldimine structure is formed, and ammonia is produced as a byproduct.

2.5. Structure of L-Methionase

L-methionine-γ-lyase (MGL), also referred to as L-methionine methanethiol-lyase or L-methionine demethylase, is a pyridoxal phosphate (PLP)-dependent enzyme that plays an

essential role in catalyzing the cleavage of carbon-sulfur bonds in L-methionine. This catalytic activity enables the conversion of L-methionine into α-ketobutyrate, methanethiol, and ammonia, a reaction central to amino acid metabolism and targeted cancer therapy (N. Z. Khalaf *et al*., 2023).

The enzyme functions by stabilizing zwitterionic carbanion intermediates, thereby reducing the energy barrier required for bond cleavage (With *et al*., 2016). L-methionine, when supplemented in the growth medium, has been observed to induce the biosynthesis of MGL, particularly in species like *Pseudomonas putida*.

MGL typically exists as a homotetramer, composed of four identical subunits. The N-terminal domain, the PLP-binding (pyridoxal) domain, and the C-terminal domain make up each subunit (Figure. 2.3) Structural studies of *P. putida* MGL reveal detailed three-dimensional organization, where the active site includes a PLP moiety covalently bound to a conserved lysine residue within the enzyme.

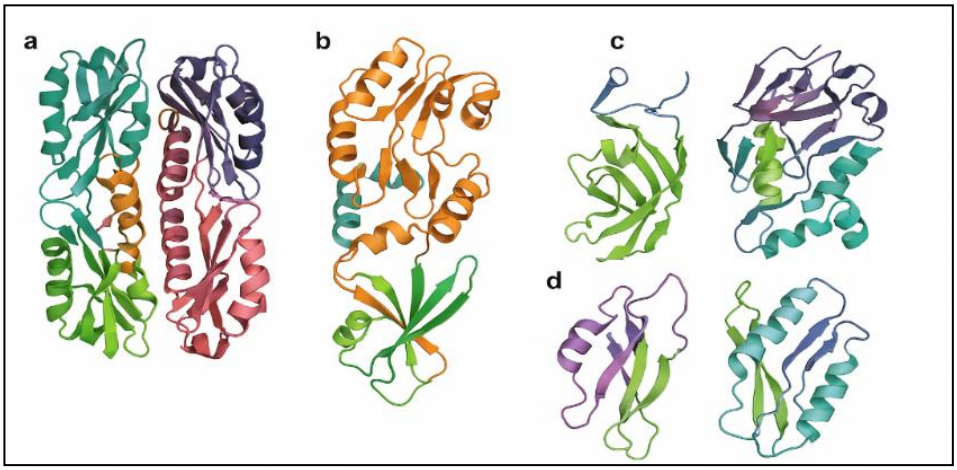

Figure 2.3. Three-dimensional structure of *P. putida* L-methionase (Batool *et al*., 2022)

L-methionase is also essential for the production of L-cysteine via the trans-sulfuration pathway in humans (Jeelani *et al*., 2017). In yeast, external supplementation of both methionine and cysteine is required for their biosynthesis, whereas microbes such as *E. coli* and plants are capable of synthesizing sulfur-containing amino acids using inorganic sulfate through the de novo cysteine biosynthesis pathway (Ebid Abdelsalam Fergany & Balbol, 2022).

The MetaCyc database outlines organism-specific pathways for methionine synthesis:

- **Trans-sulfuration-I pathway** is utilized by *Escherichia coli* K-12, converting homoserine to methionine.
- **Methionine biosynthesis-II pathway** is followed by *Arabidopsis thaliana*.
- *Corynebacterium* glutamicum (Reershemius & Vorlop, 2008), *Leptospira meyeri*, and *Saccharomyces cerevisiae* are known to exhibit the methionine biosynthesis-III route (Ogawa *et al.,* 2022).
- **Methionine salvage-I pathway** is employed by *Klebsiella oxytoca* and *Klebsiella pneumoniae*.

Super pathways for methionine biosynthesis, combining features of other pathways, are utilized by organisms like *Pseudomonas aeruginosa*, *Pseudomonas putida*, and *Saccharomyces cerevisiae* (B. Sharma *et al*., 2014).

2.6. Source of L-Methionase

L-methionase is found in many different bacteria, although it is especially expressed in *Pseudomonas* species. Its expression is typically induced by the supplementation of L-methionine in the culture medium. Numerous organisms, including *Pseudomonas putida, Citrobacter freundii, Trichomonas vaginalis*, and *Entamoeba histolytica,* have been found to have crystal structures of L-methionase.

This enzyme has been isolated from a broad range of biological sources, including bacteria, protozoa, fungi, archaea, and plants, emphasizing its evolutionary and functional significance across diverse life forms.

The diverse distribution of L-methionase suggests its crucial role in sulfur metabolism and potential applications in biotechnology and therapeutic industries. A detailed summary of various organisms from which L-methionase has been isolated is offered in (Table 2.1).

Table 2.1 Sources of L-Methionase Enzyme

Source Type	Organism	Reference
Bacteria	*Bacillus subtilis*	(Kharayat & Singh, 2018)
	Brevibacterium linens	(Ipek *et al*., 2023)
	Clostridium tetani	(Morozova *et al*., 2017)
	Clostridium sporogenes	(Pokrovsky *et al*., 2018)
	Citrobacter intermedius	(Lin *et al*., 2017)
	Citrobacter freundii	(Ronda *et al*., 2011)
	Porphyromonas gingivalis	(Yoshimura *et al*., 2000)
	Pseudomonas ovalis	(Kannan *et al*., 2021)
	Pseudomonas putida	(Hoffman, 2015)
	Streptomyces variabilis	(Kavya & Nadumane, 2020)
	Treponema denticola	(Fukamachi *et al*., 2005)
	Corynebacterium glutamicum	(Bonnarme *et al*., 2000)
Protozoa	*Trichomonas vaginalis*	(Tokoro *et al*., 2003)
	Entamoeba histolytica	(Jeelani *et al*., 2017)
Plant	*Arabidopsis thaliana*	(Reda *et al*., 2023)
Archaeon	*Ferroplasma acidarmanus*	(Baumler *et al*., 2007)
Fungus	*Aspergillus* sp. RS-1a	(El-Shora *et al*., 2021)
	Geotrichum candidum	(Bonnarme, Lapadatescu, *et al*., 2001)
	Aspergillus flavipes, *Aspergilluscarneus*,	(El-Shora *et al*., 2021) (Khalaf & El-Sayed, 2009).
	Fusarium solani, *Penicillium notatum*,	(S. A. El-Sayed *et al*., 2017)
	Chaetomium globosum	(Shimaa *et al*., 2016)
	Candida tropicalis	(M. H. Selim, Karm Eldin, *et al.,* 2015)

2.7 Screening of Fungal Isolates for L-Methionase Production

Qualitative screening of fungal isolates for L-methionase production is a critical first step in identifying strains with promising enzyme activity. This method offers a rapid, cost-effective means of evaluating numerous isolates, allowing researchers to distinguish between producers and non-producers of the enzyme efficiently (Kumar *et al*., 2011).

Early screening plays a pivotal role in conserving both time and resources while providing initial insights into the enzymatic capabilities of various fungi (El-Sayed *et al*., 2019).

Simple indicator-based media are widely used in this phase to facilitate visual detection of L-methionase activity. These media typically undergo color changes in response to enzymatic reactions, offering an easy and direct method for detecting enzyme presence (Sahu *et al*., 2020). This visual method is especially valuable for screening large numbers of isolates without relying on expensive analytical tools.

As a preliminary assessment tool, qualitative screening lays the groundwork for further quantitative and optimization studies. It supports a structured research process by narrowing down high-yielding strains for detailed evaluation and potential application in biotechnology and therapeutics (Selim *et al*., 2021).

Overall, this method represents a strategic and foundational approach to advancing L-methionase research, with significant implications for therapeutic enzyme development and industrial processes.

2.8 L-Methionase Activity Assay Methods

Quantitative assays are essential for accurately measuring enzymatic activity by evaluating reaction rates and the production of specific products. *L*-methionase, a pyridoxal-5-phosphate (PLP)-dependent enzyme, catalyzes the conversion of *L*-methionine into methanethiol, α-ketobutyrate, and ammonia.

This enzyme has garnered considerable interest in the extension of cancer therapy, owing to its ability to selectively target and deprive tumor cells of methionine, an amino acid vital for the proliferation of rapidly dividing cells (Javia *et al.*, 2024), (Sharma *et al.*, 2014). Quantitative evaluation methods facilitate the identification of potent microbial strains or engineered systems that demonstrate elevated enzyme activity, a key requirement for therapeutic development and industrial-scale production (Kotramada *et al.*, 2020), (Selim *et al.*, 2016), (Aldawood & Al-Ezzy, 2024).

2.8.1 Ellman's Method:

Ellman's method quantifies L-methionase activity based on the detection of methanethiol, a product of L-methionine decomposition. The method uses 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), which reacts with free sulfhydryl groups to produce a yellow-colored compound, 2-nitro-5-thiobenzoate (TNB). This color change is measurable at 412 nm and indicates methanethiol production (Sharma and Kanwar, 2015). DTNB is commonly incorporated into agar media, where colonies expressing L-methionase can be visually identified by the surrounding yellow halo. The high specificity of DTNB for thiol groups under neutral pH, along with its rapid reactivity and strong absorbance, make it a preferred reagent for this assay (Ashkan *et al.*, 2023; Kulikova *et al*., 2017).

2.8.2 Nicotinamide Adenine Dinucleotide (NADH) Method:

This method involves monitoring the decrease in NADH absorbance at 340 nm, which correlates with the production of α-ketobutyrate, a direct product of L-methionine degradation. The assay provides a reliable estimate of enzymatic conversion efficiency (Kulikova *et al*., 2017).

2.8.3 MBTH Method:

The 3-methyl-2-benzothiazolonehydrazone (MBTH) method is a colorimetric assay used to measure α-ketobutyrate levels produced during L-methionine degradation. Upon reacting with MBTH and incubating at 50 °C for thirty minutes, a stable azine derivative is formed, which exhibits maximum absorbance at 320 nm. This method offers high sensitivity and is commonly used in spectrophotometric analyses of L-methionase activity (Soda, 1968; Goyer *et al*., 2007; Muharram, 2016; Sato & Nozaki, 2009; Khan, 2011; Kannan *et al*., 2021).

2.8.4 Nessler's Method:

Named after Julius Nessler, this method detects ammonia, one of the by-products of L-methionine cleavage by L-methionase. The assay uses Nessler's reagent (K_2HgI_4), which reacts with ammonia to form a yellow to brown precipitate. The intensity of the color, measured spectrophotometrically at 480 nm, correlates with the amount of ammonia released, thereby indicating enzymatic activity (Kharayat & Singh, 2018; Sanjotha & Manawadi, 2017; Tokoro *et al*., 2003).

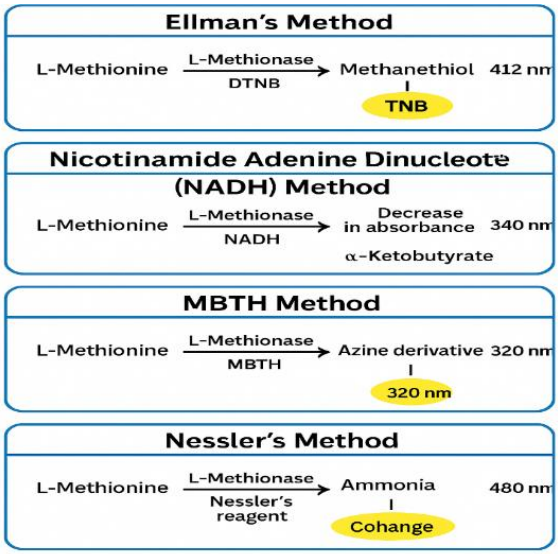

Figure 2.4. Schematic Representation of Various L-Methionase Activity Assay Methods (Sato & Nozaki, 2009; Gojgini *et al*., 2021)

2.9 Production of L-Methionase Enzyme

2.9.1 Optimization of L-Methionase Production

Optimization of environmental and nutritional parameters is essential to enhance the yield of L-methionase during fermentation processes. The one-factor-at-a-time (OFAT) method remains a widely used approach for this purpose. Although it can be time-consuming, OFAT is effective for identifying the individual influence of each variable by varying one factor while keeping others constant, thus determining the high and low thresholds critical for enzyme production (Alshehri, 2020).

2.9.2 Optimum Culture Medium

Optimization of the culture medium plays a crucial role in maximizing L-methionase production. Various carbon and nitrogen sources have been investigated to improve enzyme synthesis from fungal isolates, particularly *Aspergillus spp***.** (M. Hendy *et al.*, 2021). Research has indicated that the choice of carbon source significantly influences enzyme yield. For instance, glucose has been identified as the most effective carbon source for *Trichoderma harzianum*, with enhanced enzyme production observed when glucose and lactose were both included in the medium using statistical optimization techniques (Salim *et al*., 2019).

Similarly, nitrogen source optimization has shown a profound impact on L-methionase biosynthesis. Kavya and Nadumane (2020) reported that several nitrogen sources, such as peptone, yeast extract, ammonium sulfate, and potassium nitrate (at 1% w/v concentration),

were individually tested and in various combinations to optimize L-methionase production from *Methylobacterium sp*. JUBTK33. They employed response surface methodology (RSM), a statistical optimization tool, to effectively determine the best combination of nutrients that significantly boosted enzyme activity.

Overall, strategic selection and optimization of media components are fundamental steps toward achieving high-yield production of L-methionase, which is an essential for its biotechnological and therapeutic applications.

2.9.3 Optimum pH for L-Methionase Production

The production and activity of microbial enzymes are highly sensitive to the pH of the surrounding environment. pH influences the surface charges of amino acids within the enzyme structure, which can either enhance or inhibit enzymatic activity, ultimately affecting both enzyme production and microbial growth dynamics.

For L-methionase production, the optimal pH varies slightly depending on the microbial species used:

- *Aspergillus fumigatus* and *Aspergillus flavipes* demonstrated maximum L-methionase production at a neutral pH of 7.0. This finding was established through statistical optimization techniques such as the Plackett-Burman design and Central Composite Design (Sanjotha & Manawadi, 2017).
- In bacterial species, *Pseudomonas stutzeri* and *Escherichia coli*, maximum levels of L-methionase production were observed within a slightly alkaline pH range of 7.5 to 8.0 (Kharayat & Singh, 2019; Ebid Abdelsalam Fergany & Balbol, 2022).
- Similarly, in the case of *Trichoderma viride,* the optimum pH for L-methionase production was found to be 8.0, yielding an enzyme activity of 1077.23 ± 906.106 IU/g following a submerged fermentation process (Dhankhar *et al*., 2020; Baghdadi & Danial, 2022).

Maintaining an optimal pH not only ensures maximum enzymatic activity but also stabilizes the growth environment for microbial strains, leading to improved L-methionase yield. Slightly alkaline conditions are generally favorable for many L-methionase-producing microorganisms, emphasizing the importance of precise pH control in fermentation systems.

2.9.4 Optimum Temperature for L-Methionase Production

Temperature plays a crucial role in regulating enzymatic activity, primarily by influencing the ionization states of the enzyme's active site and its substrate. These changes directly impact the rate of the biochemical reaction. The effectiveness of temperature on enzyme activity is governed by a number of factors, including the enzyme structural stability, the persistence and concentration of the substrate, and the duration of thermal exposure.

In that case of L-methionase, determining the optimal temperature for its production is essential for maximizing enzymatic yield. A study conducted on *Hafnia alvei* involved culturing the organism at various incubation temperatures 25°C, 30°C, 35°C, 37°C, 40°C, and 45°C. Following incubation, the crude extract from each condition was evaluated using a standard L-methionase activity assay. The results identified 37°C as the optimal temperature, at which the enzyme exhibited the highest specific activity (Alshehri, 2020).

This optimum aligns with the mesophilic nature of most L-methionase-producing organisms, where moderate temperatures not only preserve the structural integrity of the enzyme but also support robust microbial growth and metabolism.

2.9.5 Optimum Incubation Period for L-Methionase Production

The incubation period is a critical parameter influencing microbial growth, metabolic activity, and ultimately, L-methionase production. As the duration of incubation increases, there is generally a corresponding rise in enzyme activity, biomass yield, and medium pH, until an optimal point is reached. Beyond this period, enzyme activity may plateau or decline due to nutrient depletion or the accumulation of inhibitory metabolic by-products.

According to Ekpenyong *et al*. (2017), a bacterial culture identified as *Pseudomonas sp.* was incubated for different time intervals ranging from 24 to 120 hours, with L-methionase activity assessed at 24-hour intervals. The study revealed that maximum enzyme activity (0.68 U/mL/min) was achieved after 24 hours of incubation, indicating rapid enzyme synthesis during the early log phase of growth.

In contrast, *Aspergillus sp.,* a fungal isolate, demonstrated optimal L-methionase production during 7th day of incubation. This longer incubation requirement is characteristic of filamentous fungi and was determined using both One-Factor-At-a-Time (OFAT) and statistical optimization methods (El-Sayed, 2009).

These results demonstrate the organism-specific nature of incubation period optimization, emphasizing the need for tailored protocols based on microbial physiology and growth kinetics.

2.9.6 Optimum Substrate Concentration for L-Methionase Production

Substrate concentration plays a crucial role in regulating the biosynthesis of enzymes, including L-methionase. An appropriate concentration of the substrate not only provides the necessary precursors for enzyme production but also helps in maintaining cellular metabolic balance. Excessive substrate levels may lead to substrate inhibition, metabolic burden, or the formation of unwanted by-products, whereas insufficient substrate can limit microbial growth and enzymatic expression.

Numerous studies have shown the significance of adjusting the amount of L-methionine in the culture medium with respect to the formation of L-methionase. Alshehri (2020) reported that *Hafnia alvei* exhibited maximum L-methionase production when cultured in a medium supplemented with 1.5 g/L of L-methionine, beyond which no significant increase in the activity of enzyme was detected. Similarly, Ebid Abdelsalam Fergany and Balbol (2022) highlighted that optimal L-methionine concentrations were necessary to induce L-methionase production in *Trichoderma viride*, with the highest activity recorded at 2.0 g/L.

Additionally, Dhankhar *et al*. (2020) demonstrated that while an initial increase in substrate concentration proportionally enhanced enzyme activity in *Pseudomonas sp.*, concentrations above 2.5 g/L led to a decline, possibly due to substrate toxicity or feedback inhibition mechanisms. In the particular case of fungal isolates like *Aspergillus flavipes*, Sanjotha and Manawadi (2017) found that moderate concentrations of L-methionine (around 1.0–1.5 g/L) were sufficient to achieve peak enzyme activity, without causing inhibitory effects.

Therefore, determining the optimum substrate concentration is fundamental to maximizing L-methionase yield while avoiding potential negative impacts on microbial growth and enzyme stability. Careful substrate optimization ensures an efficient, economically viable production process for biotechnological and therapeutic applications.

2.9.7 Optimum Inoculum Size for L-Methionase Production

Inoculum size plays a vital role in determining the efficiency and yield of microbial enzyme production, including L-methionase. An appropriate inoculum size ensures the rapid establishment of microbial growth, balanced nutrient consumption, and effective enzyme

secretion. Conversely, an excessively low inoculum can delay microbial growth, prolong the lag phase, and result in insufficient enzyme production. However, an overly large inoculum can lead to early nutrient depletion, oxygen limitation, and metabolic stress, ultimately lowering the enzyme yield (Alshehri, 2020).

Many studies have shown the importance of optimizing inoculum volume for maximum L-methionase production. For instance, Alshehri (2020) reported that *Hafnia alvei* showed optimal L-methionase activity when a 5% (v/v) inoculum size was utilized in submerged fermentation conditions. Increasing the inoculum size beyond 5% led to a decline in enzyme activity, probably because of the excessive microbial load consuming available nutrients rapidly and generating toxic metabolites.

Similarly, Kharayat and Singh (2018) highlighted that in *Trichoderma viride* cultures, a 3–5% inoculum size was ideal for achieving maximum L-methionase production. A higher inoculum concentration negatively affected biomass and enzyme yield, possibly due to increased competition for dissolved oxygen and nutrient sources.

Moreover, Baghdadi and Danial (2022) reported that an optimal inoculum size of 4% favored maximal L-methionase activity from *Pseudomonas stutzeri*, while deviations from this concentration led to a significant decrease in enzymatic output.

These results indicate that careful optimization of inoculum size is crucial for maximizing L-methionase production. Optimizing the inoculum not only enhances enzyme yield but also ensures economic feasibility by reducing the fermentation time and improving process efficiency for therapeutic and industrial applications.

2.9.8 Optimum Carbon and Nitrogen Sources for L-Methionase Production

The selection of appropriate carbon and nitrogen sources is critical in maximizing L-methionase production. Carbon sources serve as the primary energy supply for microbial growth and metabolism, while nitrogen sources are essential for protein synthesis, including enzyme production. The characteristics and purpose of these nutrients significantly influence the metabolic pathways involved in enzyme biosynthesis.

2.9.8.1 Carbon Sources

Different microorganisms exhibit varying preferences for carbon sources when producing L-methionase. Simple sugars such as glucose are commonly preferred due to their easy assimilation and rapid energy release. Salim *et al*. (2019) reported that glucose was the most effective carbon source for enhancing L-methionase production in *Trichoderma*

harzianum under submerged fermentation conditions. Similarly, M. Hendy *et al.* (2021) discovered that the supplementation of glucose and lactose significantly improved L-methionase production in *Aspergillus* species when optimized through statistical methodologies.

In contrast, complex carbon sources or polysaccharides may sometimes inhibit enzyme production by introducing catabolite repression effects, where easily metabolized sugars suppress the synthesis of secondary metabolites like enzymes.

2.9.8.2 Nitrogen Sources

Nitrogen sources, whether organic or inorganic, are equally important for optimizing enzyme yield. Organic nitrogen sources like yeast extract, peptone, and casein hydrolysate have shown superior results in promoting L-methionase production. Kavya and Nadumane (2020) reported that peptone and yeast extract at 1% (w/v) concentration alone or in combined with significantly boosted enzyme production by *Methylobacterium sp.* JUBTK33.

Inorganic nitrogen sources like ammonium sulfate and potassium nitrate have also been explored. Although they are cost-effective and readily available, their efficiency in supporting enzyme production often falls short compared to organic sources. Nonetheless, combining both organic and inorganic nitrogen sources has been suggested to achieve balanced growth and maximize enzymatic output (Kavya & Nadumane, 2020).

Therefore, glucose and lactose serve as the optimal carbon sources, while peptone, yeast extract, and ammonium sulfate have been recognized as the preferred nitrogen sources for efficient L-methionase production across various microbial strains.

Optimizing carbon and nitrogen sources not only enhances enzyme yield but also ensures economic viability and scalability of the production process, which is critical for both industrial and therapeutic applications.

2.9.9 Plackett-Burman Design and Central Composite Design for L-Methionase Enzyme Production

Statistical experimental designs are indispensable tools for the efficient optimization of biotechnological processes, including the production of L-methionase. Among these, the Plackett-Burman Design (PBD) and Central Composite Design (CCD) have been widely used to systematically screen and optimize key factors influencing enzyme yields.

2.9.9.1 Plackett-Burman Design (PBD)

The Plackett-Burman Design is primarily employed for the initial screening of a significant quantity of variables to identify those that significantly affect the production process. In L-methionase production, PBD allows researchers to determine the most influential factors among variables such as pH, temperature, carbon source, nitrogen source, inoculum size, incubation time, and substrate concentration.

Sanjotha and Manawadi (2017) applied PBD to optimize L-methionase production from *Aspergillus flavipes*. Their findings revealed that pH, duration of incubation, and glucose concentration were the critical parameters positively influencing enzyme activity. Similarly, Dhankhar *et al.* (2020) demonstrated that factors such as carbon and nitrogen source concentration had a significant impact on L-methionase production in *Pseudomonas* species. The use of PBD streamlines the optimization process by reducing the number of experimental trials required, thereby saving time and resources while ensuring statistically reliable results.

2.9.9.2 Central Composite Design (CCD)

Following the initial screening by PBD, the Central Composite Design is often used for a more refined optimization. CCD is a part of Response Surface Methodology (RSM) and is particularly suited for modeling and analyzing problems where a response is influenced by several variables. It helps in understanding the interactions between factors and finding the optimum levels for maximum enzyme production. For *L*-methionase, CCD facilitates the precise optimization of significant factors identified through PBD.

In a study conducted by *Salim et al.* (2019), CCD was employed to optimize *L*-methionase production by *Trichoderma harzianum*. Variables such as glucose concentration, *L*-methionine concentration, and incubation time were fine-tuned to achieve maximum enzyme yield (Salim *et al.*, 2019). Similarly, CCD was applied by *Kharayat et al.* to optimize media components like glucose, NaCl, and casein enzymic hydrolysate for improved *L*-methionase yield from *Pseudomonas stutzeri* (Kharayat & Singh, 2019).

The method has also been extended to other fermentation targets in *T. harzianum*, like endoglucanase production, demonstrating its broad applicability in enzyme biotechnology (Bagewadi *et al.*, 2017), (Das *et al.*, 2021), (Othman *et al.*, 2021). CCD enables the construction of mathematical models and response surface plots, providing greater understanding of the relationships among the variables and aiding in process scale-up for industrial applications.

The combined application of Plackett-Burman Design for factor screening and Central Composite Design for detailed optimization provides a systematic, efficient, and cost-effective approach for maximizing *L*-methionase production. This dual-strategy optimization is crucial for developing industrial-scale processes, improving yield, and advancing the therapeutic applications of *L*-methionase.

2.10 L-Methionase Enzyme Purification

Methionine gamma-lyase (MGL) is synthesized by bacteria, protozoa, archaea, and plants, whereas fungi primarily produce extracellular MGL. Consequently, the separation of L-Methionase from non-fungal microbial sources typically necessitates cell disruption through chemical, enzymatic, or mechanical methods (Biochem *et al*., 1976). Among these sources, *Pseudomonas putida* has been considered as one of the most efficient producers of MGL (Dias & Weimer, 1998).

Several organisms, including *Arabidopsis thaliana* (Goyer *et al*., 2007), *Aspergillus flavipes* (El-Shora *et al*., 2021; S. A. Khalaf & El-Sayed, 2009), *Candida tropicalis* (M. H. Selim, Karm Eldin, *et al*., 2015), *Streptomyces variabilis* (M. H. Selim *et al*., 2016), *Brevibacterium linens*, and *Pseudomonas putida* (Peron *et al*., 2003; Takakura *et al*., 2004; El-Sayed *et al*., 2017), been employed for L-methionase extraction through sonication and homogenization techniques. Notably, L-methionase production from fungal sources, such as *Aspergillus flavipes*, requires a significantly longer cultivation period (8–10 days), whereas bacterial sources like *Pseudomonas putida* achieve comparable enzyme production within just 1–2 days (Abou Zeid *et al*., 2023).

Microorganisms are considered highly valuable for commercial enzyme production due to their ability to grow in inexpensive media and produce enzymes rapidly.

The crude protein from culture supernatants was subjected to ammonium sulfate precipitation, fractionated into three saturation ranges: 0–30%, 30–70%, and 70–90% (Reda *et al*., 2023). Following precipitation, proteins were collected via centrifugation at 10,000 × g for 20 minutes at 4°C. The resulting protein pellets were dialyzed three times over 24 hours against a potassium phosphate buffer (25 mM, pH 7.2) to remove residual salts (Sanjotha & Manawadi, 2017).

The dialyzed protein, containing L-methionase activity, was then loaded onto a 2.25 cm Diethylaminoethyl (DEAE) Sephadex anion-exchange column, pre-equilibrated with 25 mM potassium phosphate buffer (pH 7.0). Unbound proteins were eluted by washing the column

twice with two bed volumes of the same buffer. Bound proteins were subsequently eluted using a linear gradient of 0.1–1.0 M NaCl at a flow rate of 60 mL/h. Protein concentration in collected fractions was determined spectrophotometrically by measuring absorbance at 280 nm. Protein concentration was also quantified using the Folin-Lowry method with bovine serum albumin as the standard (Aldawood & Al-ezzy, 2024).

Table 2.2 Summary of L-Methionase Purification Steps and Conditions

Purification Step	Method Used	Conditions/Parameters	References
Cell Disruption (Non-Fungal)	Sonication or Homogenization	Mechanical/chemical disruption methods to release intracellular MGL	(Biochem *et al.,* 1976; Dias & Weimer, 1998)
Crude Extract Preparation	Ammonium Sulfate Precipitation	Saturation ranges: 0–30%, 30–70%, 70–90% at 4°C, 10,000 × g, 20 min	(Reda *et al*., 2023)
Purification Step	Dialysis	3 exchanges over 24 h against 25 mM potassium phosphate buffer (pH 7.2)	(Sanjotha & Manawadi, 2017)
Cell Disruption (Non-Fungal)	DEAE-Sephadex Column	25 mM phosphate buffer (pH 7.0); linear gradient of 0.1–1.0 M NaCl, 60 mL/h flow rate	(Aldawood & Al-ezzy, 2024)
Crude Extract Preparation	Spectrophotometry (280 nm)	Folin-Lowry method using bovine serum albumin as standard	(Aldawood & Al-ezzy, 2024)
Salt Removal	Sephacryl-S-300 HR Column	25 mM phosphate buffer (pH 7.0); flow rate 30 mL/h; 2 mL sample volume	(Reda *et al.,* 2023)

Both bound and unbound protein fractions were evaluated for L-methionase activity. Active fractions were combined, concentrated, dialyzed, and further purified through gel filtration chromatography. For this step, a Sephacryl-S-300 HR matrix (2 × 50 cm column) was utilized, with fraction elution carried out using 25 mM potassium phosphate buffer (pH 7.0) at a flow rate of 30 mL/h and a sample volume of 2 mL. Protein concentrations were assessed at 280 nm, and active fractions demonstrating L-methionase activity were freeze-dried and stored at -80°C (Reda *et al*., 2023).

2.10.1 Biochemical Characterization of L-Methionase

2.10.2 Effect of pH on L-Methionase Activity

Enzymes, as complex biological macromolecules, are highly sensitive to fluctuations in pH. Each enzyme exhibits a specific optimal pH range wherein its catalytic efficiency reaches a maximum. The effect of pH on enzyme activity is multifaceted, influencing several crucial aspects such as:

1. The binding affinity of the enzyme to its specific substrate.
2. The catalytic effectiveness of the enzyme.
3. The ionization state of both the enzyme active site and the substrate.
4. Structural stability and conformational integrity of the protein (Dayanand & Nadumane, 2023).

In the evaluation of L-methionase activity across varying pH conditions, enzyme assays were conducted using buffers spanning a pH range from 3.0 to 9.0. Among the tested conditions, the highest L-methionase activity was recorded in Tris-HCl buffer at pH 8.0–9.0, achieving a maximum specific activity of 1.42 U/mL/min (Kotramada Bopaiah *et al*., 2020). For comparison, the control experiment conducted at pH 7.0 yielded the lowest enzymatic activity, indicating a clear preference for a slightly alkaline environment.

Previous research supports these findings. For instance, L-methionase production from *Aspergillus ustus* was significantly enhanced as the alkalinity of the medium increased, with the highest productivity achieved at pH 8.5 (Abu-Tahon & Isaac, 2016). Interestingly, in the current study, the enzyme exhibited negligible activity at acidic pH values ranging between 3.0 and 5.0, suggesting that extreme acidic conditions destabilize the enzyme's active conformation.

Conversely, a recent study by Mohkam *et al.* (2020) reported that a recent bacterial isolate, *Alcaligenes* sp., displayed optimal L-methionase activity at pH 6.0, which lies within the acidic range. This deviation highlights species-specific differences in pH tolerance and optimal catalytic conditions.

2.10.3 Effect of pH Stability on L-Methionase Activity

The stability of enzymes across various pH levels is crucial for their industrial and therapeutic applications. pH stability refers to an enzyme ability to maintain structure and activity under prolonged exposure to different pH conditions. L-methionase generally shows optimal stability in slightly alkaline environments.

Kotramada Bopaiah *et al*. (2020) reported that L-methionase retained maximum stability between pH 8.0 and 9.0 in Tris-HCl buffer, while acidic conditions (pH 3.0–5.0) significantly reduced its activity. Similarly, Abu-Tahon and Isaac (2016) found that *Aspergillus ustus*-derived L-methionase maintained stability near pH 8.5, with substantial activity loss outside this range. Interestingly, Mohkam *et al*. (2020) demonstrated that L-methionase from *Alcaligenes* sp. remained stable at a lower pH of 6.0, although prolonged exposure to extreme pH conditions still caused partial denaturation.

Overall, maintaining conditions close to the enzyme optimal pH is critical for preserving L-methionase functionality during production and application.

2.10.4 Effect of Temperature on L-Methionase Activity

The functional properties of enzymes with respect to temperature are governed by the concept of enzyme temperature optimum. Enzymatic activity, thermal stability, and exposure time during experiments act carefully to determine this optimum, which provides limited but significant data on the extent to which enzymes respond to variations in temperature (El-Sayed & Shindia, 2011a).

L-methionase activity was evaluated at various incubation temperatures: 25°C, 28°C (room temperature), 37°C, 40°C, and 50°C. The highest enzyme activity was observed at 37°C, followed closely by activity at 28°C (Zolfaghar *et al*., 2019). A decline in activity was noted upon incubation at higher temperatures, although activity remained relatively stable between 40°C and 50°C.

L-methionase isolated from *Hafnia alvei* and *Alcaligenes* sp. exhibited optimal activity at 35°C under controlled conditions (Mohkam *et al*., 2020). Similarly, *Candida tropicalis*-derived L-methionase demonstrated peak activity at 45°C (M. H. Selim, Karm Eldin, *et al*., 2015), whereas L-methionase purified from cheese lactic acid bacteria exhibited an optimum temperature of 37°C (Bonnarme, Lapadatescu, *et al*., 2001).

2.10.4 Effect of Temperature Stability on L-Methionase Activity

Temperature stability is critical for maintaining the structural integrity and catalytic function of enzymes like L-methionase during industrial and therapeutic applications.

Studies show that L-methionase retains high activity between 30°C and 37°C (Kotramada Bopaiah *et al*., 2020). Activity declines gradually above 40°C due to thermal denaturation. L-methionase from *Alcaligenes* sp. also exhibited good stability at 35°C but showed reduced activity at higher temperatures (Mohkam *et al*., 2020). Strains such as *Candida tropicalis* maintained significant activity at 45°C (M. H. Selim, Karm Eldin, *et al.,* 2015), although prolonged exposure above the optimal range eventually led to denaturation (Bonnarme, Lapadatescu, *et al*., 2001).

Overall, L-methionase demonstrates moderate thermal stability, with peak performance under mesophilic conditions (30°C–37°C), making it suitable for various biotechnological applications.

2.10.5 Effect of Metal Ions on L-Methionase Activity

Metal ions play a crucial role in the functionality of many enzymes, acting as essential cofactors that enhance or inhibit catalytic activities. For L-methionase, the effect of metal ions varies depending on the microbial source and the type of ion involved.

Studies have shown that the activity of L-methionase derived from *Brevibacterium linens* was significantly stimulated by Na^+ and K^+ ions have been shown to positively influence *L*-methionase activity (Su *et al.*, 2016; Wallis *et al.*, 2020).

Similarly, *L*-methionase from *Aspergillus flavipes* exhibited enhanced activity when exposed to Co^{2+}, Mn^{2+}, Cu^{2+}, and Mg^{2+} ions (Kebeish & El-Sayed, 2012; El-Sayed *et al.*, 2016). Among these, Na^+ at a concentration of 10 mM particularly boosted *L*-methionase activity, while Mg^{2+}, K^+, and Ni^{2+} also had positive effects.

Conversely, several metal ions have shown strong inhibitory effects. For example, Zn^{2+}, Mn^{2+}, and Cu^{2+} significantly inhibited *L*-methionase activity from *Brevibacterium linens* (Sharma *et al.*, 2019). Inhibitors like bithionol and DL-propargylglycine were reported to reduce *L*-methionase activity in *Trichomonas vaginalis* (Gustin, 2014).

In other organisms, sulfhydryl reagents such as iodoacetic acid and β-mercaptoethanol inhibited *L*-methionase in *Lactococcus lactis* and *Aspergillus flavipes* (Khalaf *et al.*, 2023; El-

Sayed *et al.*, 2012). Additionally, *L*-methionase from *A. flavipes* was inhibited by EDTA, NaN_3, Li_2^+, Cd^{2+}, DMSO, and β-mercaptoethanol (El-Sayed *et al.*, 2014).

Natural inhibitors have also been recognized. For instance, myrsinoic acid B suppressed *L*-methionase activity in periodontal bacteria such as *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, and *Treponema denticola* (Ishikawa *et al.*, 2021; Foo *et al.*, 2016; Yoshimura *et al.*, 2002; Fukamachi *et al.*, 2005).

Furthermore, agents like SDS, β-mercaptoethanol, PMSF, and Fe^{2+} negatively affected *L*-methionase from *A. flavipes* (El-Sayed & Shindia, 2011b). Strong inhibitors including DL-propargylglycine, hydroxylamine, and guanidine hydrochloride were also reported to reduce enzyme activity.

In *Candida tropicalis*, *L*-methionase was highly sensitive to inhibition by Cd^{2+}, Cu^{2+}, Cr^{2+}, Zn^{2+}, Fe^{2+}, Ba^{+}, and Co^{2+} (Selim, Karm Eldin, *et al.*, 2015). Similarly, *L*-methionase activity in *Streptomyces* species was inhibited by Cr^{2+}, Fe^{2+}, Ni^{2+}, Cd^{2+}, PMSF, β-mercaptoethanol, and SDS, with Hg^{2+}, Cu^{2+}, and iodoacetate showing particularly strong inhibitory effects at a 10 mM concentration (Kavya & Nadumane, 2020; Selim, Elshikh, *et al.*, 2015).

Additionally, in *Streptomyces variabilis*, inhibition was observed in the form of PMSF, EDTA, and tris-glycine buffers (Steck *et al*., 2022). The inhibiting effect of Cu^{2+} suggests that oxidation of the active site's sulfhydryl (SH) groups to disulfides occurs, while EDTA inhibition supports the hypothesis that L-methionase functions as a metalloenzyme (A. S. A. El-Sayed, 2009).

Notably, complete inactivation of L-methionase has been achieved with thiol-reactive agents like iodoacetate (Faleev *et al*., 1996), underscoring the importance of sulfhydryl groups for enzymatic activity.

Table 2.3 Biochemical Characterization of L-Methionase

Parameter	Key Findings	References
Optimal pH for Activity	Highest activity at pH 8.0-9.0 (Tris-HCl buffer); no activity at pH 3.0-5.0	Kotramada Bopaiah et al., 2020; Abu-Tahon & Isaac, 2016
pH Stability	Stable activity maintained between pH 8.0 and 9.0; decreased activity outside this range	Kotramada Bopaiah et al., 2020; Mohkam et al., 2020
Optimal Temperature for Activity	Highest activity at 35-37°C depending on source; room temperature (28°C) shows moderate activity	Zolfaghar et al., 2019; M. H. Selim et al., 2015
Temperature Stability	Stable activity maintained at 30-37°C; activity decreases beyond 40°C due to denaturation	Kotramada Bopaiah et al., 2020; Bonnarme et al., 2001
Effect of Metal Ions - Activators	Na^{+}, K+, Co2+, Mn2+, Cu2+, Mg2+ enhance activity in various microbial sources	Su et al., 2016; Kebeish & El-Sayed, 2012
Effect of Metal Ions - Inhibitors	$Zn^{2+,}$ Cd^{2+}, Cu^{2+}, Cr^{2+}, Fe^{2+}, Hg^{2+}, SDS, EDTA, β-mercaptoethanol, iodoacetate inhibit activity	H. Sharma et al., 2019; A. S. A. El-Sayed, 2009
Natural Inhibitors	Myrsinoic acid B inhibits L-methionase in periodontal bacteria	Ishikawa et al., 2021; Foo et al., 2016

2.11. L-Methionase Requirements in Cancer Cells

Tumor cells exhibit uncontrolled and rapid growth in comparison with healthy cells. Many cancers human cell lines display an increased demand for L-methionase to support elevated protein synthesis and regulate DNA expression (Cavuoto & Fenech, 2012; Benavides *et al*., 2007; Kim & Park, 2003; Cellarier *et al*., 2003; Machover *et al*., 2002; İpek et al., 2023). L-methionase is converted into S-adenosylmethionine (SAM), a critical methyl donor involved in methylation of DNA is an epigenetic process linked to cancer development (Hens *et al*., 2016; Bird, 2002; Moore *et al*., 2013; Das & Singal, 2004; Sharma *et al*., 2014; Bach *et al*., 2017).

A diet high in L-methionine has been related to a higher chance of prostate cancer due to elevated SAM levels, leading to DNA hypermethylation in regulatory regions, including tumor suppressor genes (Hullo *et al*., 2007). CpG islands, which consist of cytosine-guanine sequences, are susceptible to methylation (Warren & Weiner, 2002). While methyl-binding proteins (MBPs) help regulate gene transcription under normal conditions, cancer cells exploit this mechanism to silence tumor suppressor genes (Parry & Clarke, 2011).

In cancer, CpG islands often become abnormally hypermethylated, leading to transcriptional silencing inherited by daughter cells (Das & Singal, 2004; Estécio & Issa, 2011). This aberrant methylation is a hallmark of various cancers, including breast, colon, lung, and brain tumors (Sproul & Meehan, 2013).

DNA methylation maintenance involves UHRF1 and DNA methyltransferases (DNMTs) (Finkelstein & Martin, 1984). UHRF1 facilitates the binding of DNMT1 to methylated DNA and regulates genes such as RB1 during the G1 phase (Unoki *et al.,* 2008; A. Kim & Benavente, 2024). Pyrosequencing assays have detected methylation changes in genes like CDKN2A, RASSF1, and DNMT1 (De Chiara *et al*., 2020; Kumar *et al*., 2020).

Moreover, Overexpression of UHRF1 in the hepatocytes of zebrafish induces hypomethylation, senescence, and hepatocellular carcinoma (Cai *et al*., 2023). Techniques like Restriction Landmark Genomic Scanning (RLGS) have helped assess methylation in malignancies (Scalea *et al.,* 2020).

L-methionase also downregulates protein kinase genes in breast (MCF-7) and prostate (LNCaP) cancer cells, exhibiting antiproliferative effects (Benavides et al., 2007). Furthermore, site-specific hypermethylation of tumor suppressor genes and hypomethylation of oncogenic microRNAs (miRNAs) are critical events in cancer progression (Ma *et al*., 2023; S. Chen *et al.,* 2022; D. Chen *et al*., 2021).

Normal cells utilize L-methionine synthase to generate L-methionine from homocysteine (Hcy), while tumor cells lack significant L-methionine synthase activity (Chaturvedi *et al*., 2018; Ogawa *et al*., 2022). This metabolic defect underpins the L-methionine dependent of tumor cells (Sharma *et al*., 2014).

L-methionine is vital for synthesizing vitamins, antioxidants (glutathione, trypanothione), DNA stabilizers, coenzymes, and for the initiation of protein synthesis (abozeid, 2023; Adeshakin *et al.*, 2021; Jeelani *et al*., 2017).

In cancer models, L-methionine depletion arrests tumor cells at the S/G2 phase, promoting apoptosis (Rajanala *et al*., 2019; Lu *et al.*, 2002). However, despite the effectiveness of L-methionine-free diets, practical limitations persist (Wallis *et al*., 2020; Lu & Epner, 2000).

Additionally, cancer cells exhibit the Warburg effect an increased reliance on glycolysis despite oxygen availability (Danhier *et al*., 2017; Iheagwam *et al*., 2022). GLUT and SGLT transporters mediate elevated glucose uptake in tumor cells (Jonsson *et al*., 2019).

Overexpression of GLUT1 correlates with cancer aggressiveness in hepatocellular carcinoma, oral cancer, pancreatic carcinoma, and gastric cancer (Shang *et al*., 2020; Botha *et al*., 2021; Du *et al*., 2022; Zhou *et al*., 2020).

Targeting enzymes involved in metabolism such as glucose transporters, pyruvate kinases, and glutaminases enhances therapeutic efficacy. Overactive fatty acid and glutamine metabolism further support tumor growth (Van Trimpont *et al*., 2022).

Furthermore, activation of the AKT pathway and ENTPD5 enhances glycolysis and lactate production, crucial for biomass expansion (Akakura *et al*., 2014; Loh *et al*., 2023; Vellard, 2003).

Ion channels, ATP synthase, and ABC transporters, including ABCG2, are dysregulated in cancers, contributing to altered metabolism and drug resistance (Galber *et al*., 2020; He *et al*., 2021; Wang *et al*., 2021; Esparza-Moltó & Cuezva, 2018)

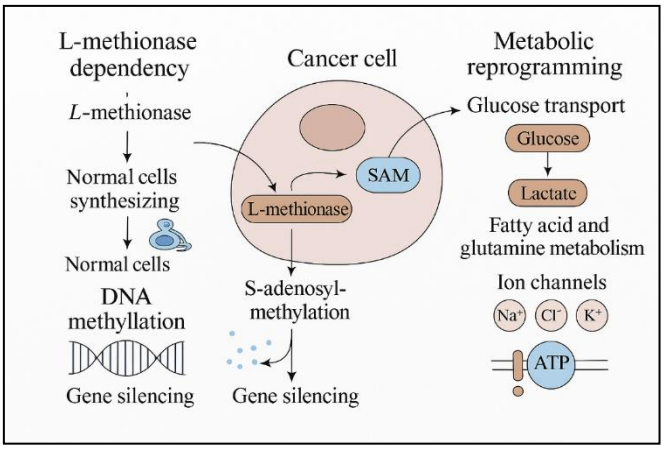

Figure 2.5. Overview of L-methionase role, DNA methylation, and metabolic reprogramming in cancer cells (Liu *et al*., 2022)

2.12. Utilization of L-Methionase in Cancer Therapy

2.12.1 Combinational Therapy

The therapeutic application of *Pseudomonas putida* L-methionase (PpLM) for depleting plasma L-methionine has been extensively researched (El-Sayed *et al*., 2017). PpLM has demonstrated potent anti-proliferative effects against multiple cancer types, including Lewis lung carcinoma, human colon carcinoma, glioblastoma, and neuroblastoma (Choi *et al*., 2024).

One major limitation of conventional cancer treatments, such as small-molecule drugs, is their reduced efficacy due to cancer stem cell's ability to expel these drugs before cell death, resulting in disease recurrence (Plana *et al*., 2022).

A promising strategy for overcome this issue involves using recombinant PpLM to deplete L-methionine from tumors (Chandra & Hong, 2015). Studies show that treatment with recombinant PpLM significantly suppresses tumor growth both *in vitro* and *in vivo*, including in xenografted nude mouse models, while having minimal impact on normal cells (Pokrovsky *et al*., 2018). Moreover, the introduction of the PpLM gene into lung cancer cells in humans using retroviral vectors has effectively reduced cell proliferation (Alshehri, 2020).

Recombinant PpLM has also been used to utilized intracellular and extracellular L-methionine. In combination with chemotherapeutic agents like cisplatin, 5-fluorouracil (5-FU), BCNU, and vincristine, PpLM demonstrated synergistic anti-cancer effects in models of colon, lung, and brain tumors (Hoffman & Yano, 2019). Additionally, adenovirus-mediated transfection of the L-methionase gene into tumor cells inhibited their growth *in vitro* (Pokrovsky *et al*., 2023).

Another innovative strategy combines L-methionase with selenomethionine (Setmet), a non-toxic prodrug. The L-methionase enzyme converts Setmet into a cytotoxic compound methylselenol which triggers apoptosis and reduces tumor growth in rodent models, prolonging survival (Sharma *et al.,* 2014).

Selenomethionine Reaction Pathway:

Selenomethionine + H_2O ⟶ (via MGL) ⟶ α-Ketobutyrate + Ammonia (NH_3) + Methylselenol (CH_3SeH)

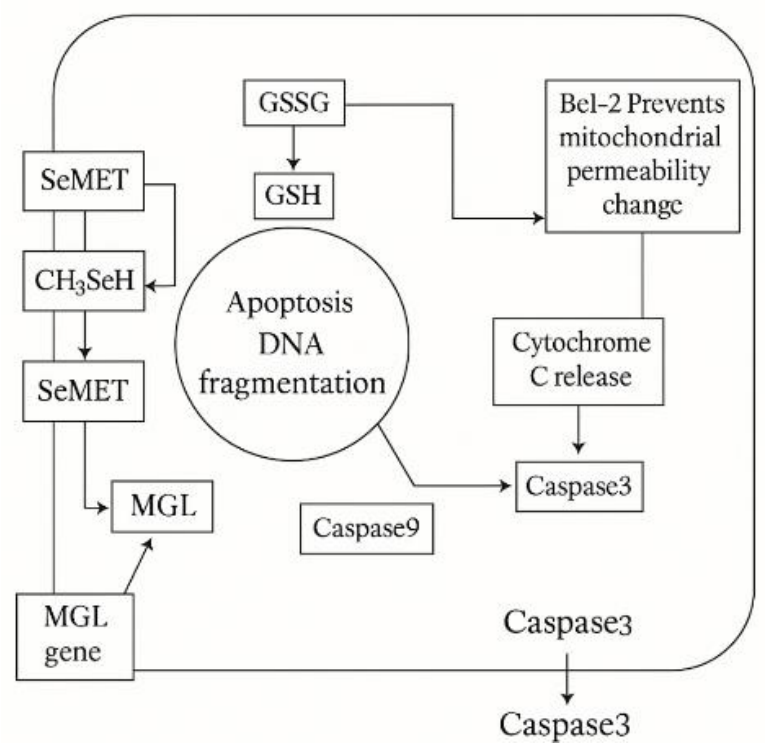

Figure 2.6. L-Methionase–Setmet combo induces ROS-mediated apoptosis in tumor cells (El-Sayed *et al*., 2021)

L-methionase catalyzes the conversion of non-toxic Setmet into methylselenol (Kaiser, 2020), which oxidizes thiol groups, generating toxic superoxides that induce apoptosis, primarily through mitochondrial pathways. Methylselenol can diffuse into neighboring tumor cells, causing oxidative stress and mitochondrial membrane disruption (Esparza-Moltó & Cuezva, 2018); Zuazo *et al*., 2012).

Treatment with Setmet in L-methionase-transduced cells inhibits tumor growth (Varlamova & Turovsky, 2021). Remarkably, methylselenol is effective at very low concentrations in inducing cell cycle arrest and apoptosis. It also modulates the expression of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs), thereby inhibiting tumor cell migration (Radomska *et al*., 2021; Varlamova & Turovsky, 2021).

Methylselenol-induced apoptosis has been observed in a number of cancer models, including murine melanoma B16F10, fibrosarcoma HT1080, colon cancer HCT-116, and prostate cancer LNCaP cells (Abozeid, 2023); (Dhankhar *et al*., 2020). In HCT116 cells,

methylselenol is more effective than in normal NCM460 cells. It also reduces prostate-specific antigen (PSA) levels in LNCaP cells. ROS generated by methylselenol promote cell proliferation at low concentrations but cause apoptosis at high levels (Fernandes *et al*., 2012).

Methylselenol induces thiol oxidation, generating ROS that led to mitochondrial swelling, cytochrome c release, caspase cascade activation, and apoptosis (Font *et al*., 2018).

Setmet is safe in normal cells because of the lack of L-methionase, but shows strong anti-prostate cancer activity when combined with L-methionase. Adenovirus-mediated transfection of tumor cells with the L-methionase gene increases Setmet sensitivity (Piastowska-Ciesielska *et al*., 2014).

The combination of the L-methionase gene, its enzyme, and Setmet presents a promising approach for targeting L-methionine-dependent tumors (Varlamova & Turovsky, 2021).

2.12.2 Use of Fusion Protein in Targeting Cancer Cells

Oxidative stress in tumor microenvironments causes the translocation of phosphatidylserine (PS) to the outer membrane of vascular endothelial cells within tumor-associated blood vessels a phenomenon not typically observed in healthy tissue (Chang *et al.*, 2020). This biological distinction provides an exploitable target for therapeutic intervention.

A fusion protein (FP) composed of L-methionase conjugated to human Annexin-V has been developed to selectively bind to these PS-expressing endothelial cells when administered systemically. This FP achieves two therapeutic functions simultaneously: it enzymatically converts the non-toxic prodrug selenomethionine (SeMet) into cytotoxic methylselenol, and it blocks the availability of L-methionine to tumor cells, which are highly dependent on this amino acid for growth and proliferation (Suganya *et al*., 2017a); (With *et al*., 2016).

One of the principal advantages of this therapeutic approach is that the fusion protein does not need to be directly delivered to the tumor cells. Instead, intravenous administration is sufficient, as the FP circulates and selectively accumulates in tumor vasculature via its high-affinity binding to PS (Mertens *et al*., 2015).

Another innovative construct, known as ATF-MGL-FP, utilizes the amino-terminal fragment of urokinase plasminogen activator (uPA) to direct L-methionase activity specifically to the outside of cancer cells. This fusion protein has demonstrated potent anti-proliferative and

anti-migratory effects, reinforcing its utility in targeting L-methionine-dependent tumors (Masucci *et al*., 2022).

Annexin-V, a key component of these fusion constructs, is among the most well-characterized PS-targeting agents. It binds membrane phospholipids in a calcium-dependent manner, providing a molecular bridge between membrane signaling pathways and therapeutic intervention (Benavides *et al*., 2014); (Lizarbe *et al*., 2013).

Collectively, these fusion protein strategies offer a promising platform for targeted cancer therapy, particularly for tumors that exhibit L-methionine dependency and elevated PS exposure

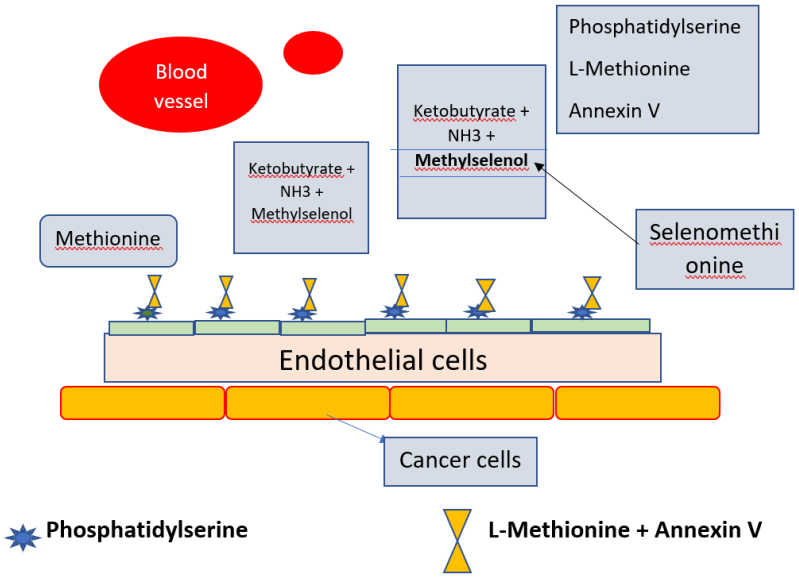

Figure 2.7. Cancer cell targeting by PS-binding molecules connected to human Annexin-V (Hassan *et al.,* 2023)

2.13. Modification of L-Methionase to Reduce Its Side Effects

Recombinant and PEGylated L-methionase have demonstrated strong anti-tumor effects in various cancers, including lung, colon, brain, prostate, and melanoma (Kulikova *et al*., 2017); (El-Sayed *et al*., 2012). Treatment lowers plasma L-methionine to under 2 µM, a level critical for tumor suppression. Side effects are generally mild, such as reduced appetite, minor weight loss, and temporary blood changes (Guo *et al*., 1993).

To enhance safety and stability, PEGylation was applied to L-methionase, which extended its half-life by 6–19 times but slightly reduced its L-methionine-depleting ability

(Takakura *et al*., 2006); (Tan *et al*., 1998). Co-factors like pyridoxal phosphate (PLP), oleic acid, and dithiothreitol (DTT) further improved enzyme efficiency (Abozeid, 2023); (Dhankhar *et al*., 2020).

PEGylation also reduced enzyme immunogenicity, making it more suitable for therapeutic use. Advanced strategies like "neutral drift" deimmunization help eliminate T-cell epitopes while preserving activity (Hoffman, 2015).

2.14. Cell Carrier-Based Drug Delivery System

A significant challenge in using L-methionase for cancer therapy is its effective delivery to tumor sites while preventing premature degradation, avoiding clearance by phagocytic cells, and extending its circulation time (With *et al*., 2016). To address this, various endogenous circulating cells such as erythrocytes, monocytes, macrophages, lymphocytes, neutrophils, platelets, leukocytes, dendritic cells, stem cells, and extracellular vesicles have been investigated as carriers for targeted cancer therapy (P. H. D. Nguyen *et al.*, 2022).

Erythrocytes, produced in the bone marrow and circulating for approximately 100-120 days, have been particularly useful due to their natural biocompatibility and prolonged lifespan. Since L-methionase is a pyridoxal phosphate-dependent enzyme with a relatively short half-life, encapsulating it within erythrocytes significantly enhances its stability and therapeutic potential (Sato & Nozaki, 2009). This method not only protects the enzyme from degradation but also reduces immune system recognition, extending its in vivo half-life from less than 24 hours to approximately 8-9 days (Dhankhar *et al.*, 2020).

Moreover, PEGylation techniques have been utilized to further increase L-methionase stability, with studies showing an extended half-life of approximately 38 hours in murine models (Hoffman, 2015). Innovations like Ery-Met (erythrocyte-encapsulated L-methionase) and Ery-ASP (erythrocyte-encapsulated asparaginase) have been successfully explored against the human leukemia HL-60 cell line, which depends on L-methionine and asparagine metabolism for proliferation (Kwon *et al.*, 2009).

2.15. *In Vitro* and *In Vivo* Evaluation of L-Methionase

2.15.1 *In Vitro* Evaluation Methods

In vitro evaluation methods are essential in the early stages of anticancer drug discovery. These laboratory-based techniques allow for high-throughput screening of cytotoxic

compounds, identification of mechanisms of action, and prediction of therapeutic potential with reduced ethical concerns and costs compared to in vivo models. With ongoing advancements, *in vitro* assays increasingly reflect the complexity of the tumor microenvironment, providing more predictive results for clinical translation.

2.15.2 Cytotoxicity and Cell Viability Assays

Cytotoxicity assays remain the gold standard in screening anticancer compounds. Common assays include the MTT, resazurin (Alamar Blue), and trypan blue exclusion tests, which quantify cell viability through mitochondrial activity, membrane integrity, or dye exclusion. These assays provide dose-response curves and IC_{50} values, enabling comparison across compounds (Ediriweera, Tennekoon, & Samarakoon, 2018).

2.15.3 Apoptosis and Cell Death Detection

Evaluation of apoptosis is critical in anticancer testing as many therapies aim to trigger programmed cell death. Techniques such as Annexin V/PI staining, caspase activity assays, and TUNEL assays are used to measure apoptotic events. These tools can distinguish between early apoptosis, necrosis, and late-stage cell death (Reddy V. *et al*., 2024).

2.15.4 Multicomponent Coculture Models

Conventional monoculture systems often fail to capture tumor complexity. Recent approaches use 3D coculture systems that include cancer cells and stromal components such as fibroblasts and immune cells. These models better replicate the tumor microenvironment and allow for more accurate predictions of drug sensitivity and resistance (Yamazoe, Hagihara, & Kobayashi, 2016).

2.15.5 Clonogenic and Sphere-Forming Assays

Clonogenic assays assess the ability of single cells to survive treatment and proliferate into colonies. This method is especially useful for evaluating long-term cytotoxic effects and cancer stem cell behavior. Sphere-forming assays in low-attachment cultures provide additional insight into the behavior of tumor-initiating cells (Inoue *et al.,* 2004).

2.15.6 Mechanistic and Molecular Assays

Advanced assays assess specific molecular targets or pathways affected by anticancer agents. These include Western blotting, qPCR, immunocytochemistry, and reporter gene assays

to evaluate gene/protein expression, signaling cascades, and epigenetic changes (Del Bene *et al*., 2009).

2.16. *In Vivo* Evaluation Methods

In vivo methods are essential in anticancer drug development to evaluate pharmacokinetics, therapeutic efficacy, safety, and tumor response within the complex biological systems of living organisms. These studies provide predictive insight into how drugs will behave in humans and are a vital bridge between in vitro testing and clinical trials.

2.16.1 Xenograft Models

Xenograft models involve implanting human tumor cells or tissues into immunodeficient mice, enabling researchers to assess tumor growth inhibition and drug efficacy. These include cell line-derived xenografts (CDX) and patient-derived xenografts (PDX), the latter offering more clinically relevant tumor biology. These models are increasingly used for evaluating small molecules, antibody-drug conjugates, and immunotherapies (Chen, Sun, & Li, 2019).

2.16.2 Syngeneic and Genetically Engineered Mouse Models (GEMMs)

Syngeneic models use mouse tumor cells in immunocompetent mice, preserving immune-tumor interactions and making them useful for immunotherapy research. Genetically engineered mouse models (GEMMs) mimic human oncogenesis through tissue-specific gene modifications, offering long-term observations of tumor development and drug response under natural immune conditions (Vasyutina *et al*., 2025).

2.16.3 Orthotopic and Metastatic Models

Orthotopic models involve implanting tumor cells into the organ of origin (e.g., pancreas, brain), enabling a more accurate simulation of tumor environment and metastatic behavior. These models are crucial for studying drug penetration, angiogenesis, and metastasis factors less observable in subcutaneous models (Sebastian, Jaykar, & Gomathi, 2020).

2.16.4 Imaging-Based Monitoring and Biomarkers

Modern in vivo studies utilize advanced imaging tools (e.g., PET, MRI, bioluminescence) to monitor tumor growth and drug efficacy over time. Additionally, surrogate biomarkers such as secreted enzymes (e.g., alkaline phosphatase) have been used to track tumor

burden in real-time and evaluate drug response without sacrificing the animal (Bao, Selvakumaran, & Hamilton, 2000).

2.16.5 Natural Compound and Novel Agent Testing

New anticancer compounds from natural sources, such as *Centella asiatica* and synthetic metal complexes, are first tested in vivo to assess tumor regression, toxicity, and survival impact. For example, rhenium complexes and asiatic acid have demonstrated significant in vivo antitumor activity in zebrafish and rodent models (Sovari et al., 2023); (Ridho et al., 2024).

2.16.6 Anticancer Activity of L-Methionase

L-methionase, also known as methionine γ-lyase (MGL), is a pyridoxal-5′-phosphate (PLP)-dependent enzyme that degrades methionine into α-ketobutyrate, methanethiol, and ammonia. Its anticancer potential lies in its ability to induce methionine starvation, a condition that selectively affects tumor cells, many of which are methionine-dependent due to their altered metabolism. Normal cells, which can utilize homocysteine remethylation pathways, are less sensitive to methionine depletion, making MGL a tumor-targeted therapy.

In *in vitro* studies, L-methionase has shown broad cytotoxicity against various human cancer cell lines. For example, recombinant MGL from *Pseudomonas putida* significantly reduced viability in 21 different cancer cell lines, including those from lung, breast, prostate, colon, and CNS tumors, while having minimal impact on normal cells (Tan *et al.*, 2010). Similarly, MGL purified from *Trichoderma harzianum* showed potent antiproliferative activity against MCF-7 (breast cancer) and HepG2 (liver cancer) cell lines, with low IC_{50} values, demonstrating the enzyme's efficacy in vitro (Salim *et al.*, 2020).

To improve stability and pharmacokinetics, MGL has been chemically modified. In one study, *Aspergillus flavipes* MGL conjugated with β-cyclodextrin exhibited enhanced thermal stability and catalytic efficiency, with significantly increased cytotoxicity against HCT-116 and MCF-7 cells (El-Sayed *et al.*, 2024). Another modification, dextranization, improved the proteolytic resistance and half-life of MGL, further boosting its therapeutic window (El-Sayed *et al.*, 2016).

In *in vivo* models, MGL has shown significant tumor suppression. In a study using A549 lung cancer xenografts, MGL from *Clostridium sporogenes* was found to synergize effectively with doxorubicin, leading to enhanced tumor regression compared to either agent alone (Pokrovsky

et al., 2019). Another innovative approach involved pairing MGL with a daidzein-propiin prodrug system. This combination therapy demonstrated strong tumor-selective effects in pancreatic, colon, and prostate cancer xenografts, achieving tumor inhibition rates of up to 70% (Qoura *et al.*, 2022).

Moreover, a comparative evaluation of MGL's anticancer activity in both 2D and 3D cell culture models revealed that although 3D spheroids were more resistant than monolayers, MGL still significantly inhibited growth in pancreatic, colon, and breast cancer cells without affecting healthy fibroblasts (Karshieva *et al.*, 2024).

2.17. Future Prospects for L-Methionase

The future outlook for L-methionase-based therapies is highly promising, with several transformative strategies under active investigation that could significantly enhance its clinical utility:

2.17.1 Gene Therapy Approaches

Gene therapy offers a novel means to deliver L-methionase genes directly into tumor tissues using viral vectors, such as adenoviruses or lentiviruses. This strategy achieves consistent methionine depletion while reducing systemic toxicity by permitting localized, continual production of the enzyme across the tumor microenvironment (Javia *et al*., 2024).

2.17.2 Targeted Nanoparticle Delivery System

Nanotechnology-based drug delivery systems, including polymeric nanoparticles and liposomes, offer enhanced specificity and reduced immunogenicity by encapsulating L-methionase for targeted release at tumor sites. Nanocarriers can be modified with tumor-targeting ligands, improving biodistribution and minimizing off-target effects (Onagun & Stephen, 2024).

2.17.3 Combination with Immune Checkpoint Inhibitors

Emerging studies suggest that methionine depletion by L-methionase can reprogram the tumor microenvironment, enhancing the efficacy of immune checkpoint inhibitors like anti-PD-1 and anti-CTLA-4 antibodies. Combining L-methionase with immunotherapies holds great potential for achieving synergistic tumor eradication (Sénéchal *et al.,* 2019).

2.17.4 Personalized Oncology and Biomarker-Driven Therapies:

Future developments in personalized oncology could involve the use of molecular biomarkers to identify tumors with high methionine dependency. Stratifying patients based on these biomarkers would optimize therapy efficacy, ensuring that L-methionase is administered to the most responsive individuals (zhu *et al*., 2025).

L-methionase also holds potential for therapeutic applications beyond cancer, particularly in the areas of obesity, aging, and heart disease, as noted by (*Iyengar et al.* 2016; *Lee et al.* 2016; and *Ables et al.* 2015).

2.18 Application of L-Methionase Enzyme

2.18.1 heart disease

Elevated serum total homocysteine (tHCY) levels are a major cardiovascular risk factor, with studies showing a strong association with myocardial infarction, stroke, and other vascular diseases (Smith & Refsum, 2021). Some studies establish tHCY as an independent, strong, and significant risk factor with little or no correlation with other known or suspected risk factors. Moderate and severe hyperhomocysteinemia because of changes in genes coding for methylenetetrahydrofolatereductase cystathionine–β-lyase are nonresponsive to vitamin therapy (Al Mutairi, 2020) (Paganelli *et al*., 2021).

Research on hyperhomocysteinemia treatment using an enzyme for HCY degradation is promising, with L-methionase showing higher activity on HCY compared to natural substrate methionine, according to various studies (Froese *et al*., 2019).

2.18.2 Aging

Aging has also been associated with changes in DNA methylation, with estrogen receptor gene hypermethylation in the colon linked to cancer and hematopoietic neoplasms, and IGF2 gene promoter methylation being significantly enhanced (Kitada *et al.*, 2021; Lauinger & Kaiser, 2021).

2.18.3 Obesity

(Tashiro *et al.*, 2020) suggest L-methionase may regulate obesity, as dietary methionine deprivation in rats-controlled weight gain. Hormonal control, including leptin, neuropeptide Y, and a specific melanocortin receptor, is crucial for obesity regulation. L-methionase has significant potential for use as an anticancer drug.

2.18.4 As an Anti-Cancer Agent

L-methionase has become a promising anti-cancer agent due to its ability to selectively target cancer cell metabolism. -methionine (L-Met), the first amino acid added during protein translation, is also an essential building block for cysteine production (Kulikova *et al.*, 2017).

It plays vital roles in the synthesis of vitamins, antioxidants, DNA stabilizers, epigenetic modulators, coenzymes, proteins, polyamines, and components involved in oxidative stress response, iron-sulfur cluster biosynthesis, and methylation reactions (Rajpara *et al.*, 2024). Additionally, L-Met is central to regulating gene expression.

Tumor cells, characterized by uncontrolled and rapid proliferation, exhibit a heightened dependency on L-Met to sustain their elevated protein synthesis rates and support DNA methylation and gene regulation (Swisher *et al.,* 2009). Many studies have confirmed that many human cancers cell lines display a metabolic defect: they are unable to synthesize L-Met endogenously due to a lack of L-Met synthase expression. In contrast, normal cells possess L-Met synthase, which enables them to convert homocysteine into L-Met using methyl donors such as methyl tetrahydrofolate and betaine (Machover *et al*., 2019).

This differential capability renders tumor cells vulnerable to L-Met depletion. L-methionase, by catalyzing the depletion of L-Met, effectively starves cancer cells of this essential nutrient, inhibiting their growth and proliferation without significantly affecting normal cells. This unique mechanism of action has led to the investigation and application of L-methionase as a therapeutic agent in treating various L-Met-dependent cancers (Wanders *et al*., 2020; Kaiser, 2020; Parnami, 2021; Cellarier *et al*., 2003)

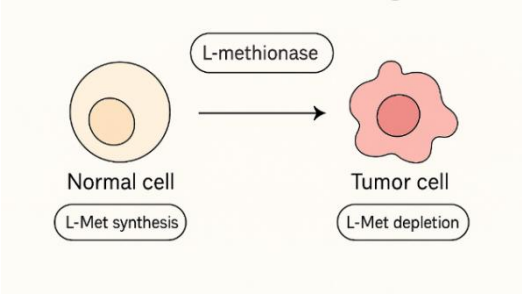

Figure 2.8. L-Methionase as an Anti-Cancer Enzyme (El-Sayed *et al.*, 2024)

2.18.5 As an Hyperhomocysteinemia and Cardiovascular Disease

Elevated serum homocysteine (Hcy) levels have been recognized as a important risk factor for numerous pathological conditions, including cardiovascular disease, coronary artery disease, and neurodegenerative disorders such as Alzheimer's disease (Machover *et al*., 2019). Homocysteine is a sulfur-containing amino acid formed during the demethylation of dietary L-methionine, which is particularly abundant in animal protein (Lu & Epner, 2000). It serves as a key intermediate in the transsulfuration pathway, where L-methionine is metabolized into cysteine.

In healthy individuals, plasma Hcy concentrations range between 5.0 and 15.0 μmol/L. When levels exceed 100 μmol/L, the condition is classified as severe hyperhomocysteinemia. Numerous clinical studies have documented a strong association between elevated Hcy levels and the incidence of myocardial infarction, stroke, and vascular dysfunction (*Hullo et al.*, 2007; *Smith & Refsum*, 2021).

Efforts to manage hyperhomocysteinemia have explored enzymatic degradation of Hcy as a therapeutic approach. L-methionase enzymes from various microbial and fungal sources exhibit relaxed substrate specificity, demonstrating 1.8- to 9.0-fold higher catalytic activity toward Hcy than toward their native substrate, L-methionine. This property highlights their potential utility in reducing plasma Hcy levels.

Moreover, L-methionase shows promise in managing metabolic disorders such as homocystinuria, which is characterized by a deficiency of cystathionine β-synthase, leading to the accumulation of both L-methionine and Hcy in blood and urine. Enzymatic degradation of Hcy via L-methionase offers a promising therapeutic route for alleviating the toxic effects of elevated homocysteine in such disorders (Ables *et al*., 2015); (Paganelli *et al*., 2021); (Finkelstein & Martin, 1984); (Seiflein & Lawrence, 2001).

2.18.6 In the Food Industry

L-methionase plays a crucial role in the food industry, particularly in the process of the distinctive aroma and flavor profiles of traditional fermented foods, such as cheese. This enzyme catalyzes the degradation of L-methionine (L-met), leading to the release of volatile sulfur compounds that are crucial for sensory characteristics.

Several cheese-ripening microorganisms, including *Brevibacterium linens* (*İpek et al.*, 2023; *Amarita et al.*, 2004), *Pseudomonas* spp., *Geotrichum candidum* (Bonnarme, Lapadatescu*, et al.,* 2001; Bonnarme, Arfi, *et al.,* 2001), and Lactococcus spp. (Mohkam *et al.*, 2020), have been identified as contributors to flavor and texture development during cheese maturation have been observed to produce L-methionase. These microbes convert L-methionine into methanethiol and 2-oxobutyrate. Methanethiol is further metabolized into compounds such as dimethyl disulfide, dimethyl trisulfide, and various thioesters, all of which contribute significantly to the aroma profile of ripened cheeses.

Simultaneously, 2-oxobutyrate can undergo condensation with active acetaldehyde derived from pyruvate, forming intermediates like 2,3-pentanedione. This compound is a key contributor to the buttery and nutty flavors characteristic of many aged cheeses (Dias & Weimer, 1998; Bonnarme *et al*., 2000; and Bonnarme, Lapadatescu, *et al.,* 2001.

2.18.7 Targeting Drug-Resistant Parasitic Infections

Antimicrobial resistance poses a significant threat to global health, as pathogens increasingly evade the effects of conventional therapeutic agents. To combat this, novel strategies involving therapeutic enzymes like L-methionase are being explored. L-methionase is particularly promising due to its absence in humans and presence in various pathogenic organisms, making it an ideal selective drug target.

Current treatments for parasitic infections often rely on FDA-approved 5-nitroimidazole drugs such as metronidazole and tinidazole. However, resistance to metronidazole has been observed in organisms such as *Trichomonas vaginalis* (Lockwood & Coombs, 1991; Tokoro *et al*., 2003; Gustin, 2014). and *Clostridium difficile*, including hypervirulent strains, which remain difficult to manage despite their partial susceptibility to metronidazole and vancomycin. As a result, there is an urgent need to identify alternative therapeutic targets for these anaerobic pathogens (Suganya *et al.,* 2017b; Abozeid, 2023).

Moreover, L-methionine depletion has shown antiviral effects in *Pneumocystis carinii*-induced pneumonia, as this pathogen exhibits a strong dependence on L-methionine. These results support the potential of L-methionase-based therapies as viable alternatives for treating drug-resistant infections (Suganya *et al*., 2017a).

2.18.8 As a Silencing in Potatoes

Increasing L-methionine levels in potato tubers can enhance the desirable aroma of baked and fried potatoes. Genetic engineering approaches aimed at boosting L-methionine content have often resulted in elevated production of α-ketobutyrate due to catabolism mediated by L-methionase.

In *Solanum tuberosum*, the gene *stMGL1* encodes an active L-methionase enzyme that degrades L-methionine. Silencing this gene leads to the accumulation of free L-methionine in potato tubers, thereby improving their aroma potential during culinary processing (F. T. Liu, 2018).