Benha University

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Botany & Microbiology Department

Bacterial L-Methioninase Enzyme and it's Prospective Application as Anticarcenomic Agent

A thesis

Submitted to Botany and Microbiology Department, Benha University in Partial Fulfillment of the Requirements for the Master Degree of Science in Microbiology

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First of all, my thanks to

"ALLAH"

The Kind and the Merciful for giving me the power to perform and complete this work

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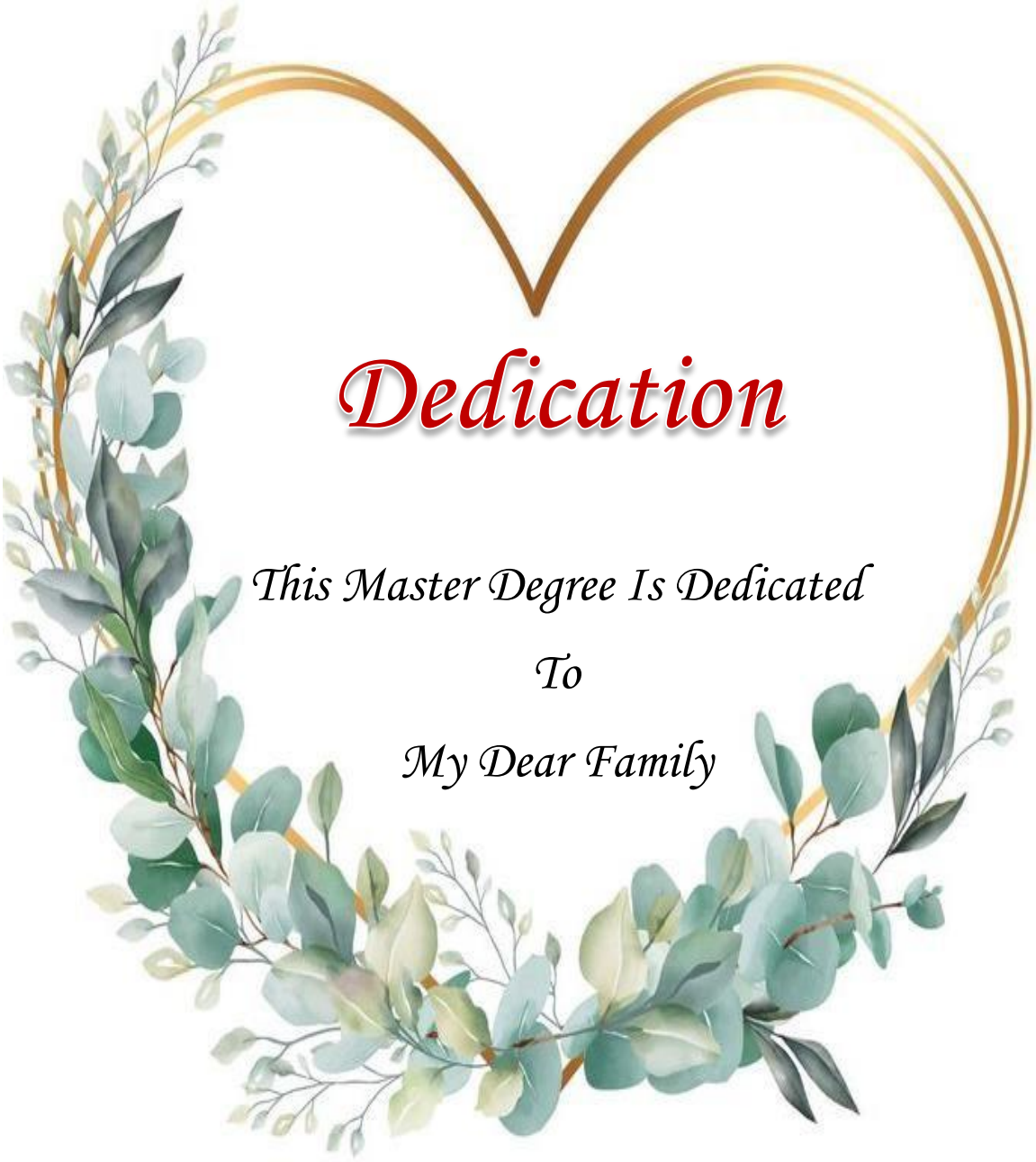

Dedication

This Master Degree Is Dedicated

To

My Dear Family

Benha University

Faculty of Science

Botany & Microbiology Department

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List OF Abbreviations

The Abbreviation	The Description
Ala	Alanine
Arg	Arginine
Asp	Aspartic acid
BAP	Benzylaminopourine
BD	Butanedione
BSA	Bovine serum albumin
Cys	Cysteine
Cyst	Cysteine
DEAE- acetate	Diethyl amino ethyl acetate
DMSO	Dimethyl sulfoxide
E	Enzyme
EC	Enzyme Commission
EGTA	Ethylene glycol tetra acetate
ES	Enzyme-substrate complex
FBS	Foetal bovine serum
GA	Glutaraldehyde
GA_3	Gibberellic acid
Glu	Glutamic acid

The Abbreviation	The Description
Gly	Glycine
GSH	Reduced glutathione
His	Histidine
Hydro.Pro	Hydroxy proline
IC_{50}	The half maximal inhibitory concentration
Ile	Isoleucine
JA	Jasmonic acid
KDa	Kilo dalton
Kin	Kinetin
Leu	Leucine
Lys	Lysine
MA	Maleic anhydride
MATII	Methionine adenosyl transferase II
MCF-7	Human breast adenocarcinoma cell line
Met	Methionine
NBS	N-bromosuccinimide
NEM	N-ethylmaleimide
O.D	Optical density
PAGE	Polyacrylamide gel electrophoresis
PEI	Polyethyleneimine

The Abbreviation	The Description
Phe	Phenylalanine
PLP	Peridoxal-5-phosphate
PMSF	Phenylmethylsulfonyl fluoride
Pro	Proline
PVA	Polyvinyl alcohol
RPMI	Roswell park memorial institute
SA	Succinic anhydrides
SAH	S-adenosyl homocysteine
SAHH	S-adenosyl-L-homocysteine hydrolase
SAM	S-adenosyl methionine
SDS-PAGE	Sodium dodecyl sulfate - polyacrylamide gel electrophoresis
SE	Standard error
Ser	Serine
SSF	Solid-state fermentation
T.cell	Type of white blood cell called lymphocyte
Thr	Threonine
TNM	Trinitromethane
Trp	Tryptophan
Tyr	Tyrosine
UV	Ultraviolet
Val	Valine

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Abstract

L-methioninase was isolated and purified from *Staphylococcus sciuri* using ammonium sulphate, DEAE-cellulose, Sephadex G-200 and Q-Sepharose with specific activity of 168.7 U mg^{-1} protein and purification fold of 177.5. The SDS-PAGE exhibited a single band at 48 kDa and this confirms purity of the L-methioninase. Analysis of amino acids of the purified enzyme exhibited variation in the content of the amino acids. The most dominant amino acids were cysteine, aspartic acid, and methionine. The values of V_{max} and K_m of the purified enzyme were 33.3 U mg^{-1} protein and 1.7 mM. The optimal pH and the optimal temperature of the enzyme were 8.0 and 40 ºC. The phytohormones activated the L-methioninase with different rates. The four reagents of the active groups namely N-bromosuccinimide (NBS), butanedione (BD), N-ethylmaleimide (NEM) and N-trinitromethane (TNM) inhibited L-methioninase in a concentration-dependent manner confirming the necessity of tryptophanyl, arginyl, sulfhydryl and tyrosyl groups for L-methionine catalysis. L-methioninase exhibited appreciable anticancer effect against HeLa and MCF-7 cell lines.

1. Introduction

Introduction

Each year, millions of individuals face the devastating impact of cancer, whether through cancer-related deaths or the difficulties associated with treatment. In addition to the financial burden of cancer treatment, there is a need for the development of new drugs and improvements in existing drugs owing to factors such as drug resistance observed in various types of cancer. The conventional treatment strategies of surgery, chemo, ionization/radiation and ultrasound therapies lack complete effectiveness in reducing cancer and usually cause serious side effects to the healthy cells of the body **(Mittelstein, 2020).** A new approach for the treatment of cancer is the use of enzymes such as L-methioninase that blocks the availability of chief nutrients required for the rapid growth of cancer cells.

1.1. L-methioninase

L-methioninase (EC 4.4.1.11) is also known as methionine-gamma-lyase, methioninase, methionine lyase, and methionine demethylase, it is a pyridoxal phosphate dependent enzyme which catalyzes α, γ-elimination of L- methionine to α-ketobutyrate, methanethiol, and ammonia **(Abdelraof *et al.*, 2019).**

Onitake (1938) was the first person to report that there were some bacterial species that produced methanethiol in the presence of L-methionine and L-cysteine. This led to a flux of research in this area such that till date many bacterial species have been reported to possess L-methioninase and it has been characterized. Examples of such bacteria are *Clostridium sporogenes, Citrobacter freundii, Pseudomonas putida, Bacillus thuringiensis, Brevibacterium linens*, etc **(Mohkam *et al.*, 2020).**

L-methionine plays a vital role in protein synthesis, methylation, and cellular functions. Unlike normal cells, cancer cells rely heavily on external methionine sources for survival owing to their altered metabolic pathways (**Hoffman, 2019a).**

By degrading the amino acid L-methionine which is essential for cancer cell growth and survival, it exhibits selective cytotoxicity toward L- methionine-dependent cancer cells while potentially sparing healthy cells **(Suganya *et al.*, 2017).**

Inhibitions of several types of tumor cell lines by enzymes has gained attention as a potential therapeutic agent against various cancer types. Cell lines, such as glioblastoma, kidney, lung, and colon cancer, could be inhibited by this type of enzyme. L-methioninase was reported widely as an antitumor factor against various malignant cell line, breast, lung, colon, kidney and glioblastoma **(Sharma *et al.*, 2014)**.

Studies have shown that the normal cells have active methionine synthase; they could grow on a medium supplemented with homocysteine, vitamin B_{12} and vitamin B_6 rather than L-methionine. This is because normal cells have the metabolic ability to convert homocysteine to L-methionine, whereas most cancer cells do not have this mechanism so it is dependent on external L-methionine supplementation from the diet **(Sharma *et al.*, 2014**).

However, L-methionine deficiency leads to the death of cancer cells. Consequently, while normal cells can function independently of L-methionine, the reliance of the majority of cancer cells on exogenous L-methionine is known asL-methionine dependence (**Hoffman, 2019a).**

2. Aim of the present work

This study is conducted with the aim of investigating the following objectives in greater depth:

1. Purification of L-methioninase from rich bacterial source.
2. Investigating the amino acid composition of the enzyme.
3. Studying the kinetics of the purified enzyme.
4. Induction of L-methioninase activity by various compounds such as phytohormones and the possible use of the enzyme as anticancer against some cancer cells.

3. Review of Literature

3.1. Enzymes

Enzymes are biocatalysts (biological catalysts) with high molecular weight protein structure, like other proteins. They consist of a large number of amino acid, there are peptide bonds between them and these amino acids form a chain one or more polypeptide chains. All enzymes are protein but all protein are not enzymes. Enzymes act by reducing the activation energy **(Al-Odat, 2024)**, thereby increasing the rate of reaction (**Fig. 1).**

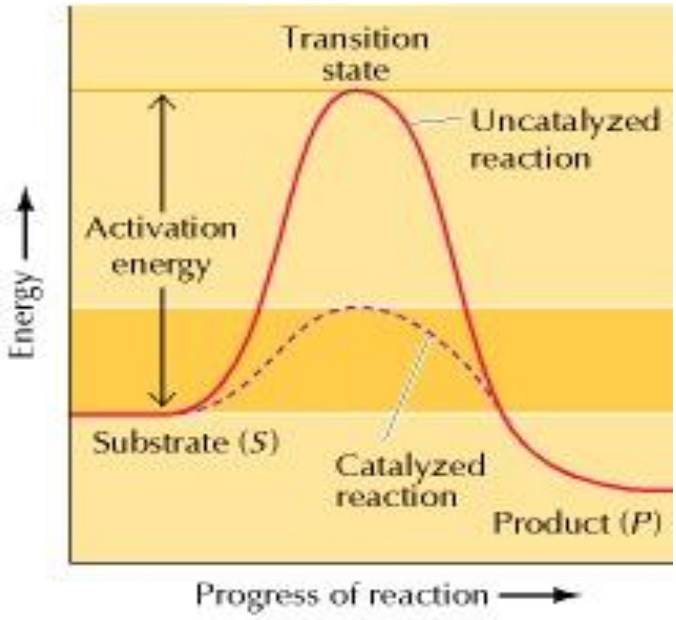


Fig. 1: Energy diagrams for catalyzed and uncatalyzed reaction (Al-Odat, 2024)

As catalysts, enzymes are required in very low concentrations, and they speed up reactions without themselves being consumed during the reaction **(Taylor, 2002)**. We usually describe enzymes as being capable of catalyzing the conversion of substrate molecules into products as follows:

$$E + Substrate \rightleftharpoons Product + E$$

Certain enzymes have the ability to convert substrate to product many times faster than others. They cannot change the equilibrium of reactions and differ from other catalysts in their specificity for the substrate. They are specific in their action, and their activity depends on the concentration of substrate and other physical conditions such as temperature and pH **(Patel *et al*., 2023).**

The catalytic activity of enzymes involves binding of their substrates to form an enzyme-substrate complex (ES)**.** The substrate binds to specific region of the enzyme, called the active site. While bound to the active site, substrates converted into the product of the reaction which, is then released from the enzyme **(Chen and Arnold, 2020)**. The enzyme-catalyzed reaction can thus be written as follows:

$$S+E \rightleftharpoons ES \rightleftharpoons E+P$$

Our bodies naturally produce enzymes. They are synthesized by living cells where RNA molecules translate information from DNA and create proteins **(Wu *et al*., 2021)**.

Enzymes speed up metabolism or the chemical reactions in our bodies without damaging the cells during the reaction and are used over and over, so that keep a person alive and well. For example, each cell in the body contains DNA. Each time a cell divides, the cell needs to copy its DNA. Enzyme help in this process by unwinding the DNA coils (**Chen and Arnold, 2020)**.

Enzymes build some substances and break other down. For example, Enzymes help the body break down larger complex molecules into smaller molecules such as glucose so that the body can use them as fuel **(Akram, 2013)**.

Enzymes can be obtained from various sources like microorganisms, plants, and animals. Microorganisms are thought to be the primary resource of enzymes due to their broad variety of activities, better yields, genetic management, and quick production in un-costly process (**Kumar *et al.*, 2020).**

More attention has been given to microbial enzymes because of their stability and activity compared to enzymes isolated from plants and animals **(Al-Odat, 2024)**.

3.1.1. Enzyme classification

Understanding enzyme classification is important for studying biochemical pathways, designing drugs, and understanding diseases caused by enzyme deficiencies. The nomenclature of enzymes was also problematic; enzymes were given trivial names to identify them. Some names were carefully chosen by groups of biochemists **(Cornish-Bowden, 2014).**

The molecular function of enzymes is defined as their ability to catalyze biochemical reactions; it is manually classified by the Enzyme Commission (EC) **(Tipton, 2022).** They were divided into 6 major classes according to the type of reaction catalyzed and a seventh, the translocases, was added in 2018 **(Tipton, 2018).**

The EC composed of four components separated by full stops **(Table 1)**. The first identifies the class of reaction catalyzed. The second number (the subclass) generally contains information about the type of compound or group involved. For the oxidoreductases, the subclass indicates the type of group in the donor that undergoes oxidation or reduction (e.g., 1.1. acts on the CH-OH group of donors). The third number, the sub-subclass, further specifies the type of reaction involved. The fourth is a serial number that is used to identify the individual enzyme within a sub-subclass (**McDonald and Tipton, 2014**).

Table 1: Summary of enzyme classification according to their reaction, with their biological importance (McDonald and Tipton, 2014).

Class	(Sub Class) Reaction	Biological importance
Oxidoreductases (EC1)	(forming carbon-oxygen bonds) Oxidation reactions involve the transfer of electrons from one molecule to another. E.g.: Lipoxidases and Dehydrogenases	Cellular respiration, photosynthesis, and detoxification processes.
Transferases (EC2)	(forming carbon-sulfur bonds). Catalyze the transfer of groups of atoms from one molecule to another. E.g.: Aminotransferase	Glycolysis, amino acid metabolism, and DNA methylation.
Hydrolases (EC3)	(forming carbon-nitrogen bonds). Hydrolysis reactions involve the cleavage of substrates by water. E.g.: Lactase, Proteases, and trypsin.	Digestion, metabolism, and cellular repair mechanisms.

Lyases (EC4)	(forming carbon-carbon bonds). Catalyze the addition of groups to double bonds or the formation of double bonds via the removal of groups	Cellular respiration, fermentation, and other biochemical processes.
Isomerases (EC5)	(forming phosphoric-ester bonds). Catalyze the transfer of groups from one position to another on the same molecule. E.g.: Topoisomerase and glucose isomerase	Glycolysis, the citric acid cycle, and the pentose phosphate pathway
Ligases (EC6)	(forming nitrogen-metal bonds).Catalyze the joining of two molecules to form a new bond. E.g.: Glutathione synthase and Aminoacyl tRNA synthetase.	DNA replication, repair, and the synthesis of large biomolecules.

Translocases EC 7	Catalyze the movement of ions or molecules across membranes or their separation within membranes. E.g.: Ubiquinone reductase, ATP synthase, Ascorbate and ferri-reductase	Nutrient uptake, waste removal, signal transduction, and the maintenance of cellular osmolarity

3.1.2. Factors affecting enzyme activity.

According to **Wiley *et al.* (2012)** the activity of enzyme is affected by the following factors:

1-Enzyme concentration.

2- Substrate concentration.

3- pH.

4-Temperature.

5- Inhibitors or activators.

3.1.3. Purification of enzymes

The purification procedure is usually necessary to isolate the enzyme. There are relatively few techniques that are available for the separation of proteins. However, by sequential use of a number of these techniques, it has been possible to isolate many enzymes in a pure state **(Copeland, 2023).**

3.1.3.1. Salting out

Salting out is a purification technique that utilizes high concentration of salts cause decreasing of the solubility, and protein precipitates **(Genck and Wayne, 2010).** Some proteins will precipitate at 50% saturation with ammonium sulphate. After removing the precipitate by filtration or centrifugation, the desired protein can be precipitated by altering the salt concentration to the level at which the desired protein becomes insoluble (**Fig. 2**).

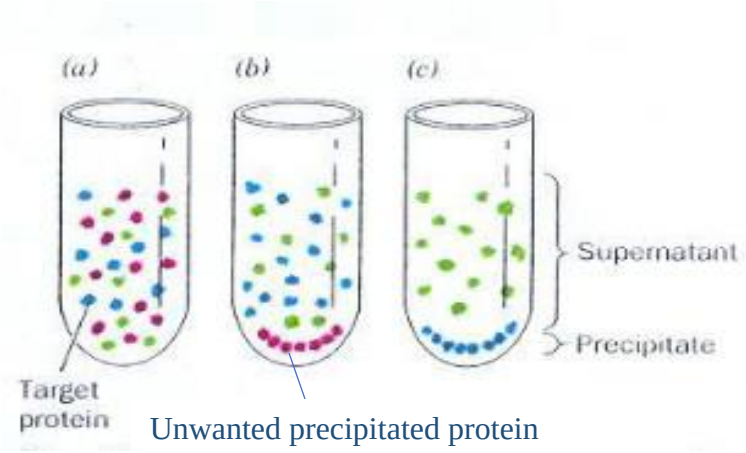


Fig. 2: Salting out technique (Genck and Wayne, 2010).

(a) The salt of choice, usually ammonium sulfate, is added to a solution of macromolecules to a concentration just below the precipitation point of the protein of interest.

(b) After centrifugation, the unwanted precipitated protein (red spheres) are discarded and more salt is added to supernatant to a concentration sufficient to salt out desired protein (green spheres).

(c) After a second centrifugation, the protein is recovered as a precipitate, and the supernatant is discarded.

This method is typically used to precipitate large biomolecules, such as proteins or DNA (**Fig. 3).** Because the salt concentration needed for a given protein to precipitate out of the solution differs from protein to protein, a specific salt concentration can be used to precipitate a target protein **(Chacon-Cortes and Griffiths, 2020).**

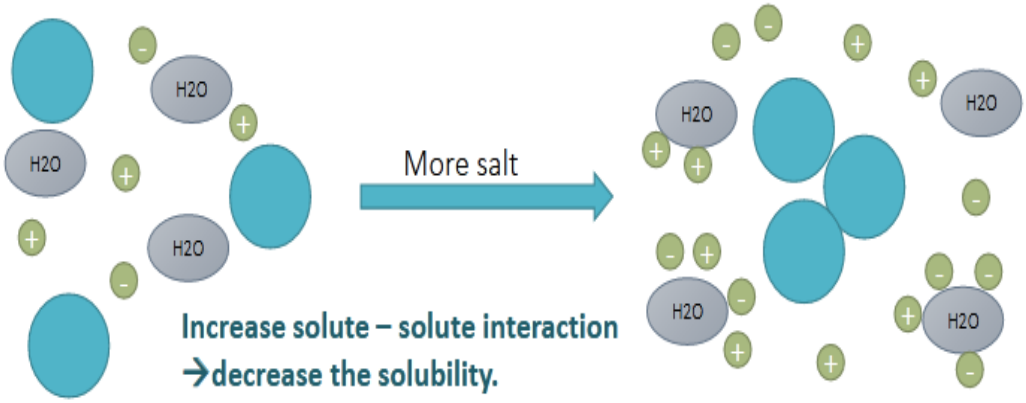


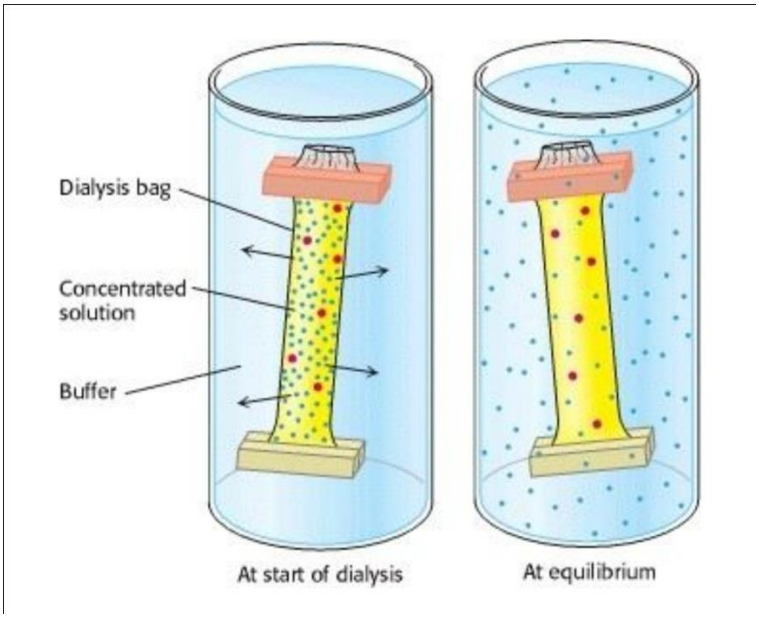
Fig. 3: Salting out mechanism(Chacon-Cortes and Griffiths, 2020).

Ammonium sulphate fractionation is an extremely effective early step in the purification of proteins. Ammonium sulphate is often used for this purpose because it is extremely soluble in water. The technique has the additional advantage of reducing the volume of material, which is often an important consideration at this stage **(Copeland, 2023)**.

However, using ammonium sulphate has a notable disadvantage. Ammonium sulphate doses contain trace quantities of heavy metals that may be sufficient to inactivate the enzyme. This problem may be overcome it easily by the use of good-quality (annular-grade) ammonium sulphate **(Patel *et al*., 2023).**

3.1.3.2. Dialysis

A dialysis membrane such as cellophane can be used to separate the globular proteins or molecules with a molecular weight of around 20, 000 Dalton (**Fig. 4**). The pore size can be changed by various mechanical and chemical treatments. The method is routinely used to separate small organic molecules , salts and organic solvents from the crude extracts **(Berg *et al*., 2002).**



(a) (b)

Fig. 4 : The separation of small and large molecules by dialysis (Berg *et al*., 2002).

(a) Only small molecules can diffuse through the pores in the bag.

(b) At equilibrium the concentrations of small molecules are nearly the same inside and outside the bag, whereas the macromolecules remain in the bag.

3.1.3.3. Gel Filtration Chromatography

Gel filtration or gel permeation chromatography is a separation method dependent upon molecular size (**Fig. 5**). The method is also known as molecular sieve, or molecular exclusion chromatography **(Stellwagen, 2009).** Its excellent reproducibility, comparatively short time and relatively inexpensive equipment make it far widely used separation method.

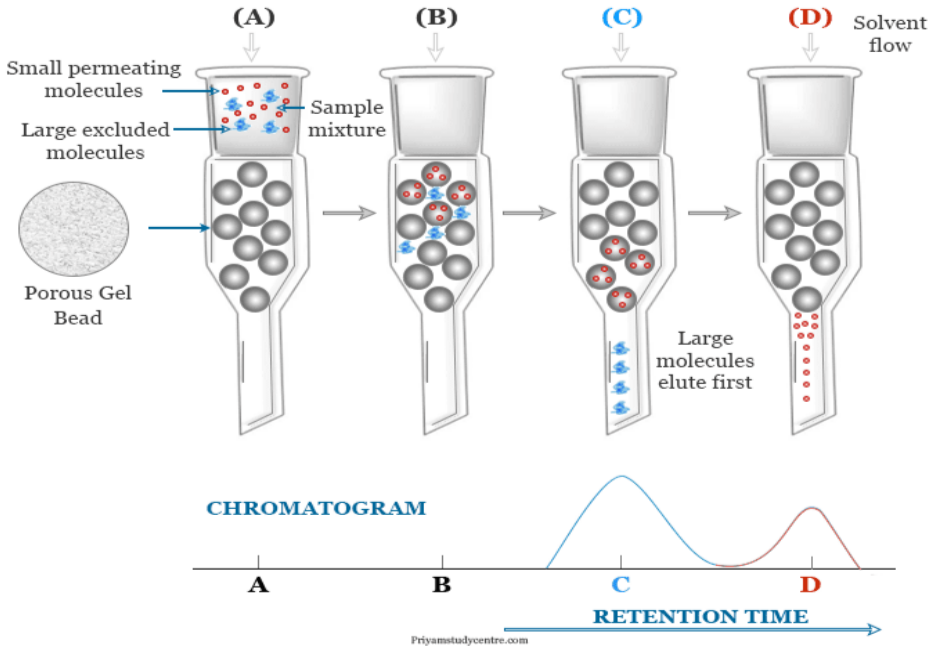


Fig. 5: Gel Filtration Chromatography (Stellwagen, 2009).

Although gel filtration using Sephadex or related materials is an extremely successful technique, there are a number of disadvantages to be considered. The amount of sample that can be applied to the gel is generally restricted to 1-2% of the total volume; with fairly simple separation, this proportion may be increased **(Crowley and Kyte, 2014).**

3.1.3. 4. Ion Exchange Chromatography (IEC)

IEC is one of the most widely used methods for enzyme purification. It separates protein molecules according to their differences in charge **(Jungbauer and Hahn, 2009).** The stationary phase (matrix) in IEC carries charged functional groups fixed by chemical bonds. The fixed groups are associated with exchangeable counter ions. In anion exchange chromatography, the fixed groups have positive charges and in cation exchange chromatography, these groups are negatively charged (**Fig. 6).**

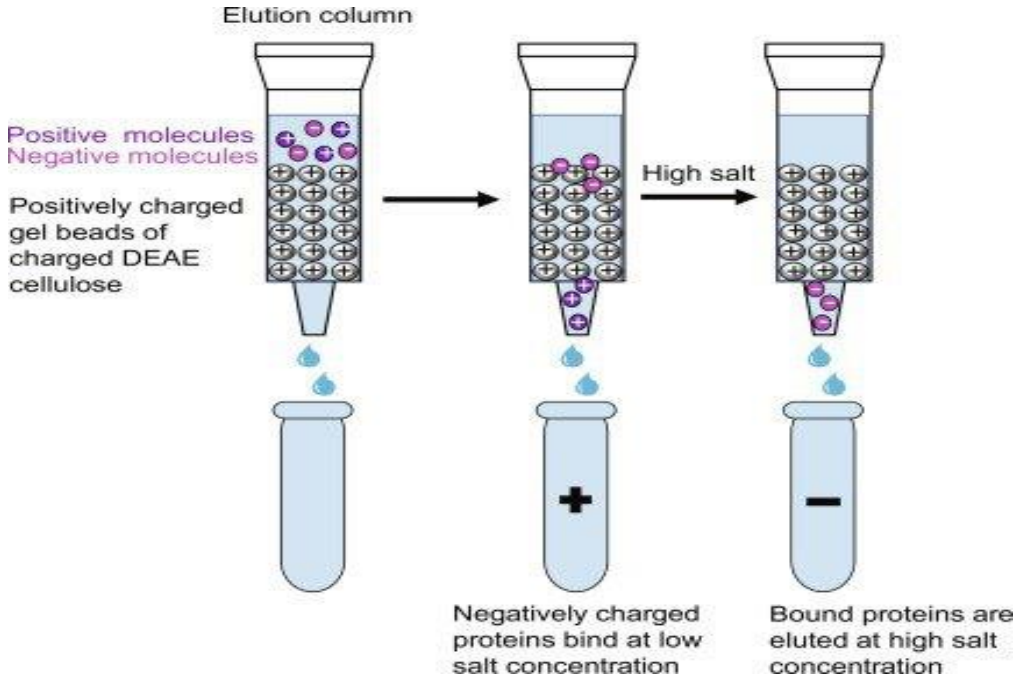


Fig. 6: Ion Exchange Chromatography (Jungbauer and Hahn, 2009).

3.1.4. Stability of enzyme

The most significant field of both essential and applied enzymology is enzyme stabilization. It is very important to understand the concepts of enzyme stabilization through studying the reasons that the enzymes can lose their biological activity **(Patel *et al*., 2023).**

The main significant goal is to achieve useful compounds through bio-catalysis in applied enzymology. Enzymes have high catalytic activity, specific activity, and the ability to function under moderate conditions. Generally, because they are unstable and are inactivated rapidly through many mechanisms, they aren't necessarily the best catalysts for actual purpose **(Dudala *et al*., 2023).**

Storage stability is the ability of the preparation to retain its activity under some specified storage conditions and can provide information about its shelf life. Operational stability is not only a function of the enzyme but also of the carrier durability and inhibitor concentrations in the analytical solution **(Maghraby *et al*., 2023).**

Stabilization of enzyme is a very useful strategy that is used to protect industrially important enzymes against inactivation caused by heat, chemicals, and other environmental factors. This can be achieved using different techniques including: enzyme immobilization, protein modification, and genetic engineering **(Boutureira and Bernades, 2015)** as shown in (**Fig. 7).**

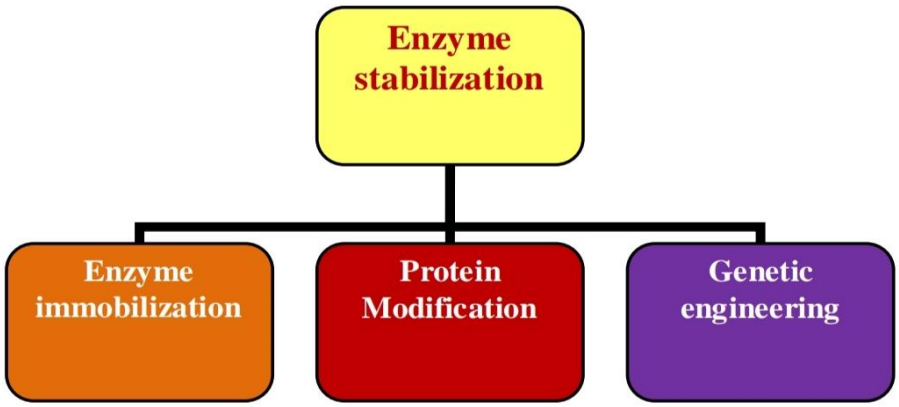


Fig. 7: Methods of enzyme stabilization (Boutureira and Bernades, 2015).

The stabilization of enzymes has received much attention in recent years. The most common methods to obtain soluble stabilized enzymes are modification of enzyme protein (chemical modifications) (**Boutureira and Bernades, 2015).**

Stabilization against thermal inactivation can be performed through immobilization such as entrapment in gels **(Crowley and Kyte, 2014).**

3.1.5. Immobilization of enzymes

Enzyme immobilization is a typical strategy that primarily aims to reduce the share of enzyme costs in process economics by enabling multiple enzyme reuses. This means that the enzyme is physically confined, often in a polymer matrix in the form of beads or membranes, in such a manner that it cannot be lost into solution **(Sheldon and van Pelt, 2013).**

The use of an immobilized enzyme also generally facilitates downstream processing because it can simply be removed by sieving, whereas considerable effort and money would have to be invested in removing a soluble enzyme from a reactor stream. Furthermore, compared to dissolved enzymes, immobilized enzymes usually show greater stability. However, there are disadvantages as well, such as reduced mass transfer or diffusion, altered kinetics, and a slight decrease in activity **(Basso and Serban, 2019).**

The advantages of enzyme immobilization can be summarized, according to **Datta *et al.* (2013)** as follows:

1) The increase of enzyme stability.
2) The enzyme support system can be easily removed from the solution without contamination by the contents of the reaction mixture.

3) A single aliquot of enzyme can be repetitively used to achieve more analyses than could be performed with the same amount of enzyme in solution.
4) It is possible to prepare unstable, sensitive, or expensive reagents using an immobilized enzyme **(Datta *et al.*, 2013)**.

The characteristics of immobilized enzymes are defined by the features of the carrier substance and by both the quality and quantity of interactions with the enzyme's surface and the substrate. Following immobilization, both enzyme stability and catalytic characteristics are typically altered, primarily as a result of the supporting matrix's changes and the microenvironment **(Goldhahn *et al.*, 2020).**

The property modification may take place by alters in the essential activity of the immobilized enzyme or by the chemical reaction between the enzyme and its substrate occurring in an environment that is independent of the overall solution. Therefore, a lack of catalytic activity is a major issue when using immobilized enzymes, particularly if the enzymes are performing on macromolecular substrates **(Guisan *et al.*, 2022).**

The activity of the enzyme may be restricted to the substrate's surface groups only due to the substrate's restricted access to the active site. The typical pattern of products produced from the macromolecular substrate may change as a result of this steric restriction **(Wahab *et al.*, 2020).**

A number of approaches can be used to prevent these steric issues, including the use of hydrophilic and inert spacer arms, the careful selection of enzyme residues engaged in the immobilization, and the selection of supports made of networks of separated macromolecular chains **(Khan, 2021).**

3.1.5.1. Choice of support and principal method

Enzyme immobilization is an approach specifically aimed at minimizing the movement of soluble enzymes in solution, as soluble enzymes can disperse and move freely. Immobilization provides support for the enzymes; when choosing an immobilization method, it is important to consider the supposed application and use first **(Fernández-Lafuente, 2017).**

3.1.5.2. Methods of enzyme immobilization

Numerous methods have been utilized previously for the immobilization of enzymes, for example: adsorption, covalent binding, entrapment, encapsulation, and cross-linking **(Brena *et al*., 2013; Basso and Serban, 2019)** as shown in (**Fig. 8).**

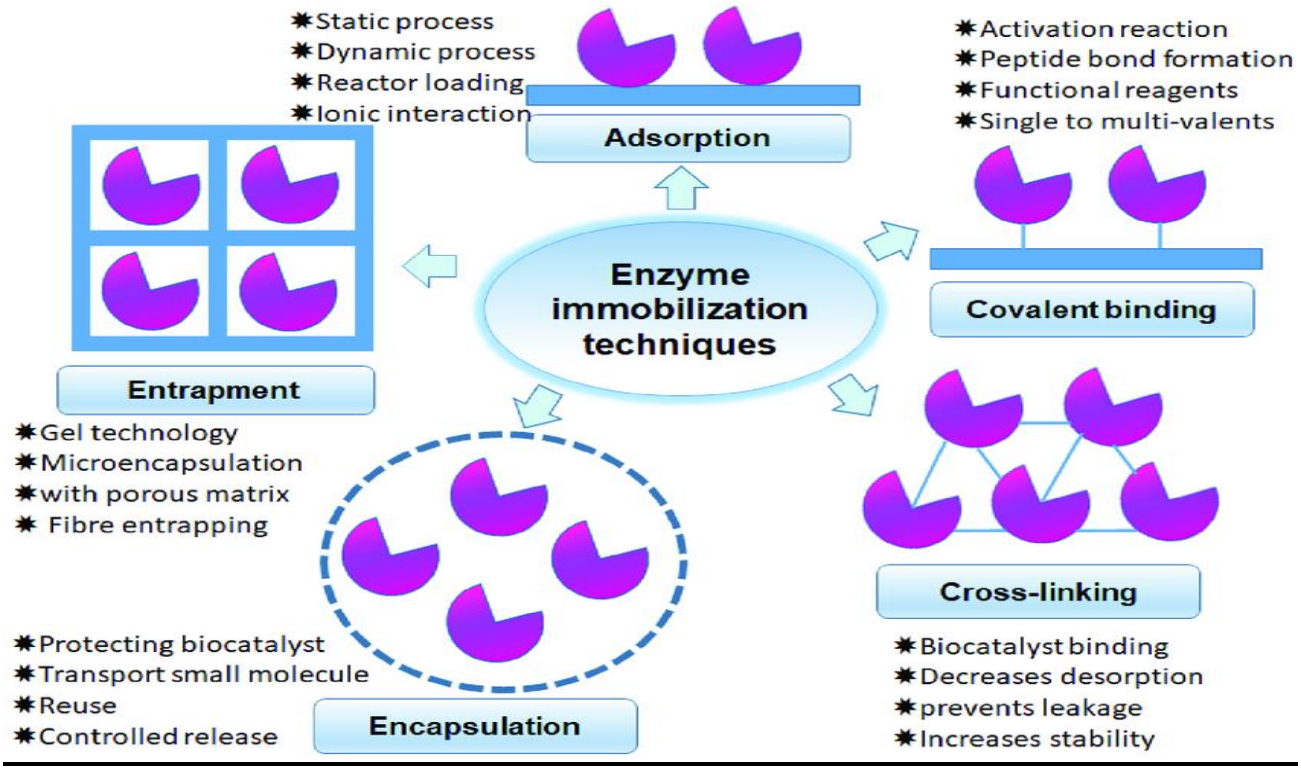


Fig. 8: Methods of enzyme immobilization (Basso and Serban, 2019).

1) Adsorption

Using adsorption as the immobilization method is the simplest strategy and involves reversible surface contact between the enzyme and transporter. Weak forces, primarily electrostatic ones, such as the forces of Van der Waals, ionic bonding, and hydrogen bonding interactions, are produced as shown in **(Fig. 9).** Hydrophobic interaction can be important, yet these forces are weak and numerous enough to allow adequate binding **(Nguyen and Kim, 2017)**.

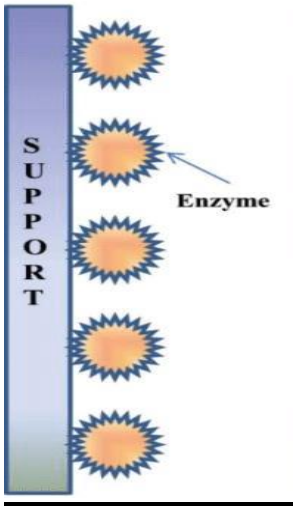


Fig. 9: Adsorption for enzyme immobilization (Sirisha *et al*., 2016).

In this procedure, the enzyme and the support material are mixed together in terms of adsorption characteristics at optimum pH, ionic strength, and other parameters for a period of time. Then, the immobilized enzyme is obtained and cleaned to eliminate the free enzyme **(Sirisha *et al*., 2016).**

Advantages of adsorption method are

1) Little or no damage to enzymes.
2) Easy, cheap, and fast.
3) No changes happened to the carriers and enzymes.
4) Reversible.

Disadvantages of adsorption method are:

1) Leakage of enzymes from the support.
2) Separation of product is not easy.
3) Nonspecific binding **(Maghraby *et al*., 2023)**.

2) Covalent binding

The method of immobilizing an enzyme through covalent binding involves creating a strong covalent bond with a carrier **(Cakmak *et al*., 2020)**.

The covalent bond is established between the functional groups on the carrier's surface and the enzyme's surface functional groups (**Fig. 10).** These functional groups include the hydroxyl group (OH) of threonine or serine, the carboxylic group (COOH) of glutamic acid or aspartic acid, the sulfhydryl group (SH) of cysteine, and the amino groups (NH_2) of arginine or lysine on the surface of the enzyme **(Hassan *et al*., 2019).**

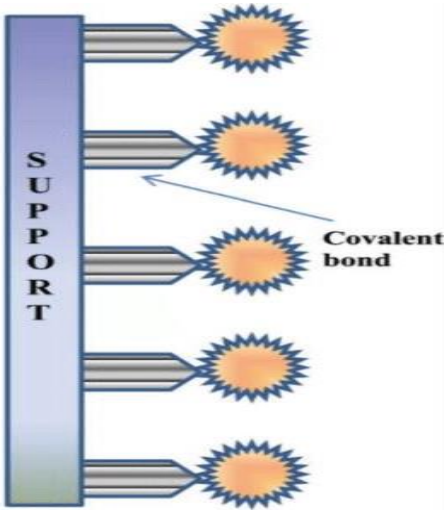


Fig. 10: Covalent binding technique of enzyme immobilization (Cakmak *et al*., 2020).

Numerous factors influence the choice of a particular carrier, and studies have shown that hydrophobicity is among the most significant variables in maintaining enzyme activity. For this reason, hydrophilic carriers like polysaccharide polymers are widely used for immobilizing enzymes, including agarose, cellulose, starch, and dextran **(Tentori *et al*., 2020)**.

Porous silica and porous glass are also common supports for enzyme immobilization. Porous silica includes tiny spherical particles melted together to form micro cavities and tiny channels. The carrier, which is highly robust and long-lasting, is typically offered as beads. There is a regular channel system in melted borosilicate glass **(Zdarta *et al*., 2018).**

Also, porous glass is long-term and resistant to solvent deformation or microbial dissolution. However, these two supports are techniques for joining an enzyme with a carrier by a covalent bond. Selecting a technique that has no influence on the enzyme is essential since it could react with the active site of the enzyme and make it inactive. Covalent bonding is done in two stages. First, a certain reagent activates the functional groups on the carrier surface; second, form a covalent bond by adding an enzyme to the activated surface of the carrier **(Cen *et al*., 2019).**

3) <u>Entrapment</u>

One of the easiest techniques of immobilization is entrapment. Calcium alginate has gained popularity as an immobilization support material. It has been utilized for the immobilization of a variety of cell types, sub-cellular organelles, multi-component systems, and enzymes (**Fig. 11)**. The physicochemical characteristics of this matrix in gel form have an important effect on the reactions of the entrapped biologically active material in the gel **(Brena *et al*., 2013).**

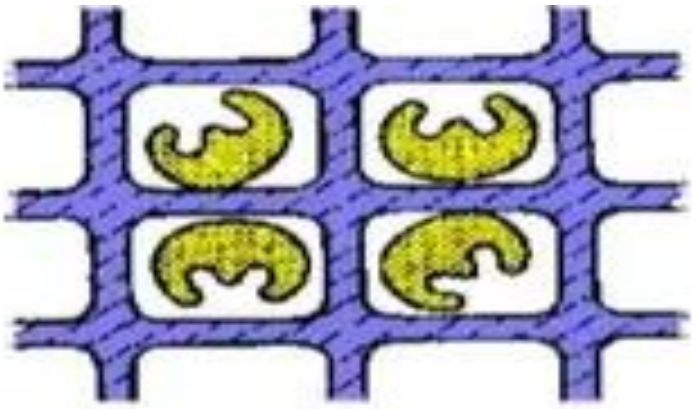


Fig. 11: Entrapment technique of enzyme immobilization (Brena *et al*., 2013).

The difference between the entrapment technique and the previous technique is that the enzyme is free in solution, but its movement is restricted by the gel lattice structure. The pore size of a gel lattice is controlled to ensure that the structure is tight enough to prevent the loss of enzymes or cells; it also allows free movement of the substrate and product. The support acts as a barrier to mass transfer, and although this has serious reaction kinetic implications, it can prevent interaction between harmful cells, proteins, enzymes, and immobilized biocatalysts **(Kalita and Sit, 2024).**

There are several major methods of entrapment, according to **Mohamed *et al*. (2015)**, as follows:

1) Lipotropic gelation of macromolecules with multivalent cations (e.g., alginate).
2) Temperature-induced gelation (e.g., agarose, gelatin).
3) Organic polymerization reaction by chemical or photochemical means (e.g., polyacrylamide).
4) Precipitation from a solvent that is immiscible (e.g., polystyrene).

4) Encapsulation

Encapsulation of enzymes as well as cells can be accomplished by wrapping the biological components inside different forms of semipermeable membranes **(Fig. 12).** It is like entrapment in that the enzymes are free in their movements, however limited in space. Vast proteins or enzymes cannot out- or inter-capsule; however, small substrates and products can go freely across the semi-permeable membrane **(Asaduzzaman and Salmon, 2022)**.

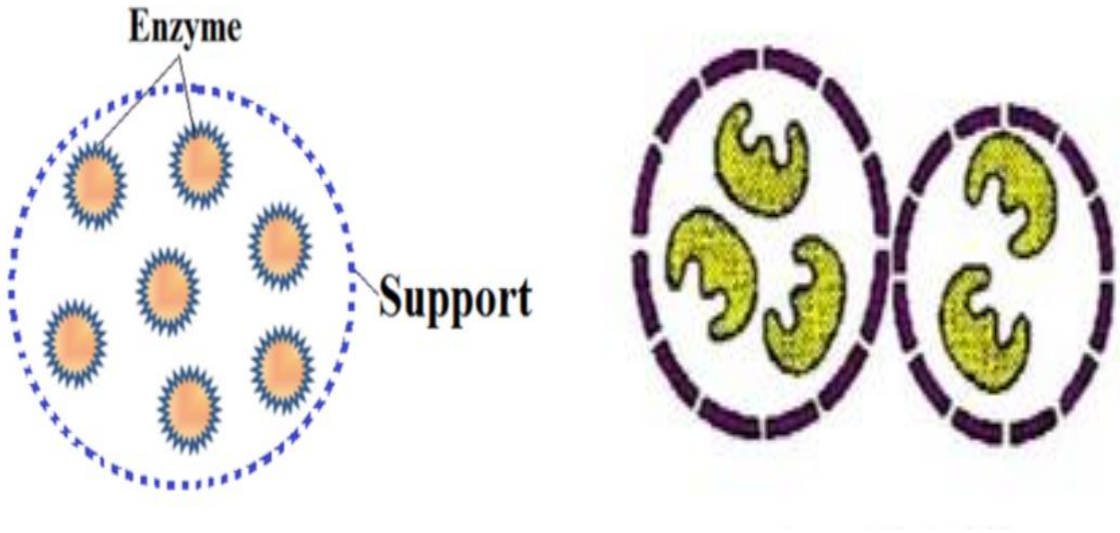


Fig. 12: Encapsulation technique of enzyme immobilization (Asaduzzaman and Salmon, 2022).

Numerous materials have been utilized to form microcapsules in the range of 10–100 µm in diameter, such as nylon and cellulose nitrate. Splitting of the membrane is an issue related to diffusion and may occur when reaction products collect quickly **(Nguyen and Kim, 2017).**

5) Cross linking

This method of immobilization depends only on enzymes, and it is support-free as it is done by joining the enzymes to each other to prepare a large, three-dimensional complex structure as shown in **(Fig. 13).** It can be done chemically or physically. The chemical type of cross-linking normally includes the formation of covalent linkages between enzymes molecules by means of a bi- or multifunctional reagent, for example, glutaraldehyde. However, limiting factors can be used in this method for living cells and many enzymes because of harmful materials **(Nisha *et al*., 2012).**

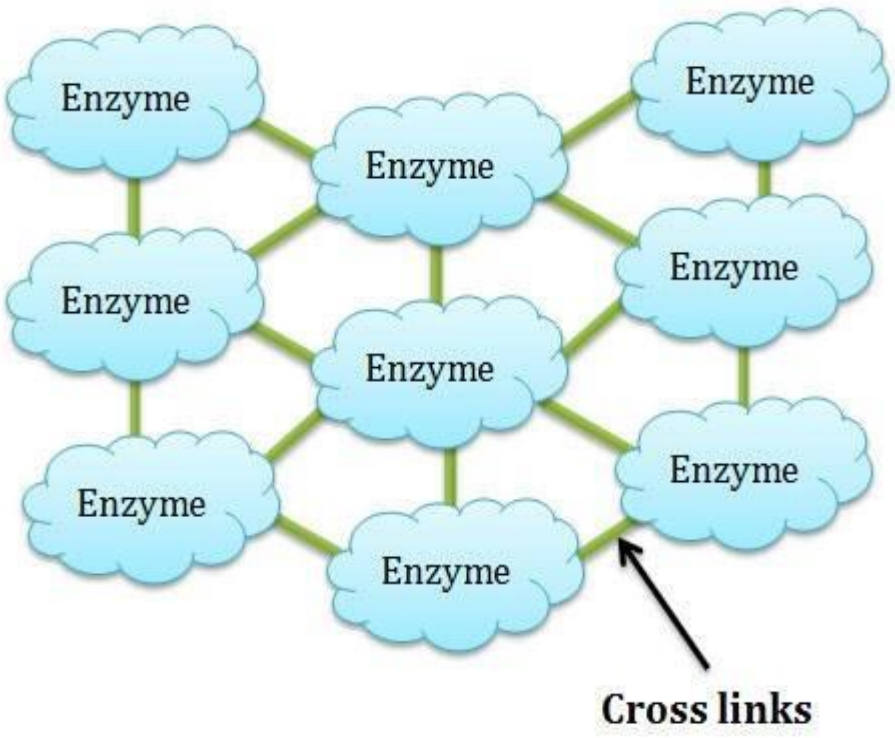
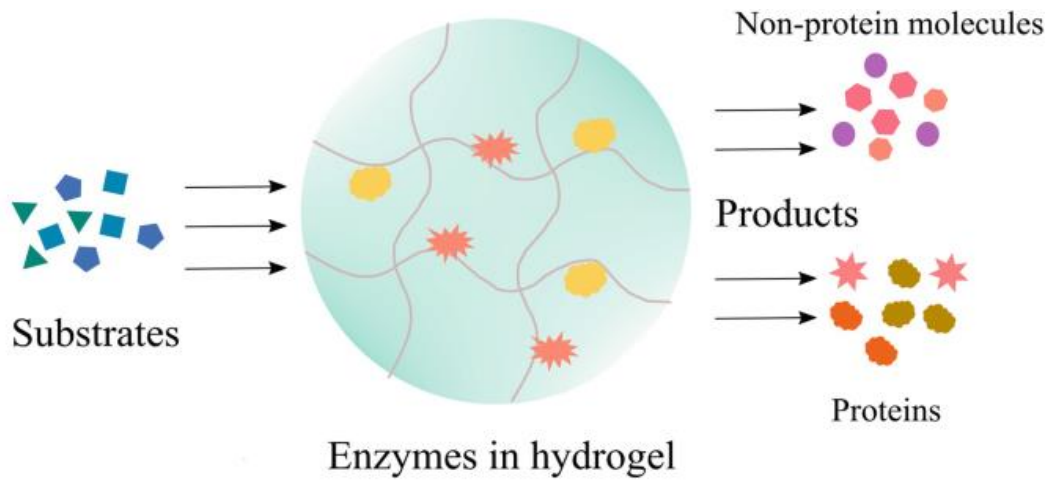


Fig. 13: Cross linking technique of enzyme immobilization (Nisha *et al*., 2012).

To minimize the close problems that can be found because of the crosslinking of a single enzyme, both albumin and gelatin have been used. This technique uses bi- or multifunctional compounds, which serve as the reagent for intermolecular cross-linking of the biocatalyst. Covalent binding or crosslinking methods are done under relatively severe conditions in comparison with those of physical adsorption or encapsulation **(Almeida *et al*., 2022)**.

6) Hydrogels

Enzymes can also be immobilized in natural or synthetic hydrogels or cryogels in non-aqueous media **(Fig. 14).** Polyvinyl alcohol (PVA) cryogels formed by the freeze-thawing method, for example, have been widely used for the immobilization of whole cells **(Tripathi *et al*., 2010).**



Hydrogels with three-dimensional porous structures and versatile functional groups are promising carriers for enzyme immobilization. The hydrogel-based multi-enzymatic systems are developed for biosynthesis, including cell-free protein synthesis and non-protein synthesis, especially high value-added molecules.

Fig. 14: Hydrogels technique of enzyme immobilization (Tripathi *et al*., 2010).

However, free enzymes, owing to their smaller size, can diffuse out of the gel matrix and are consequently leached in an aqueous medium. In order to entrap free enzymes, the size of the enzyme must be increased, e.g., by crosslinking **(Meyer *et al*., 2022).**

A different strategy to stimulate enzyme size is to produce a polyelectrolyte complex. Because proteins are ampholytic, they can appear as polyanions or polycations, depending on the medium's pH. They can frequently produce complexes with polyelectrolytes that are oppositely charged **(Peppas and Hoffman, 2020).**

3.1.5.3. Changes in the catalytic properties after immobilization

The observed changes in the catalytic properties upon immobilization may also be due to changes in the three-dimensional conformation of the protein provoked by the binding of the enzyme to the matrix. These effects have been demonstrated and, to a lesser extent, exploited for a limited number of enzyme systems. Quite often, when an enzyme is immobilized, it’s operational stability at higher temperatures and in the presence of organic solvents is highly improved **(Arcus and Mulholland, 2020).**

Thus, the stabilization principle has been greatly enhanced for immobilizing enzymes. At the molecular level, actual stabilization has been shown, like in the protein immobilized case within multiple points of covalent binding. Research performed by many investigators utilizing various techniques has shown that the stabilization and the number of covalent bonds in the matrix are correlated **(Attique *et al*., 2023).**

3.1.6. Applications of Enzymes

The several advantageous of catalysis by enzymes has permitted their use in various areas of biotechnology **(Singh *et al.*, 2016).** Enzymes are used in many industries, including:

1- The food industry.

2- Pharmaceutical industry.

3-Light industry.

4-Environmental management.

3.1.7. Enzyme therapy

Enzyme therapy is meaning using enzymes as drugs for disease treatment. These enzymes have two important characteristics, which are different from traditional drugs. Firstly, enzymes frequently bind and act on their targeted sites with high affinity and specificity, and secondly these are catalytic in nature and can convert multiple target molecules to the desired products. These two features are what make the use of enzymes very reliable **(El-Shora *et al.*, 2024a).** Many such enzymes are being utilized in cancer therapy. Examples of them are L-arginase, L-methioninase, L-asparaginase, etc.

3.2. L-methionine metabolism

L-methionine is a sulfur-containing essential amino acid and is historically known as the first amino acid to be recruited to the ribosome to initiate protein synthesis in eukaryotes. L-methionine depletion in culture media drastically suppressed protein synthesis in immortalized cell lines by impairing the recognition of translation start sites **(Mazor *et al.*, 2018).**

L -methionine produces metabolites that are key to polyamine synthesis, DNA synthesis, redox balance, and methylation reactions **(Sanderson *et al.*, 2019)**. The core of L -methionine metabolism is the L-methionine cycle which comprises a series of reactions that catabolize and regenerate L-methionine.

3.2.1. L- methionine cycle:

After entering L-methionine T cells, the following reactions occur (**Fig. 15)**.

1- Conversion of L-methionine into S-adenosylmethionine (SAM) by methionine adenosyl transferase II (MATII).
2- SAM is converted into S-adenosylhomocysteine (SAH) after donating a methyl group for methylation reactions **(Sanderson *et al.*, 2019)** including histone methylation and DNA methylation. This step is mediated by Methyl transferases (MTs).
3- SAH is then hydrolyzed by S-adenosyl-L-homocysteine hydrolase (SAHH) to generate homocysteine.
4- Finally, homocysteine receives a methyl group from the folate cycle or betaine to become methionine. Also, homocysteine enter the transsulfuration pathway to produce glutathione (GSH) and control redox balance.

The methionine cycle is interconnected with three important metabolic pathways by providing substrates. These pathways include the folate cycle, the transsulfuration pathway, and the methionine salvage pathway as in **(Fig. 15) (Sanderson *et al*., 2019).**

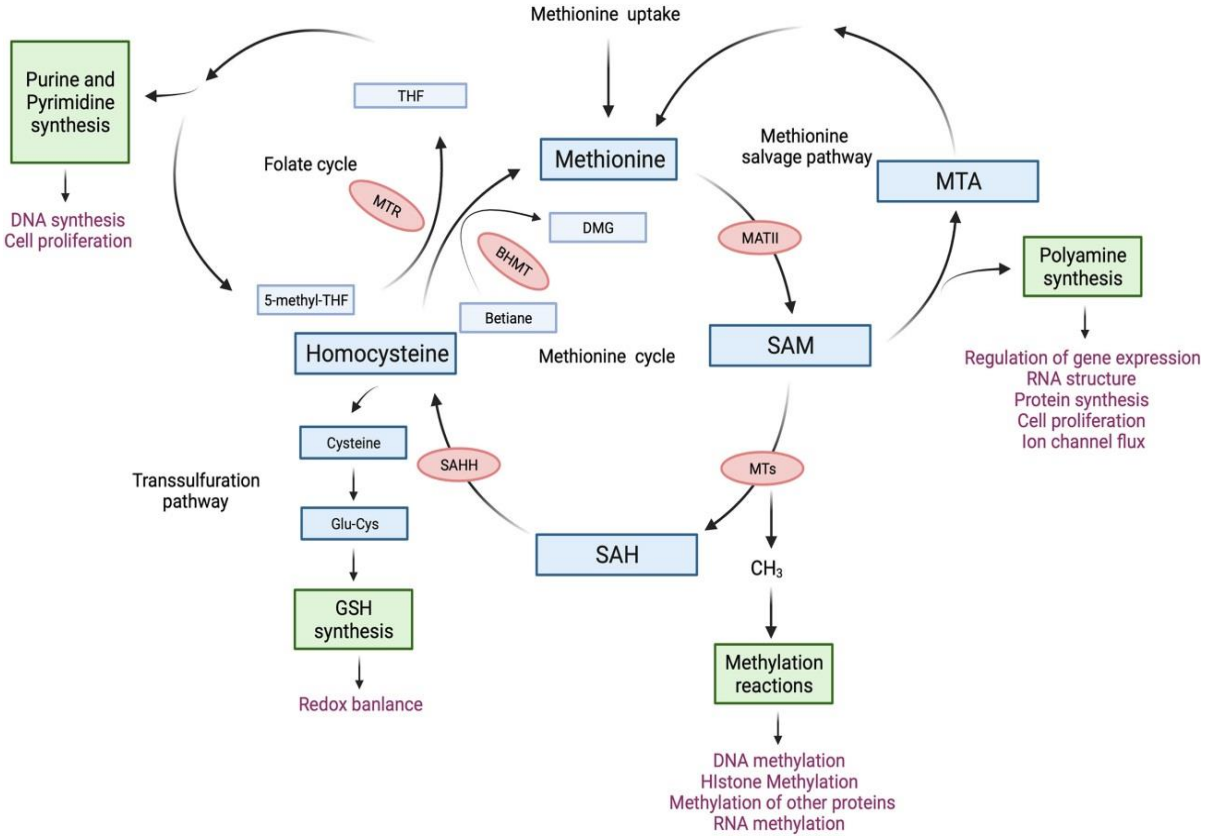


Fig. 15: L-methionine metabolism in T. cells (Sanderson *et al*., 2019).

3.3. L-methioninase

L-methioninase (EC.4.4.1.11) is a PLP-dependent (Pyridoxal 5 -phosphate) enzyme which is hydrolytic in nature. This enzyme belongs to the family of lyases, especially the class of Carbon-sulfur layers **(Abdelraof *et al*., 2019).** L-methioninase utilizes one cofactor pyridoxal phosphate **(El-Shora *et al*., 2021).** L-methioninase has high therapeutic value because of association with high immunogenicity **(Kharayat and Singh, 2018).**

L-methioninase was reported widely as an antitumor factor against various malignant cell line, breast, lung, colon, kidney, and glioblastoma **(Milad *et al*., 2020)**.

3.3.1. L-methioninase mechanism of action

This enzyme catalyzes the direct conversion of L-methionine ($C_5H_{11}NO_2S$) into α- ketobutyrate, methanethiol and ammonia **(Kharayat and Singh, 2018)** as can be seen from **(Fig. 16).**

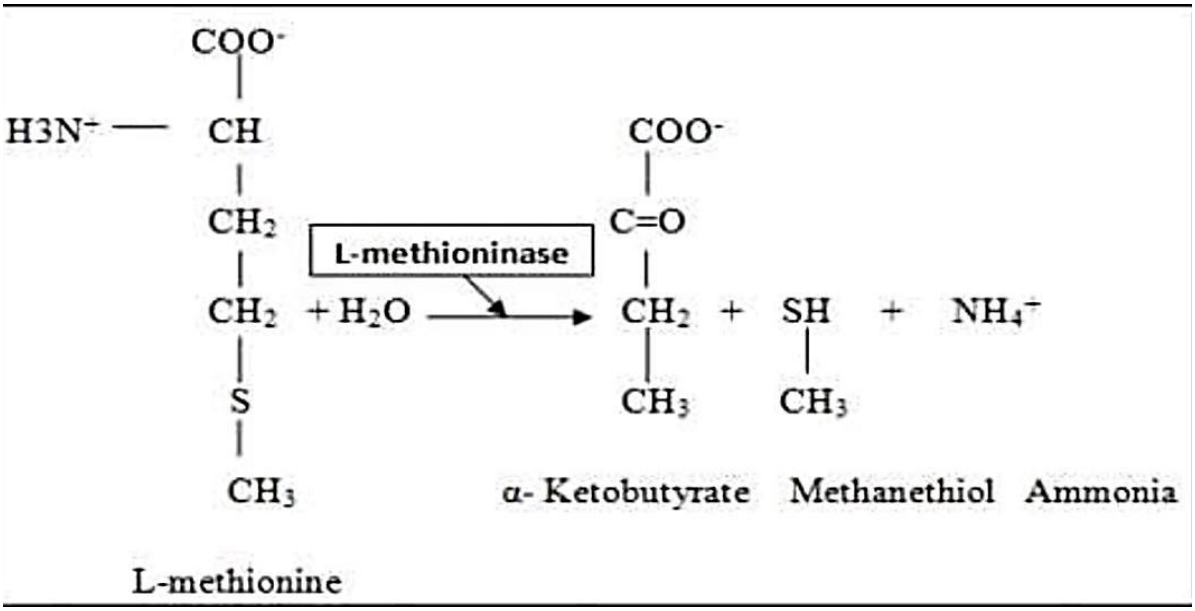


Fig. 16: Catalytic pathway for catalysis of L-methionine by L-methioninase mammals (Kharayat and Singh, 2018).

3.3.2. Sources of L-methioninase

L-methioninase was comprehensively characterized from many bacterial species as intracellular enzyme and from fungal species as intracellular and extracellular enzyme and absent in mammals **(Kharayat and Singh, 2018).** Various sources of the enzyme are compiled in **(Table 2).**

Table 2: Sources of L-methioninase (Suganya *et al*., 2017).

Bacteria	Fungi	Protozoa	Plant Sources
Achromobacter starkey	*Aspergillus niger*	*Entamoeba histolytica*	*Arabidopsis thaliana*
Aeromonas hydrophila	*Aspergillus flavipes*	*Trichomonas vaginalis*	*Cucumis melo*
Arthrobacter sp	*Aspergillus ustus*		*Solanum tuberosum*
Bacillus subtilis	*Aspergillus parasiticus*		*Catharanthus roseus*
Bacillus thuringiensis	*Clonostachys rosea*		
Brevibacterium linens	*Cladosporium*		
Clostridium sporogenes	*Cladosporoides*		
Citrobacter intermedius	*Cladosporium oxysporum*		
Citrobacter freundii	*Debaromyces hansenii*		
Clonostachys rosea	*Fusarium nivale*		
Fusobacterium nucleatum	*Saccharomyces cerevisiae*		
Ferroplasma acidarmanus	*Geotrichum candidum*		
Idiomarina sps	*Trichoderma koningii*		

The enzyme is formed by most bacteria as an intracellular enzyme. L-methioninase has also been known to be produced both by gram-positive and gram negative bacteria **(Bhawana and Priyanka, 2018).**

3.3.3. Role of L-methioninase in cancer therapy

According to the WHO website, cancer is one of the prime causes of death throughout the world, and accounts for almost 10 million deaths in 2020 alone. The most common cancer is breast, lung, prostate, colon, etc. Such cells, when malignant, require methionine in higher concentrations, so as to have high protein synthesis and DNA expression. The anti-tumor function of T cells is closely related to the metabolism of amino acids and other nutrients in T cells **(Wang *et al.*, 2022).**

Normal cells have the ability to grow on homocysteine, instead of methionine, due to their active methionine synthase. Thus, depleting cellular/plasma methionine levels using L-methioninase seems to be a promising therapeutic approach to treat cancer **(Bopaiah *et al.*, 2020).**

L-methionine plays an important role in tumor cells. These cells become L-methionine dependent. L- methionine dependency of abnormal cells can be utilized for the arrest of cancer cells by the enzymatic depletion of L- methionine. It was observed that such depletion when introduced in various carcinomas, gliomas, melanomas, etc resulted in the cell cycle of these cells getting arrested in late S/G2 phase **(Vachher *et al*., 2022).**

Therefore, L-methionine restriction with methioninase is stated to have a comprehensive selective strategy for many cancers in vitro as well as a high activity for killing cancer cells. L-methionine gets converted to S-adenosyl methionine and participates in DNA methylation, which is associated with cancer.

Higher levels of L-methionine in the body mean higher bioavailability of S-adenosyl methionine which will donate methyl groups to DNA. This can eventually lead to DNA hypermethylation of regulatory regions which could include the tumor suppressors (**Hoffman, 2019b).**

4. Materials and Methods

Materials

All of the chemicals were of analytical grade and purchased from Sigma.

4.1. The source of bacteria

Staphylococcus sciuri used in the present study was bought from Clinical Microbiology Lab in Faculty of Medicine, Mansoura University, Dakahlia Governorate, Egypt.

Staphylococcus sciuri is Gram positive cocci, it is a coagulase negative, oxidase positive and novobiocin resistant staphylococcal species. This species of bacteria is important human pathogens responsible for endocarditis, septic shock, urinary tract infections and wound infections **(Stepanovic *et al.*, 2003).**

4.2. Storage of pure culture

The bacterium is sub-cultured one every month by preparing slants using nutrient agar medium. To each of the test tubes 15 ml of nutrient medium is added in a tilted position. After the solidification of the medium, colonies of bacteria were streaked it, and incubated for 24 h at 30 ^{o}C. Then it was stored at 4 ^{o}C in a refrigerator.

4.3. Nutrient agar media (Wright, 1934)

The medium composed of peptone 10 g/l, glucose 5 g/l, yeast extract 3 g/l, NaCl 5 g/l, Agar 15-20 g/l, and distilled water 1000 ml and 0.5 g L-methionine.

4.4. General culture conditions

One milliliter of bacteria suspension was inoculated into liquid media (broth media) containing 10 g/l sucrose, 0.5 g L-methionine, 10 ml corn steep liquor, 0.1 g yeast extract, 3 g NaCl, 2 g KH_2PO_4 and 0.25 g $MgSO_4$ at pH 8.0. The medium was divided into 100- ml fractions in 250 ml flasks and autoclaved at 121 ºC for 15 min. The inoculated flasks were incubated at 37 ºC in shaker for 3 days (**Mohkam *et al*., 2020**).

4.5. Preparation of enzyme extract

Bacterial cells of 3-day old were harvested by centrifugation at 10,000 rpm for 20 min then washed by 150 mM potassium phosphate buffer (pH 8.0) after that the cells were suspended in the same buffer.

The suspended cells were disrupted by ultra-sonication with an ultrasonicator (Ultrasonic Homogenizer 4710, Cole-Palmer Instrument Co., Chicago, IL, USA) for two min at 30 sec intervals. The supernatant was collected after centrifugation at 8,000 rpm to give the crude enzyme extract (**El-Shora *et al*., 2015**).

4.6. Assay of L-methioninase

The activity of L-methioninase assay was determined by estimating the amount of ammonia liberated from L-methionine spectrophotometrically followed by Nesslerization according to **Thompsom and Morrison (1951).**

A reaction mixture, containing 0.5 ml of 1% L-methionine in 0.15 M phosphate buffer (pH 8.0 for the free enzyme), 0.5 ml of enzyme solution and 0.1 ml pyridoxal phosphate was incubated at 30 °C for 1 h.

Then the reaction was stopped by adding 0.5 ml of 1.5 N trichloro-acetic acid (TCA). To 3.7 ml distilled water, 0.1 ml of the above mixture and 0.2 ml of Nessler's reagent were added. After keeping the mixture for 20 min at room temperature, readings were observed at 480 nm using a UV-

spectrophotometer. One unit (U) of L-methioninase was defined as the amount of enzyme that liberates one µmole of ammonia per min under optimal conditions.

4.7. Purification of L-methioninase

4.7.1. Ammonium sulfate fractionation

The purification of the crude extract (supernatant) was performed at 4°C to ensure enzyme stability and activity. Ammonium sulfate was added to achieve 85% saturation, and the mixture was left for 12 h at 4°C. Following this, the solution was centrifuged at 8,000 rpm for 20 min at 4°C to collect the precipitate. The precipitate was subsequently dissolved in 0.01 M phosphate buffer at pH 8.0 and dialyzed overnight against the same buffer at 4°C, resulting in the dialyzed ammonium sulfate fraction. The enzyme activity was determined in this fraction with the protein content.

4.7.2. DEAE-cellulose chromatography

The dialysate was loaded onto a DEAE-cellulose column (2.5 x 15 cm) equilibrated with the same phosphate buffer. Elution was carried out with a linear gradient of 0 to 0.5 M NaCl in phosphate buffer at a flow rate of 1ml/2 min. Fractions containing L-methioninase activity were pooled and concentrated by precipitation at 85 % ammonium sulfate saturation.

4.7.3. Sephadex G-200 gel filtration

The concentrated dialysate was then loaded onto a 2.5 x 15 cm Sephadex G-200 gel filtration column equilibrated with the same buffer. Elution was performed with the buffer at a flow rate of 1 ml/2 min. Fractions containing L-methioninase activity were pooled and concentrated as mentioned above.

4.7.4. Q-Sepharose chromatography

The pooled enzyme was loaded onto a 2.5 x 15 cm Q-Sepharose column equilibrated with the same buffer. The column was washed with the buffer, and

a linear gradient of 0 to 1 M NaCl in the buffer was applied at a flow rate of one ml/min. Fractions containing L-methioninase activity were pooled and desalted. The purified enzyme preparation was stored at - 30 °C and used for characterization.

4.7.5. SDS-polyacrylamide gel electrophoresis

Denaturing PAGE was performed as described by **Laemmli (1970)** with 12% acrylamide gels. Polypeptides separated on the gel were stained with Coomassie Brilliant Blue R-250. The used markers were phosphorylase (97 kDa), bovine albumin (66 kDa), ovalbumin (45 kDa) and carbonic anhydrase (30 kDa). The molecular weight of L-methioninase was determined from a calibration curve.

4.8. Determination of protein

The protein content of the enzyme extract was determined by the method of **Bradford (1976).** A sample of 30 µl of the extract was added to a tube and the volume was made up to 100 µl with 0.15 M NaCl. One ml Bradford´s reagent was added and mixed well and the absorbance was measured at 595 nm. The protein concentration of the sample was determined using the calibration curve of bovine serum albumin (BSA) as standard.

4.9. Amino acid composition of L-methioninase

Acid hydrolysis of the purified enzyme was performed according to **(El-Shora *et al*., 2001).** A known weight of the purified enzyme (10 mg) was placed in a thick-walled glass hydrolysis tube, followed by the addition of 5 ml 6.0N hydrochloric acid. The tube was evacuated, sealed and put in an oven at 110 ºC for 24h. The tube was then removed from the oven and centrifuged for 5 min at 3000 rpm after which the hydrolysate supernatant was collected. Excess hydrochloric acid was evaporated using a rotatory evaporation. This process was repeated several times until the hydrolysate was freed from

hydrochloric acid. The residue was dissolved in 5 ml distilled water and the amino acids were determined using high performance amino acid analyzer, Model Backman System 7300 and Date System 7000, Column Na-A/B/D 25cm.

4.10. Effect of substrate concentration on L-methioninase activity

The effect of L-methionine on the enzyme activity was carried out using various concentrations (2, 4, 6, 8 and 10 mM of L-methionine) in the reaction mixture of the enzyme. The other factors which affect the enzyme activity including temperature, pH, and time of incubation were kept constant. Lineweaver-Burk plot was illustrated by the relation of the reciprocal of V (1/V) against the reciprocal of S (1/S) from which the K_m and V_{max} were calculated **(Michaelis and Menten, 1913)**.

4.11. Effect of pH on L-methioninase activity

The optimal pH for the enzyme activity was determined through a range from 3 to 10. The following buffers were used sodium acetate buffer (pH 4.0 - 5.0), potassium phosphate buffer (6.0 - 7.0), Tris-HCl buffer (8.0 - 9.0) and sodium bicarbonate buffer (10.0). A graph of the enzyme activity against pH was plotted, and subsequently the optimal pH for L-methioninase was determined.

4.12. Effect of temperature on L-methioninase activity

The reaction mixture of L-methioninase was incubated at different temperatures 20, 25, 30, 35, 40, 45, 50, 55 and 60 ºC. The optimal temperature was determined from the graph of enzyme activity temperature.

4.13. Effect of phytohormones on L-methioninase

The four compounds gibberellic acid (GA_3), jasmonic acid (JA), benzylaminopurine (BAP) and kinetin (Kin) were tested individually in the reaction mixture of L-methioninase at 200 µmol. Then the reaction was

stopped by 0.5 ml of 1.5 N TCA. The tubes were centrifuged at 10,000 rpm followed by assaying the enzyme activity in the supernatant.

4.14. Modification of L-methioninase by reagents of the active groups

N-bromosuccinimide (NBS), butanedione, N-ethylmaleimide, and trinitromethane (TNM) are well known as modifiers for tryptophanyl, arginyl, sulfhyryl and tyrosyl residues in the enzyme protein (**El-Shora *et al.*, 2024b**). The purified enzyme was treated with each of these compounds at 10 mM in the reaction mixture followed by determination of the enzyme activity.

4.15. Effect of chelating agents on L- methioninase activity

The effect of EGTA, phenanthroline and α- α-dipyridyl as chelating agents on L-methioninase activity was investigated at various concentrations (2, 4, 6, 8 and 10 mM) in the reaction medium followed by determining the activity of the enzyme as percentage of control.

4.16. Effect of sulfur compounds on L-methioninase activity

The influence of cysteine, N-acetyl cysteine, thioglycolate, reduced glutathione and cystamine on L-methioninase activity was investigated at various concentrations (25, 50, 75, 100 and 125 µM) in the reaction mixture. Control samples, which did not include any of these compounds, were also prepared. Enzyme activity was measured in terms of units and expressed as a percentage of the control.

4.17. Thermostability of L-methioninase

The thermostability of L-methioninase was carried out by heating the purified enzyme for 15 min in water bath in presence of 10 mM of xylitol, 10 mM of trehalose or 10% of glycol chitosan individually various temperatures (40, 45, 50 and 55 ºC) followed by determination of the enzyme activity as mentioned above.

4.18. Effect of DL-homocysteic acid, hydroxylamine, phenylmethylsulfonyl fluoride (PMSF) and DL-propargylglycine on L-methioninase activity

Each of the four compounds: DL-homocysteic acid, hydroxylamine, phenylmethylsulfonyl fluoride (PMSF) and DL-propargylglycine was tested in the reaction medium at various concentrations (2, 4, 6, 8 and 10 mM). The reaction was stopped using 5 % TCA followed by enzyme assay.

4.19. Immobilization of purified L-methioninase:

4.20.1. Immobilization of L-methioninase on carrageenan gel

a). Preparation of gel beads

Carrageenan was dissolved in distilled water (2% w/v).The solution was left standing to disengage bubble before use. The polymer solution of carrageenan was sprayed into KCl solution (2% w/v) using a syringe. The formed microcapsules were left for 3 h (**Ying *et al*., 2016**).

b). Activation of gel beads

The beads were soaked in polyethyleneimine (PEI) for 3 h and washed to remove excess PEI, put in a glutaraldehyde (GA) solution for 3 h, and then washed to remove unbound GA. The beads' color becomes orange brown because of the reaction between amino groups found in PEI and aldehyde group found in GA forming Schiff's base (–N=CH–) (**Elnashar *et al*., 2013**).

c). Covalent immobilization of L-methioninase

Covalent immobilization of L-methioninase onto the activated gel beads where the enzyme amino group (NH_2) reacted with the free C=O groups of glutaraldehyde that was found on the surface of activated gel beads forming amide bond (C= N–). About 1 g of the previously treated gel beads was loaded with 4 ml of 100 mM phosphate buffer (pH 8.0) containing 10 U of L-methioninase. This mixture was incubated in bottle (25 ml) for 16 h at room temperature using a stirrer, and then the gel beads were washed well with buffer and directly assayed for L-methioninase activity (**Amal *et al*., 2016**).

4.20.2. Immobilization on chitin

The immobilization process was conducted following the method as described by **Tiarsa *et al*. (2022).** The native enzyme (0.5 ml) was immobilized onto 0.20 g of chitin using 0.5 ml of the buffer (pH 8.0). The enzyme was incubated at 4 ^{o}C for 30 min, and then centrifuged for 10 min. An aliquot of 0.25 ml of the supernatant was taken to be used as a control. Then, 0.75 ml of L-methionine as substrate was added to the immobilized enzyme, followed by incubation at 40 ^{o}C for 30 min. The mixture was eventually centrifuged at 5000 rpm for 15 min and the immobilization efficiency was determined.

4.21. Anticancer efficiency of L-methioninase

Anticancer efficiency of L-methioninase was measured against: human breast adenocarcinoma cell line (MCF-7), and normal cell. MTT Cell Viability assay was used as described by **Hansen *et al*. (1989)**. % Cell viability = O.D of treated cells / O.D of control cells × 100. Cancer cells (5×104) were seeded in each well containing 0.1 ml of Roswell Park Memorial Institute (RPMI) medium in 96-well plates and then L-methioninase of different concentrations (20, 40, 60, 80 and 100 μgml^{-1}) was added after 24 h. MTT of 10 µl (3-(4,5-dimethylthiazol2-yl)-2,5-diphenyl tetrazolium bromide, 5 mg ml^{-1} , stock solution; Sigma) was then added to each well and cell viability was assessed after 48 h.

The medium from the well was discarded, and formazan blue crystals, which formed in the cells, were dissolved with 100 µl DMSO. The rate of color production was measured at 570 nm in a spectrophotometer. All experiments were conducted under standard laboratory illumination. The relation between the viability of cell and L-methioninase concentration was plotted in order to get the viability curve. The effective dose required to inhibit cell growth by 50 % (IC_{50} in unit per milliliter) was determined.

The tumor cell lines human breast adenocarcinoma cell line (MCF-7), cervical (HeLa) and normal lung cell line (WI-38) were chosen for the assessment of the cytotoxic potency of the naturally synthesized L-methioninase .

The inspected cell lines were credited from (Sigma-Aldrich, USA). Foetal Bovine Serum (FBS) and MTT were obtained from GIBCO, UK, and RPMI-1640 medium, DMSO, and MTT from Sigma Co., St. Louis, USA.

A colorimetric test was used to measure the cytotoxicity of living or viable cells. Cellular enzymes were responsible for turning the yellow color of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution into blue insoluble formazan crystals. The reduction in MTT color to the appropriate blue formazan is shown to be inversely correlated with the number of viable cells (**Oka *et al.*, 1992).** In this instance, mitochondrial succinate dehydrogenases generated from live or viable cell lines had an impact on the reduction step.

4.21.1. MTT solution preparation

Water (10 mg/ml), ethanol (20 mg/ml), buffer salt solution, and culture medium (5 mg/ml) were combined to create the MTT solution. The use of a 5 mg/ml solution in PBS and subsequent vortex mixing was praised. The sterilized solution was filtered and then mixed with MTT solution before being placed at -20°C.

4.21.2. MTT test procedure

In RPMI-1640 medium with 10% foetal bovine serum, the cell strains were cultured. Penicillin (100 units/ml) and streptomycin (100 µg/ml) antibiotics were introduced at 37 °C in an incubator with 5% CO_2. A 96-well plate containing cell lines was seeded with 1.0 x 10^4 cells per well and incubated at 37 °C for 48 hours with 5% CO_2. Following incubation, cells were exposed to various concentrations of the test substances before being given

another 24 h of incubation. After the drug had been administered for 24 h, MTT solution (5 mg/ml) was added, and the process was repeated for 4 h.

The produced violet formazan is then dissolved in 100 µl of DMSO, which is then added to each well. The absorbance readings were obtained at 570 nm using a plate reader, and the colorimetric analysis is obtainable by (EXL 800, USA). The reads were obtained, and after adjusting for culture and medium background, the reads were reported as corrected absorbance. The % inhibitions were calculated as follows:

$$\text{Percentage viability} = \frac{At - Ab \times 100}{Ac - Ab}$$

$$\text{Percentage inhibition} = \frac{100 - At - Ab \times 100}{Ac - Ab}$$

Where,

At=Absorbance value of tested enzyme extract

Ab=Absorbance value of blank

Ac=Absorbance value of negative control (untreated cells)

The blank sample was made by combining a medium devoid of cells with MTT solution and solubilizing buffer, whereas the negative control was made by adding untreated cells to MTT solution and solubilizing buffer.

Statistical analysis

All the data in the present study are expressed as mean ± SE obtained from three measurments.

The IC_{50} values were derived via a sigmoid-type nonlinear regression that was processed using the GraphPad 9.2.5 program.

5.Results

Results

5.1. The purification of L-methioninase

The purification of L-methioninase from *Staphylococcus sciuri* was carried out by ammonium sulphate precipitation (85%), DEAE-Cellulose, Sephadex G-200 and Q-Sepharose. The results in **Table 3** showed that the enzyme was purified with specific activity of 168.7 U mg^{-1} protein and purification fold of 177.5. The yield of purification process was 26.4%.

The profile of purified L-methioninase activity from Q-sepharose is shown in **Table 4** and **Fig. 17**. The highest activity of L-methioninase was recorded at the fraction no. 10.

The purity of L-methioninase was confirmed by sodium dodysylsulphate poly acrylamide gel electrophoresis (SDS-PAGE) which showed a single band with molecular weight of 48 K Da **(Fig. 18)**.

5.2. Amino acid composition

Amino acid composition of the purified enzyme was studied. The results are shown in **(Table 5 and Fig. 19)**. There are 18 amino acids detected cysteine (Cyst), aspartic acid (Asp) and methionine (Met) are the most dominant amino acids. They are represented by 10.7 %, 9.9 % and 9.7 %, respectively.

Lysine, proline and alanine are represented by 8.5 %, 7.4 % and 6.0 %, respectively. Hydroproline and tryptophan were not detected. The other remaining amino acids varied in their percentages.

5.3. Lineweaver-Burk plot of L-methioninase

The Plot of 1/S against 1/V is known as Lineweaver-Burk plot. This plot for L-methioninase is shown in **(Table 6 and Fig. 20)** where there is a straight line, from which V_{max} and K_m values were calculated.

The V_{max} value (maximum velocity of the enzyme under standard assay conditions) was 33.3 U mg^{-1} protein. However, the K_m value (Machealis constant which is a constant that is a measure of the kinetics of an enzyme reaction and that is equivalent to the concentration of substrate at which the reaction takes place at one half its maximum rate) was 1.7 mM for L-methioninase.

Table 3: Purification of L-methioninase from *Staphylococcus sciuri* .

Fraction	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1} protein)	Purification fold	Yield (%)
Crude extract	536	511.3	0.95	1.0	100
Ammonium sulfate (85%)	85	290.2	3.41	3.58	56.7
DEAE-Cellulose	8	181.0	22.6	23.7	35.3
Sephadex G-200	1.6	140.1	87.5	92.1	27.4
Q-Sepharose	0.8	135.0	168.7	177.5	26.4

Table 4: The profile of purified L-methioninase activity from Q - Sepharose.

Fraction number	Enzyme activity (U mg^{-1} protein)
1	1.1 ± 0.03
2	1.2 ± 0.05
3	2.0 ± 0.08
4	2.4 ± 0.07
5	2.8 ± 0.09
6	3.7 ± 0.1
7	4.6 ± 0.2
8	8.0 ± 0.3
9	10.9 ± 0.4
10	15.8 ± 0.3
11	12.8 ± 0.4
12	11.0 ± 0.3
13	8.5 ± 0.3
14	6.9 ± 0.2
15	3.7 ± 0.2

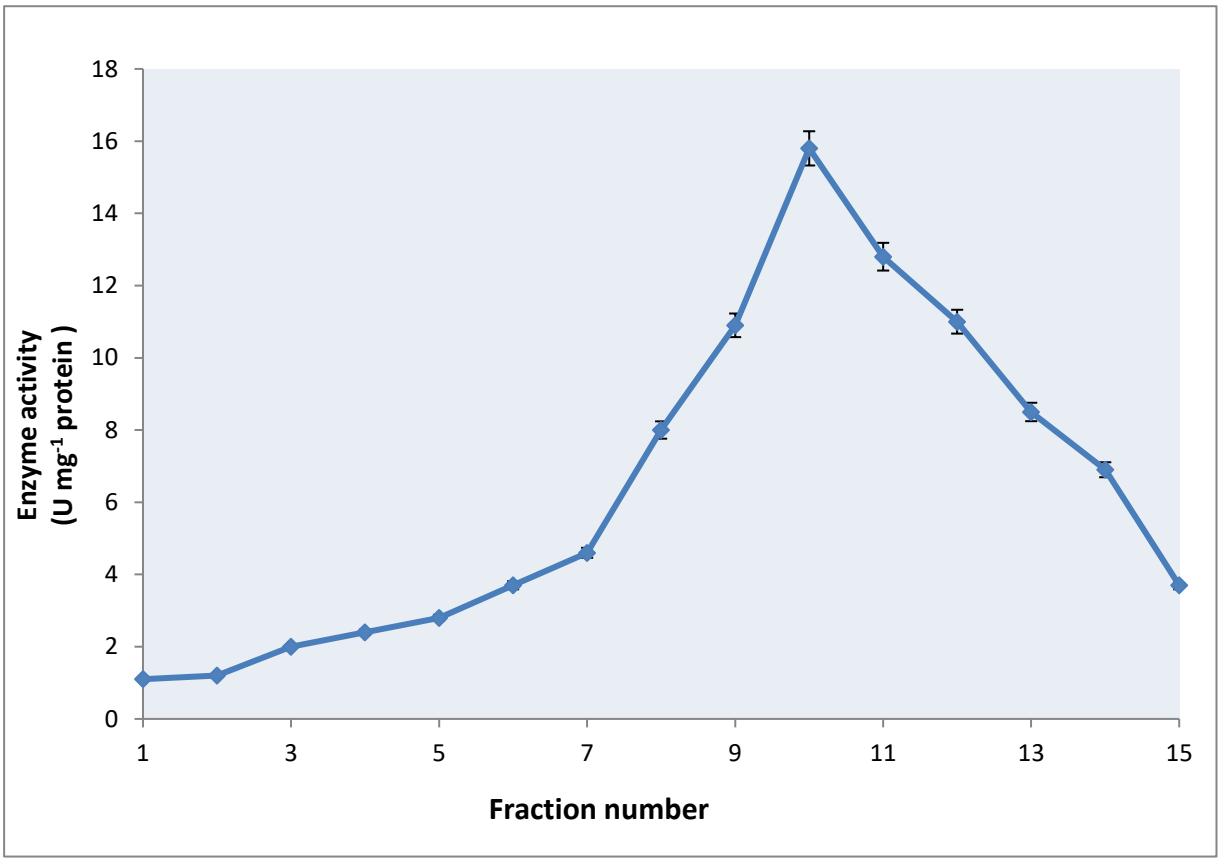


Fig. 17: The profile of purified L-methioninase activity from Q-Sepharose.

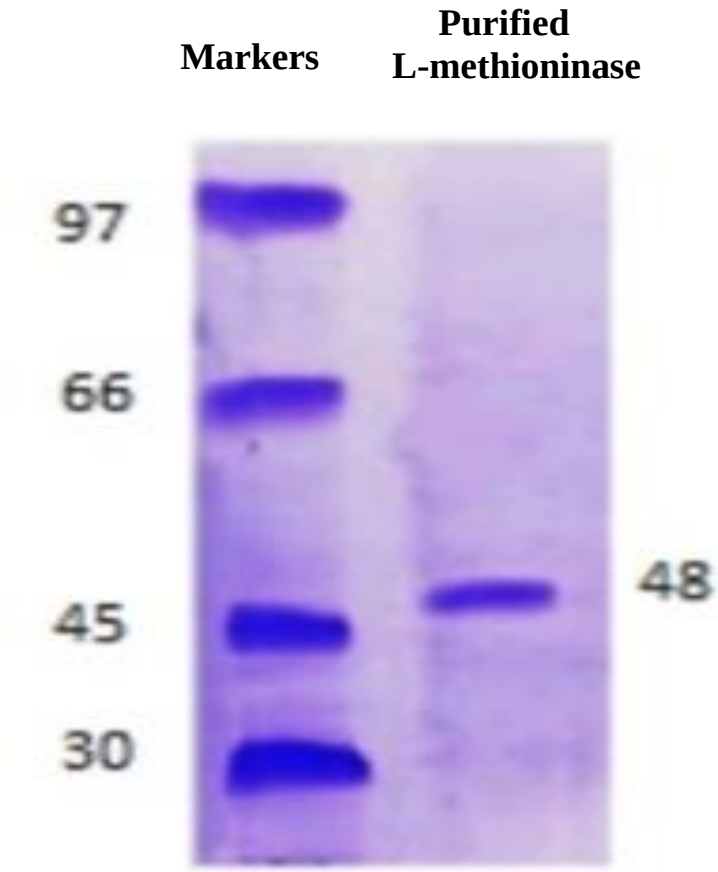


Fig. 18: SDS-PAGE of purified L-methioninase from *Staphylococcus sciuri* on Q-Sepharose.

Table 5: Amino acid composition of the purified L-methioninase.

No	Amino acid	% of total detected amino acids
1	Cys	1.8
2	Cyst	10.7
3	Asp	9.9
4	Thr	4.5
5	Ser	5.8
6	Glu	4.6
7	Pro	7.4
8	Hydro.Pro	N.D
9	Gly	5.5
10	Ala	6.0
11	Val	0.6
12	Met	9.7
13	Ile	4.3
14	Leu	7.5
15	Tyr	2.3
16	Phe	4.7
17	His	2.8
18	Trp	N.D
19	Lys	8.5
20	Arg	3.4

N.D: Not detected.

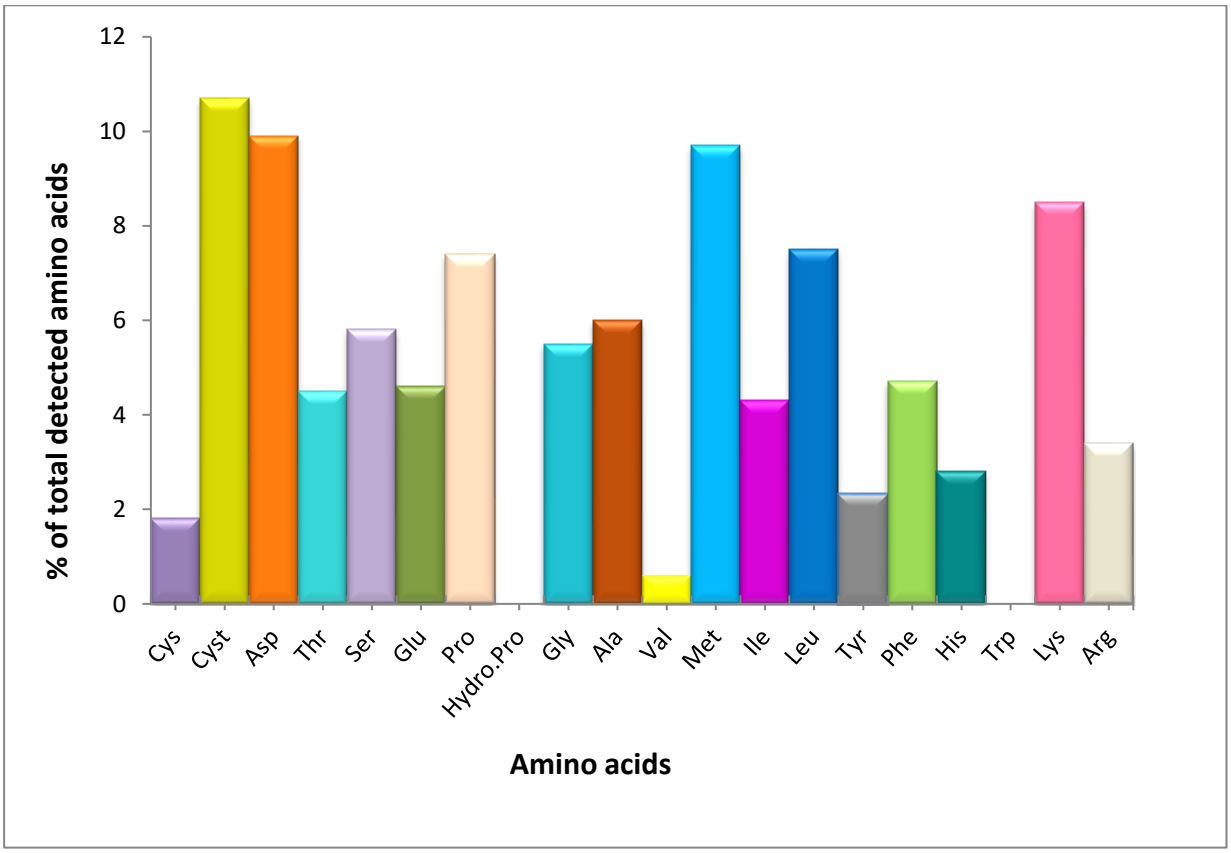


Fig. 19: Amino acid composition of the purified L-methioninase.

Table 6: Reciprocal of Substrate (S) against reciprocal of Velocity (V) of L-methioninase.

1/S	1/V
0.50	0.058
0.25	0.050
0.166	0.043
0.125	0.038
0.100	0.037
0.083	0.037

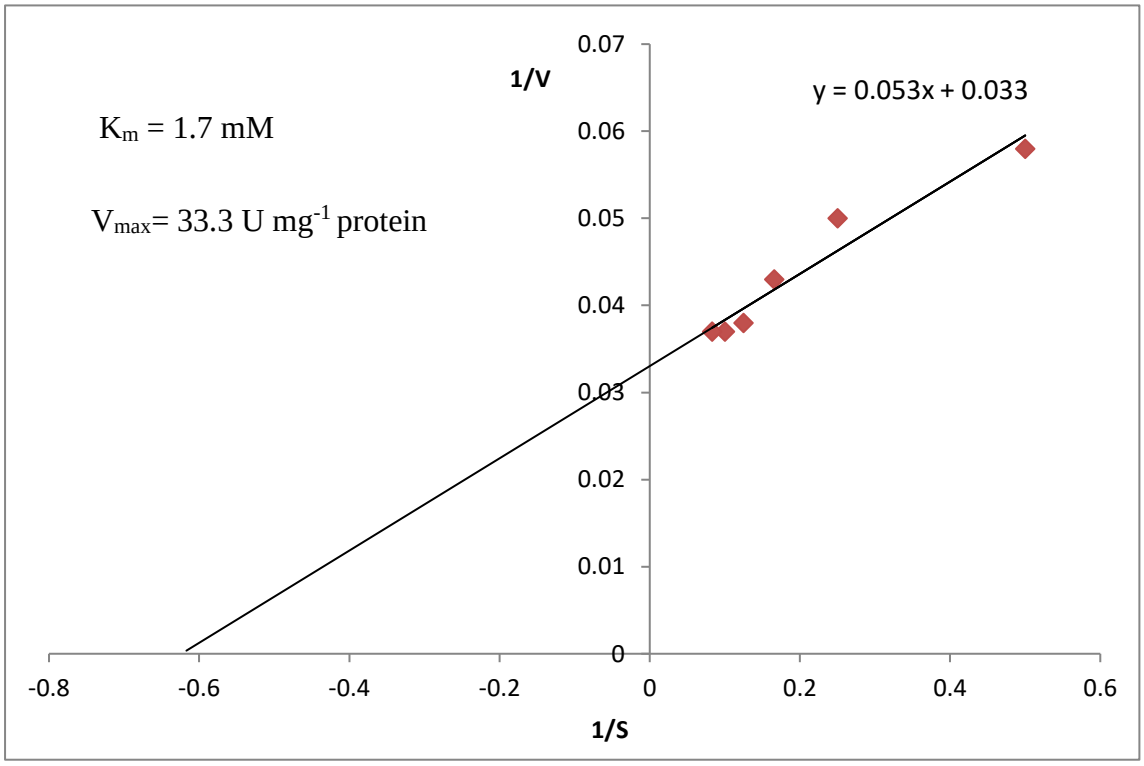


Fig. 20: Lineweaver-Burk plot of L-methioninase.

5.4. Effect of pH on L-methioninase activity

In this experiment the effect of various pH values on L-methioninase was investigated. These pH values were 3, 4, 5, 6, 7, 8, 9 and 10. From the results in **(Table 7 and Fig. 21)**, it was noticed that increasing the pH resulted in an increasing in the enzyme activity gradually up to pH 8.0 which seems to be the optimal pH value where the enzyme activity was 26.4 U mg^{-1} protein.

5.5. Effect of temperature on L-methioninase activity

The effect of temperature on purified L-methioninase was investigated at 20, 25, 30, 35, 40, 45, 50, 55 and 60ºC. The other factors affecting the enzyme activity were kept constant. The results in **Table 8 and Fig. 22** show that the optimal temperatures for L-methioninase was 40ºC, respectively. The lower temperatures than the optimal one expressed lower activities and the higher temperatures resulted in reducing the activity due to heat inactivation.

5.6. Effect of phytohormones on L-methioninase activity

The effect of phytohormones including gibberellic acid (GA_3), jasmonic acid (JA), benzylaminopurine (BAP) and kinetin (Kin) was investigated. They were used at 200 µmol in the reaction mixture.

The results in **(Table 9 and Fig. 23)** indicate enhancing of the enzyme activity depending on the type of phytohormones. Kinetin was the best stimulator where the activity is (49.5 U mg^{-1} protein), followed by gibberellic acid (39.4 U mg^{-1} protein), benzylaminopurine (31.7 U mg^{-1} protein) and jasmonic acid (20.9 U mg^{-1} protein).

5.7. The effect of reagents of active groups on L-methioninase activity

The effect of N-bromosuccinimide (NBS), butanedione (BD), N-ethylmaleimide (NEM) and trinitromethane (TNM) was carried out by including each of these compounds individually at 10 mM in the reaction

mixture under standard assay conditions followed by determination of the enzyme activity.

The results in **(Table 10 and Fig. 24)** indicate that four compounds inhibited the enzyme activity with different rates depending on the type of the reagent. NEM was the most potent inhibitor where the activity was 3.8 U mg^{-1} protein. BD inhibited the enzyme and the activity was 5.5 U mg^{-1} protein in its presence whereas TNM inhibited the enzyme and the activity was 9.3 U mg^{-1} protein. NBS expressed the least inhibitory effect where the activity was 11.6 U mg^{-1} protein**.**

Table 7: Effect of pH on the activity of purified L-methioninase.

pH	Enzyme activity (U mg^{-1} protein)
3	4.1 ± 0. 2
4	7.3 ± 0.2
5	20.2 ± 0.6
6	23.3 ± 0.4
7	24.4 ± 0.5
8	26.4 ± 0.6
9	24.3 ± 0.5
10	22.4 ± 0.6

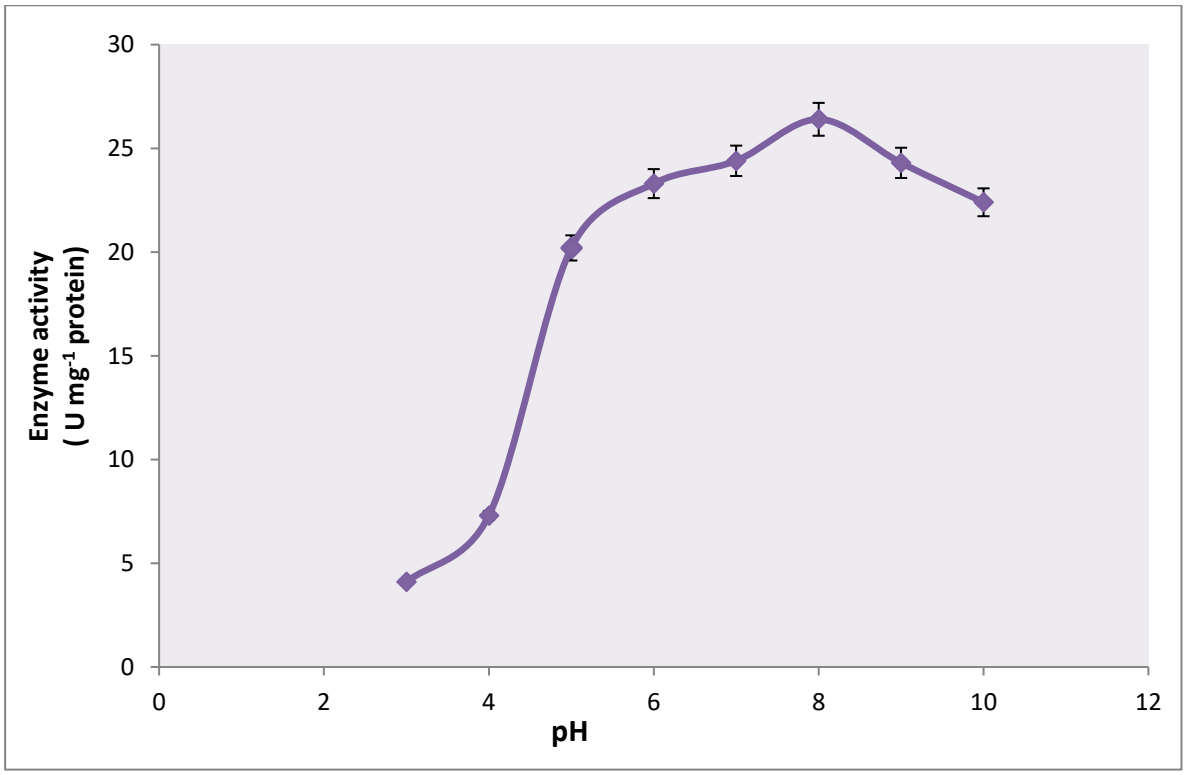


Fig. 21: Effect of pH on the activity of purified L-methioninase.

Table 8: Effect of temperature on the activity of purified L-methioninase.

Temperature (°C)	Enzyme activity (U mg^{-1} protein)
20	7.8 ± 0.3
25	8.8 ± 0.3
30	10.5 ± 0.4
35	10.7 ± 0.4
40	11.4 ± 0.5
45	10.1 ± 0.3
50	9.3 ± 0.2
55	7.3 ± 0.3
60	5.1 ± 0.3

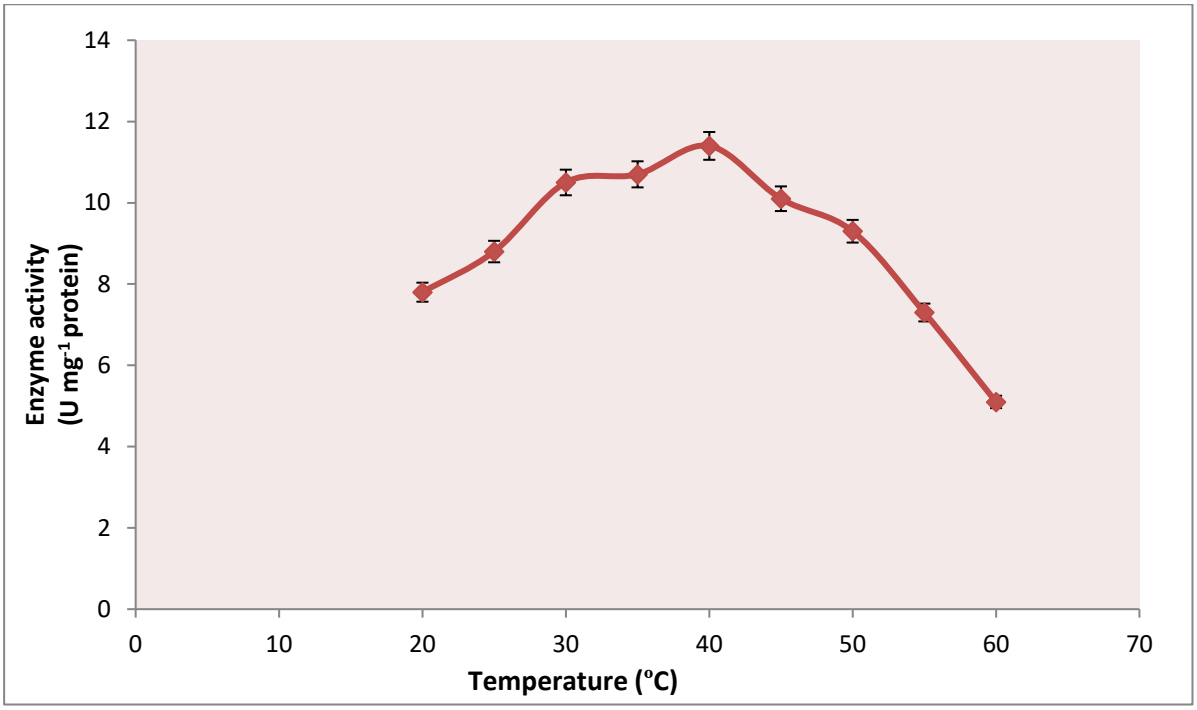


Fig. 22: Effect of temperature on the activity of purified L-methioninase.

Table 9: Effect of phytohormones at 200 µmol on L-methioninase activity.

Phytohormones (200 µmol)	Enzyme activity (U mg^{-1} protein)	% of control
Control	15.6 ± 0.6	100
Gibberellic acid (GA_3)	39.4 ± 0.9	252.6
Jasmonic acid (JA)	20.9 ± 0.4	134.0
Benzylaminopurine (BAP)	31.7 ± 0.9	203.2
Kinetin (Kin)	49.5 ± 0.6	317.3

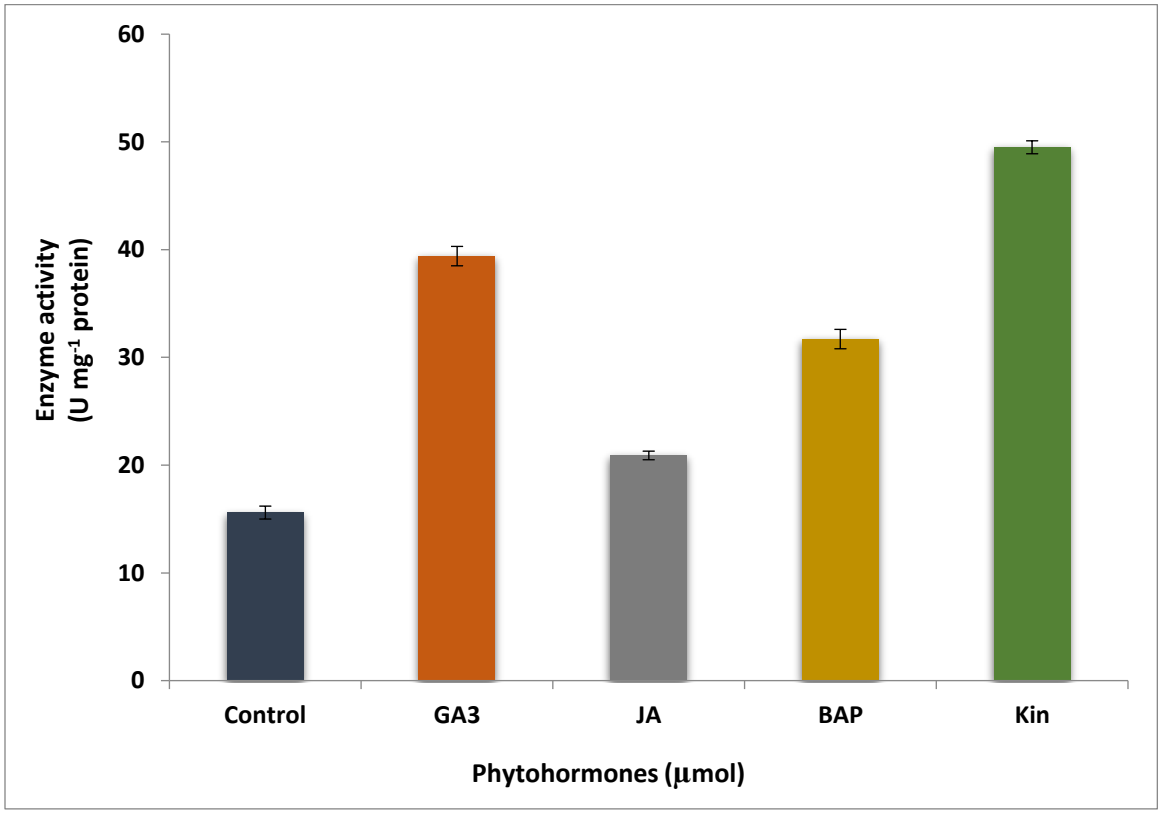


Fig. 23: Effect of phytohormones at 200 µmol on L-methioninase activity.

Table 10: Effect of reagents of different active groups at 10 mM on L-methioninase activity.

Reagents (10 mM)	Enzyme activity (U mg^{-1} protein)	% of control
Control	14.7 ± 0.6	100
N-bromosuccinimide	11.6 ± 0.5	78.9
Butanedione	5.5 ± 0.3	37.4
N-ethylmaleimide	3.8 ± 0.1	25.9
Trinitromethane	9.3 ± 0.3	63.3

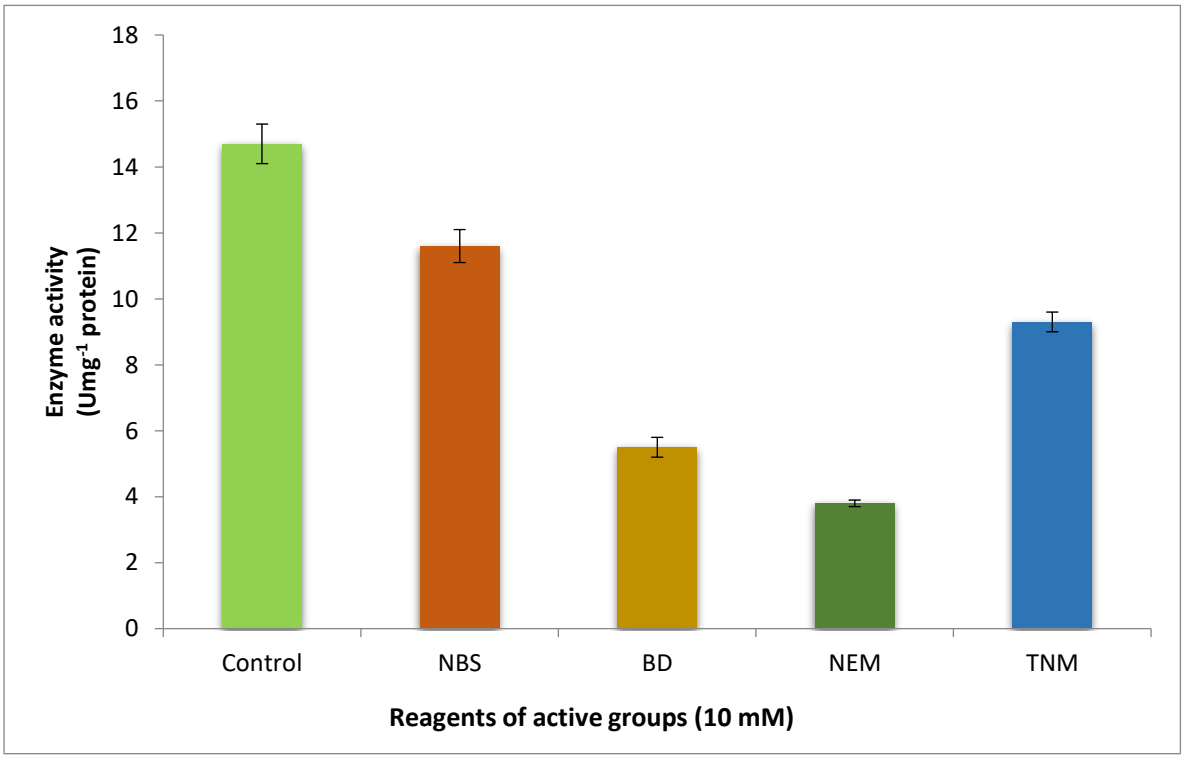


Fig. 24: Effect of reagents of different active groups at 10 mM on L-methioninase activity.

5.8. The effect of chelating agents on L-methioninase activity

The effect of chelating agents on L-methioninase activity was studied at various concentrations (2, 4, 6, 8 and 10 mM). The chelating agents include α- α-dipyridyl , phenanthroline and EGTA.

The results in **(Tables 11, 12 and 13 and Figs. 25a, 26a and 27a)** indicate that in presence of each of the above compounds, the enzyme activity decreased gradually depending on the concentration. At 10 mM the enzyme activity was 2.5, 3.9 and 2.8 U mg^{-1} protein in presence of α- α-dipyridyl , phenanthroline and EGTA , respectively.

The IC_{50} for α- α-dipyridyl, phenanthroline and EGTA as shown in **(Figs. 25b, 26b and 27b)** were calculated and its values were 5.25, 7.03 and 6.43 mM, respectively.

5.9. The effect of sulfur containing compounds on L-methioninase activity

The effect of five sulfur containing compounds including thioglycolate, N-acetyl cysteine, cysteine, reduced glutathione (GSH) and cystamine was investigated by including each compound in the reaction mixture of L-methioninase at various concentrations (25 , 50 ,75 ,100 and 125 µM) under standard assay conditions.

In case of thioglycolate **(Table 14 and Fig. 28**) the activity decreased gradually after 25 µM which induced the enzyme activity by 114.3%.

The optimum concentration of N-acetyl cysteine **(Table 15 and Fig. 29)** was 50 µM where the activity was 19.9 U mg^{-1} protein after which the activity decreased continuously and reach 9.5 U mg^{-1} protein at 125 µM representing 143.1 % of the control value. In case of cysteine **(Table 16 and Fig. 30)** the optimal concentration was 50 µM where the activity was 19.0 U mg^{-1} protein representing 116.5% of the control value.

For reduced glutathione (GSH), **(Table 17 and Fig. 31)** the optimal concentration was 75 µM and at this concentration the activity was 25. 6 U

mg^{-1} protein representing 162 % of the control value. Cystamine as another sulfur containing compound induced the enzyme activity at 25, 50 and 75 mM but at **(Table 18 and Fig. 32)** 100 µM the activity declined to 16 U mg^{-1} protein which is still higher than the control value. However, at 125 µM the activity declined to 13.8 U mg^{-1} protein and representing 92% of the control value.

Table 11: **Effect of α- α-dipyridyl on L-methioninase activity**.

Concentration (mM)	Enzyme activity (U mg^{-1} protein)	% of control
0	15.4 ± 0.6	100
2	10.0 ± 0.3	64.9
4	8.7 ± 0.3	56.4
6	5.8 ± 0.2	37.6
8	3.2 ± 0.1	20.7
10	2.5 ± 0.1	16.2

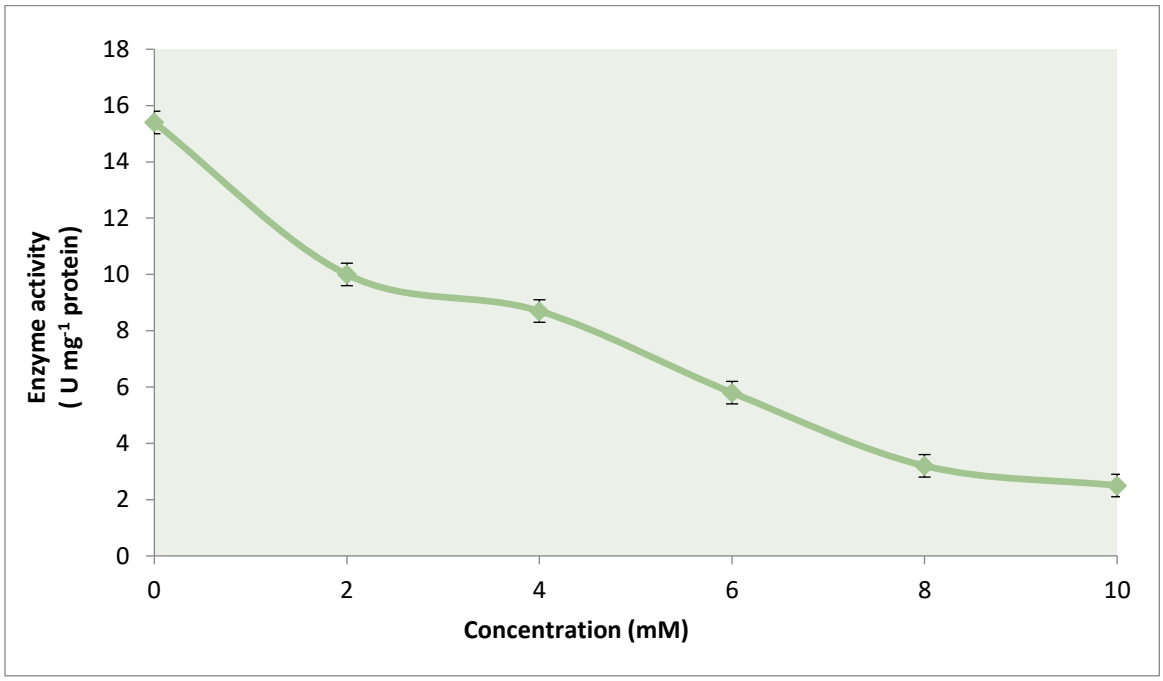


Fig. 25a: Effect of α- α-dipyridyl on L-methioninase activity.

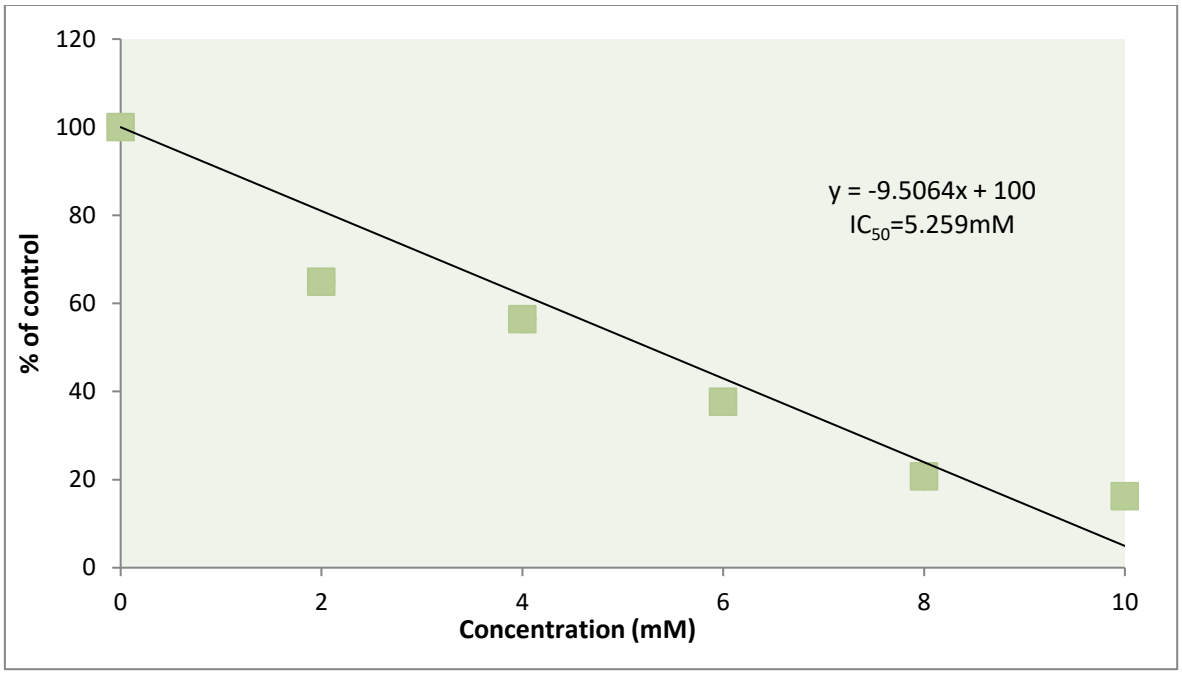


Fig. 25b: Relation between concentration of α- α-dipyridyl and % of control.

Table 12 : Effect of phenanthroline on L-methioninase activity.

Concentration (mM)	Enzyme activity (U mg^{-1} protein)	% of control
0	15.4 ± 0.6	100
2	13.4 ± 0.6	87.0
4	11.2 ± 0.4	72.7
6	9.4 ± 0.3	61.0
8	6.8 ± 0.3	44.1
10	3.9 ± 0.1	25.3

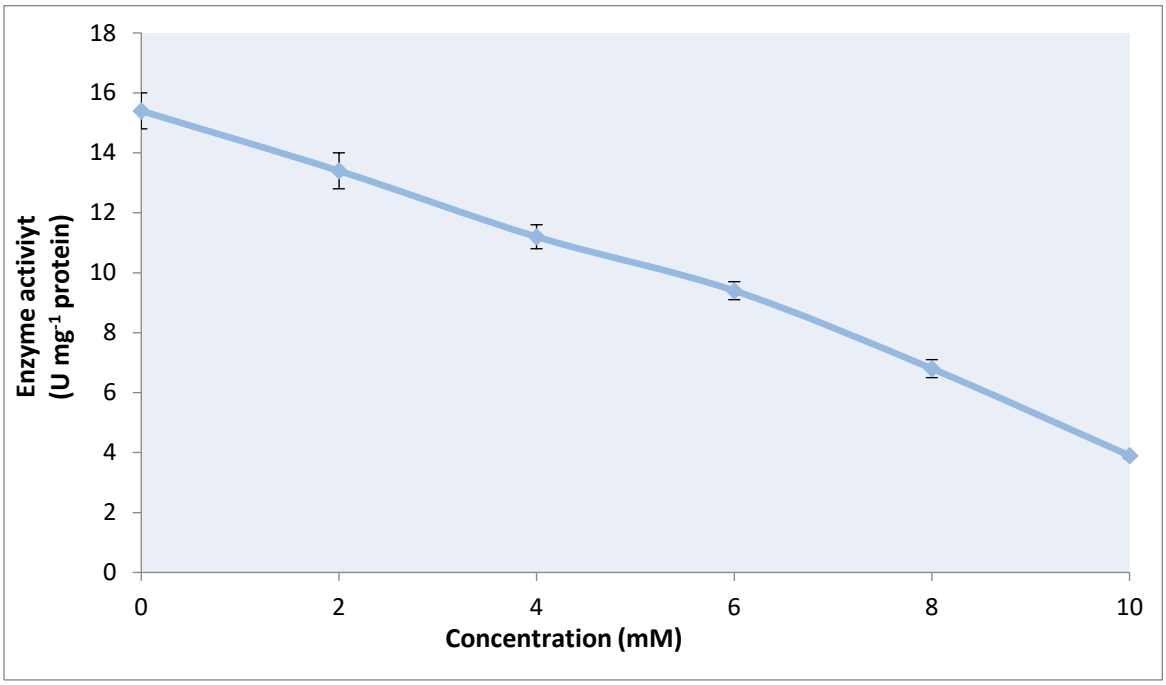


Fig. 26a : Effect of phenanthroline on L-methioninase activity.

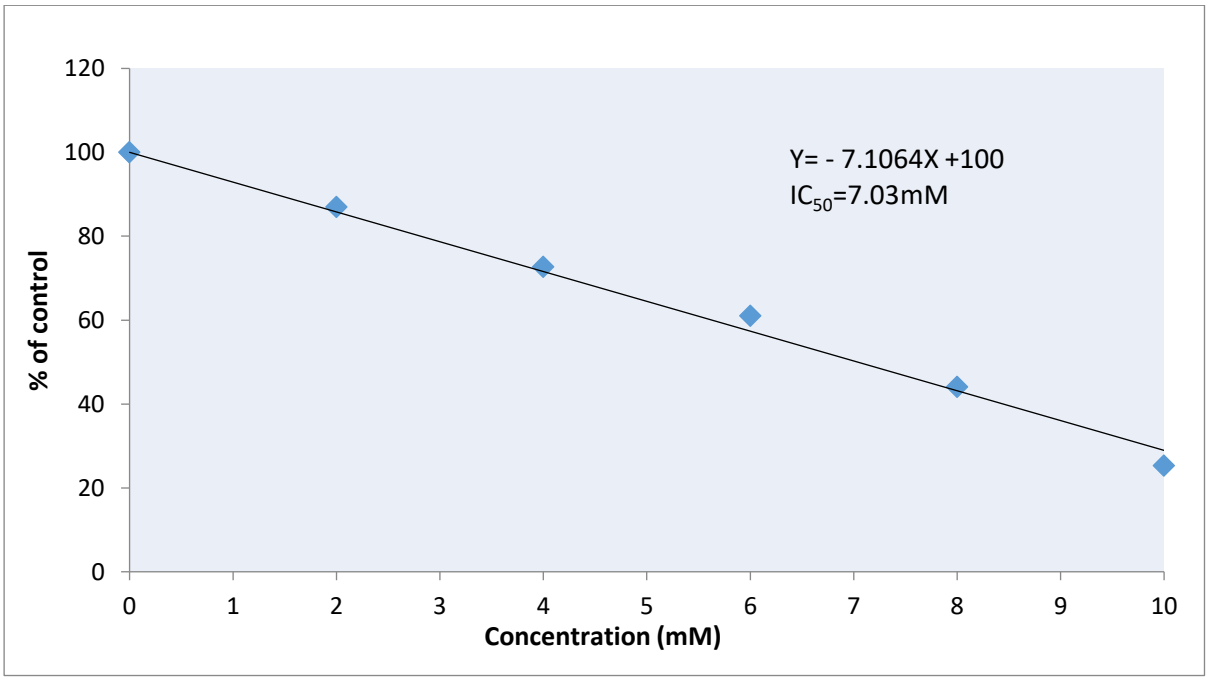


Fig. 26b : Relation between concentration of phenanthroline and % of control.

Table 13: Effect of EGTA on L-methioninase activity.

Concentration (mM)	Enzyme activity (U mg^{-1} protein)	% of control
0	15.4 ± 0.6	100
2	14.0 ± 0.6	90
4	11.8 ± 0.5	76.6
6	8.7 ± 0.4	56.4
8	5.5 ± 0.4	35.7
10	2.8 ± 0.1	18.1

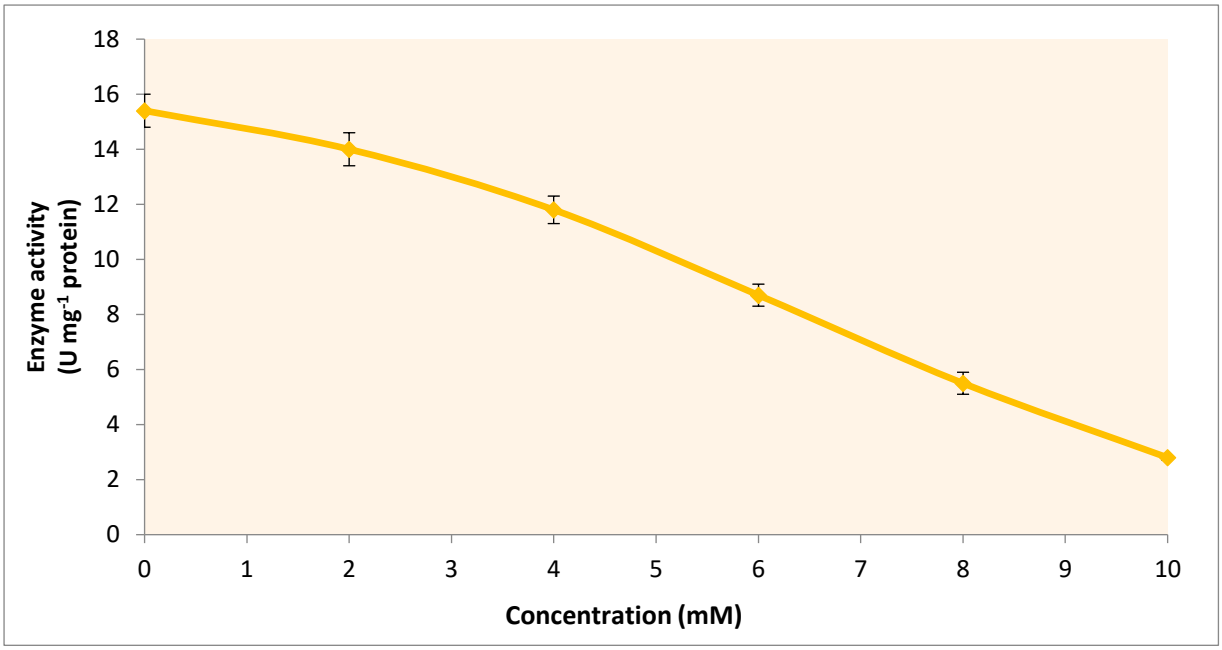


Fig. 27a: Effect of EGTA on L-methioninase activity.

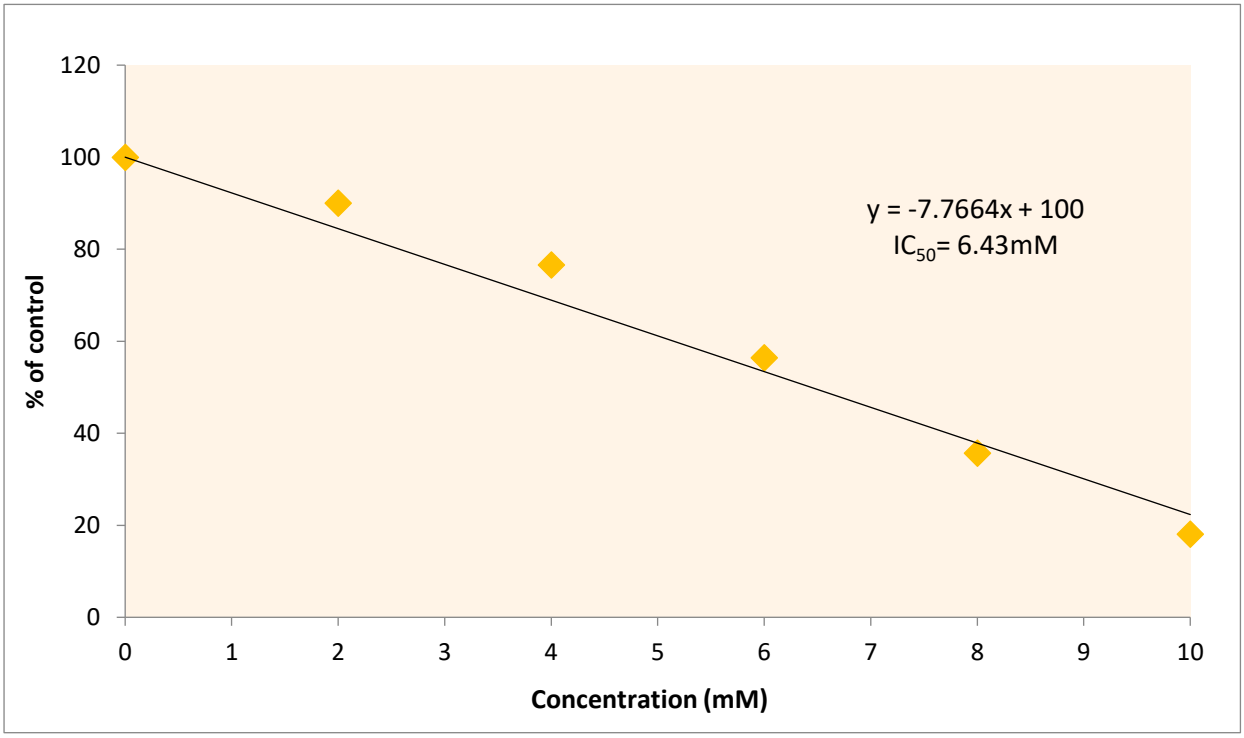


Fig. 27b: Relation between concentration of EGTA and % ofcontrol.

Table 14: Effect of thioglycolate on L-methioninase activity.

Concentration (µM)	Enzyme activity (U mg^{-1} protein)	% of control
0	13.9 ± 0.4	100
25	15.9 ± 0.3	114.3
50	13.4 ± 0.3	96.4
75	10.9 ± 0.3	78.4
100	9.0 ± 0.2	64.7
125	6.0 ± 0.1	43.1

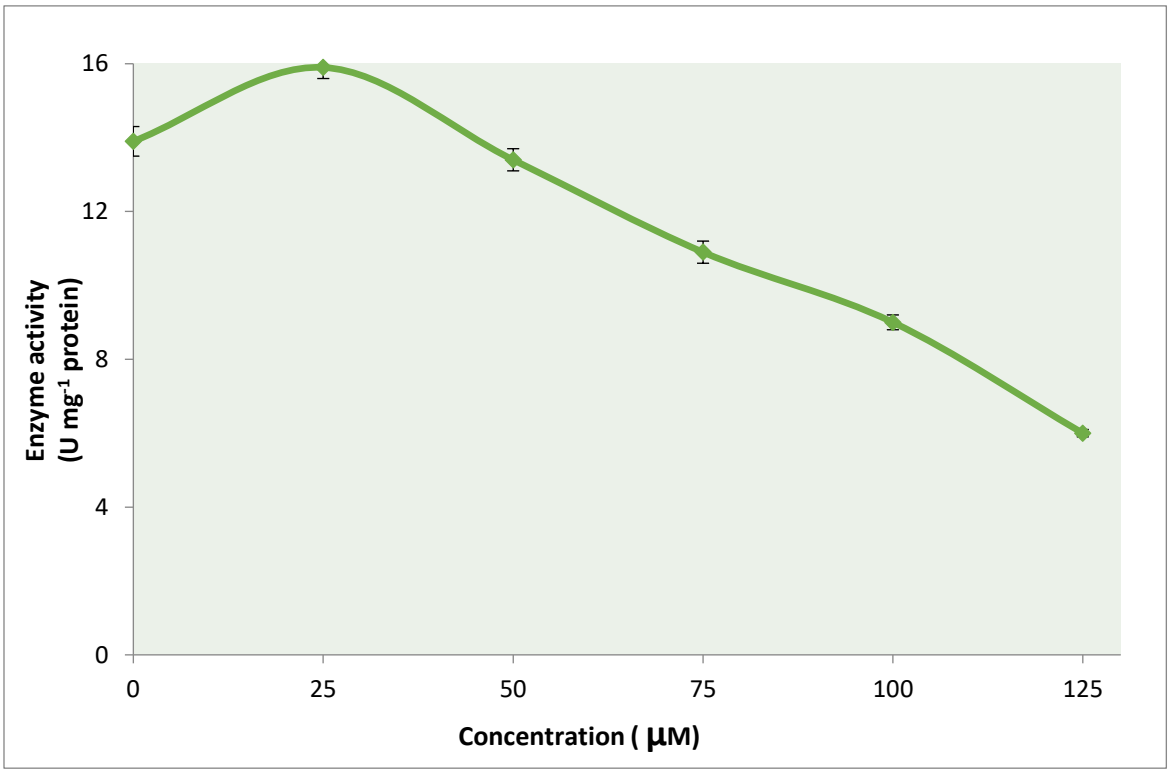


Fig. 28: Effect of thioglycolate on L-methioninase activity.

Table 15: Effect of N-acetyl cysteine on L-methioninase activity.

Concentration (µM)	Enzyme activity (U mg^{-1} protein)	% of control
0	13.9 ± 0.4	100
25	15.9 ± 0.4	114.3
50	19.9 ± 0.3	143.1
75	16.9 ± 0.3	121.5
100	10.9 ± 0.1	78.4
125	9.5 ± 0.1	68.3

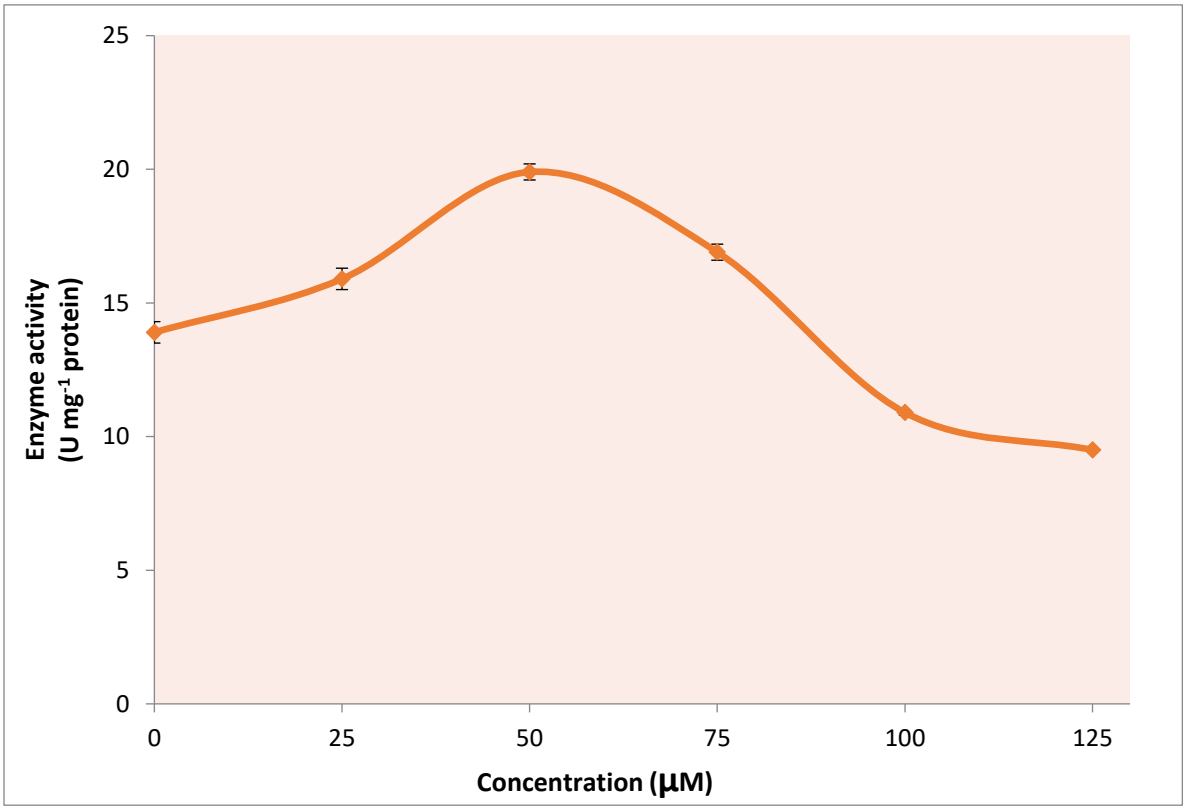


Fig. 29: Effect of N-acetyl cysteine on L-methioninase activity.

Table 16 : Effect of cysteine on L-methioninase activity.

Concentration (µM)	Enzyme activity (U mg^{-1} protein)	% of control
0	16.3 ± 0.4	100
25	18.2 ± 0.6	111.6
50	19.0 ± 0.5	116.5
75	17.7 ± 0.5	108.5
100	15.4 ± 0.6	94.4
125	13.8 ± 0.4	84.6

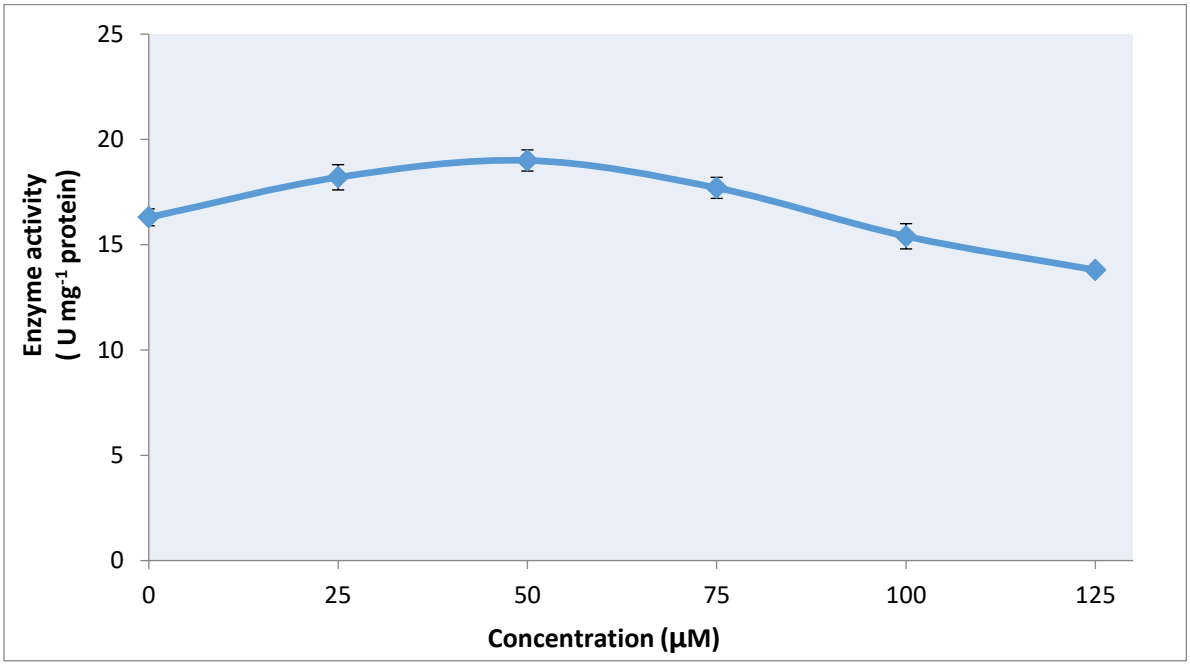


Fig. 30 : Effect of cysteine on L-methioninase activity.

Table 17: Effect of reduced glutathione (GSH) on L-methioninase activity.

Concentration (µM)	Enzyme activity (U mg^{-1} protein)	% of control
0	15.8 ± 0.5	100
25	17.9 ± 0.7	113.2
50	21.4 ± 0.8	135.4
75	25.6 ± 0.8	162.0
100	22.7 ± 0.5	143.6
125	19.0 ± 0.4	120.2

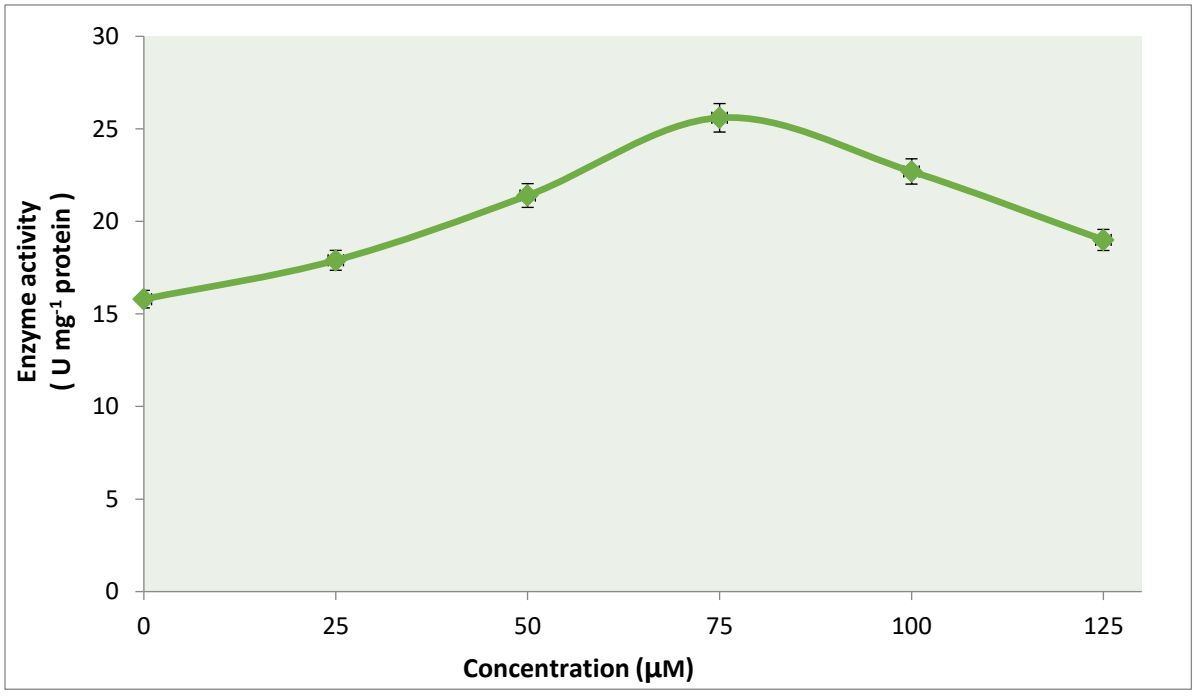


Fig. 31: Effect of reduced glutathione (GSH) on L-methioninase activity.

Table 18: Effect of cystamine on L-methioninase activity.

Concentration (µM)	Enzyme activity (U mg^{-1} protein)	% control
0	15.0 ± 0.6	100
25	17.9 ± 0.4	119.3
50	20.1 ± 0.5	134.0
75	23.0 ± 0.6	153.3
100	16.0 ± 0.4	106.6
125	13.8 ± 0.5	92.0

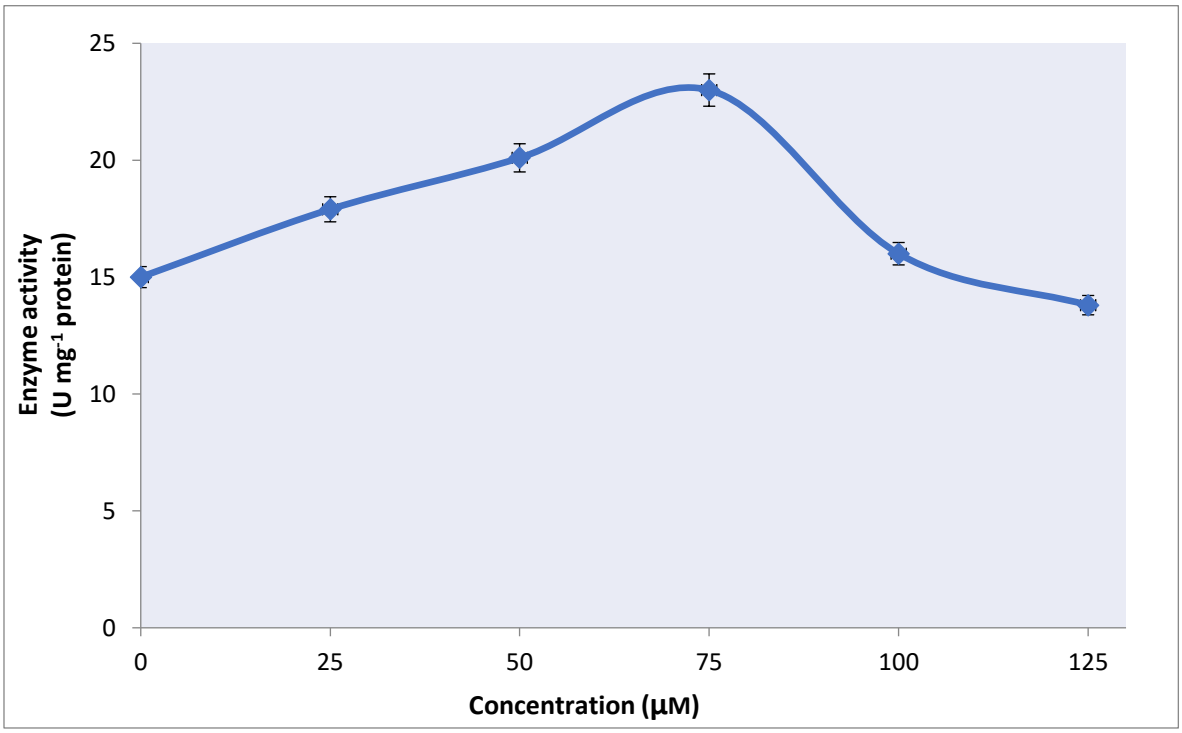


Fig. 32: Effect of cystamine on L-methioninase activity.

5.10. Thermostability of L-methioninase in presence various additives

The thermostability of L-methioninase was investigated at higher temperatures (40, 45, 50 and 55ºC) which are over the optimal one in presence or absence of 10 mM trehalose or 10 mM xylitol or 10 % w/v glycol chitosan.

The results in **(Table 19 and Fig. 33)** reveal that the enzyme was denatured at the above mentioned temperatures in absence of xylitol. The activities in its absence as percentages of control were 51%, 34% and 17 % at the above mentioned temperatures, respectively. However, in presence of xylitol the activities percentages increased to 82 %, 70 % and 44 % at the above mentioned temperature, respectively.

In presence of 10 mM of trehalose **(Table 20 and Fig. 34)** the activities percentages increased to 61%, 55% and 42% at 45, 50 and 55ºC, respectively. However, the increase in presence of xylitol was higher than that offered for the enzyme by trehalose.

In presence of (10 % w/v) of glycol chitosan **(Table 21 and Fig. 35)** which is a derivative of chitosan the activities percentages increased to 84 %, 73 % and 60 % at 45, 50 and 55ºC, respectively. Thus, it is apparent that the increase of relative activity in presence of glycol chitosan is higher than that offered for the enzyme by xylitol and trehalose.

5.11. Effect of anhydrides on the activity of L-methioninase

The activity of purified L-methioninse was measured in presence of maleic anhydride or succinic anhydride. Each of the two anhydrides was tested at 5 mM in the reaction mixture of purified L-methioninase. The results in **(Table 22 and Fig. 36)** indicate that each of the two anhydrides was inhibitor for the enzyme compared to the control to which no anhydride was added. The control sample expressed 28 U mg^{-1} protein which decreased to 21 U mg^{-1} protein in presence of succinic anhydride and to 14 U mg^{-1} protein in presence of maleic anhydride.

Table 19: Effect of 10 mM xylitol on thermostabiliy of L-methioninase at higher temperatures over the optimum in presence or absence of xylitol.

Temperature (°C)	Relative activity (%)	
	+ Xylitol	- Xylitol
40	100	100
45	82 ± 1.6	51 ± 1.1
50	70 ± 1.3	34 ± 0.6
55	44 ± 0.6	17 ± 0.4

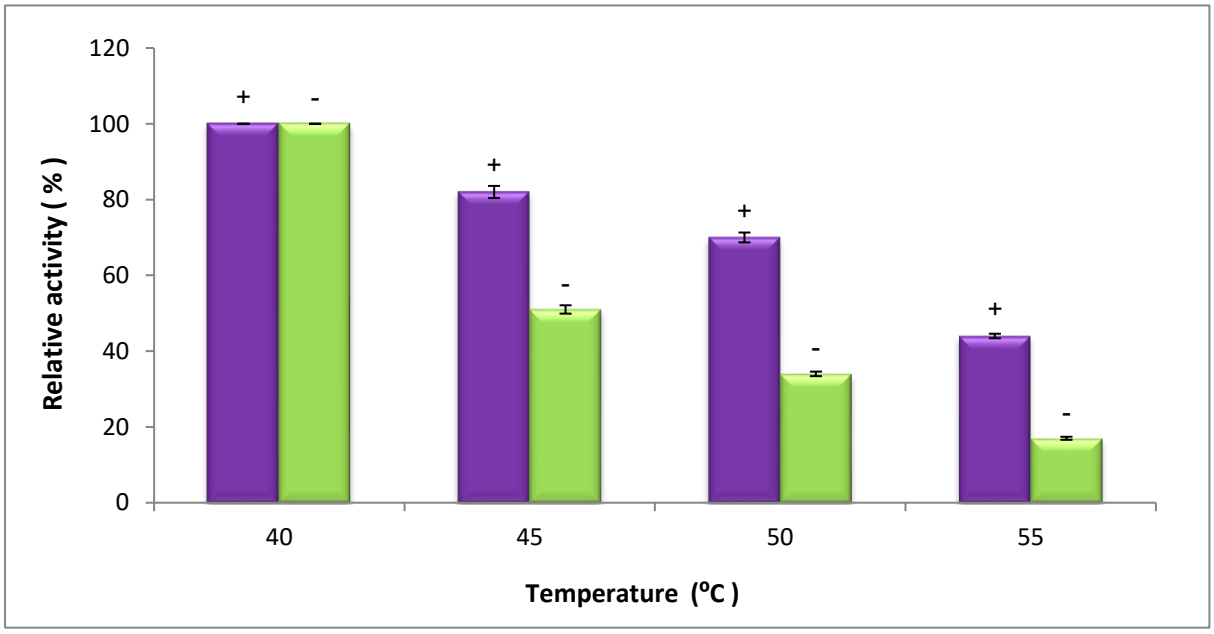


Fig. 33: Effect of 10 mM xylitol on thermostabiliy of L-methioninase at higher temperatures over the optimum in presence or absence of xylitol

Table 20: Effect of 10 mM trehalose on thermostability of L-methioninase at higher temperatures over the optimum in presence or absence of trehalose.

Temperature (°C)	Relative activity (%)	
	+ Trehalose	- Trehalose
40	100	100
45	61 ± 1.1	51 ± 1.0
50	55 ± 1.0	34 ± 0.6
55	42 ± 0.7	17 ± 0.4

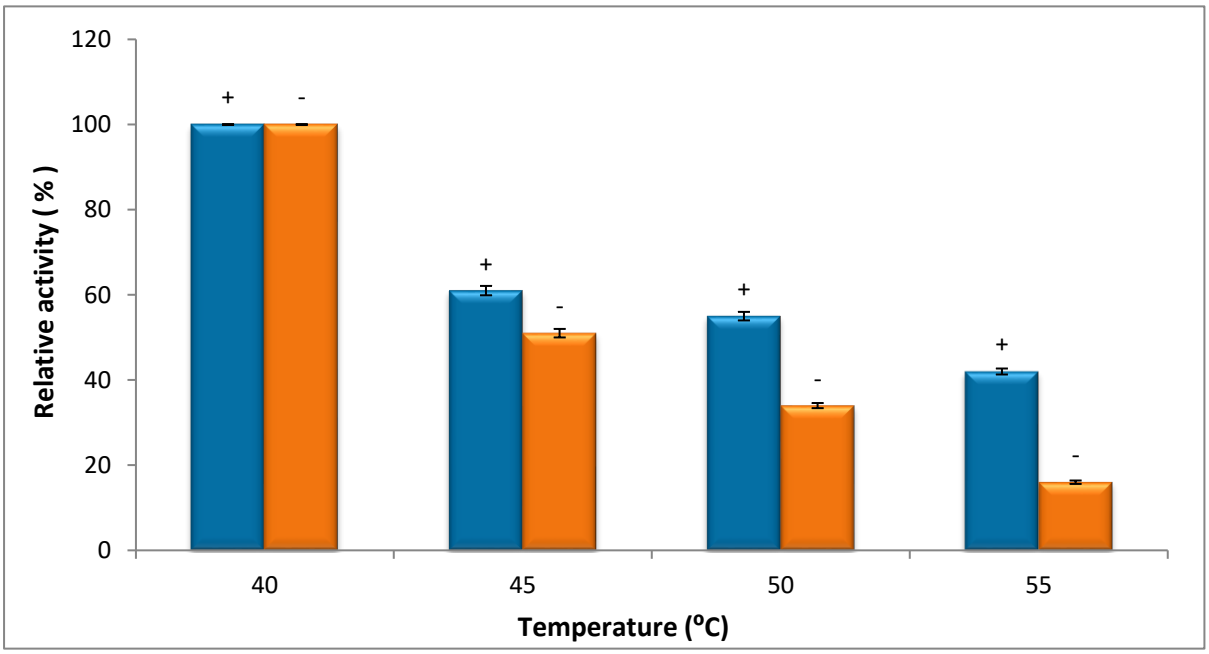


Fig. 34: Effect of 10 mM trehalose on thermostability of L- methioninase at higher temperatures over the optimum in presence or absence of trehalose.

Table 21: Effect of 10 % (w/v) glycol chitosan on thermostability of L-methioninase.

Temperature (°C)	Relative activity (%)	
	+ Glycol chitosan	- Glycol chitosan
40	100	100
45	84 ± 1.5	51 ± 1.3
50	73 ± 1.3	34 ± 0.7
55	60 ± 1.2	17 ± 0.7

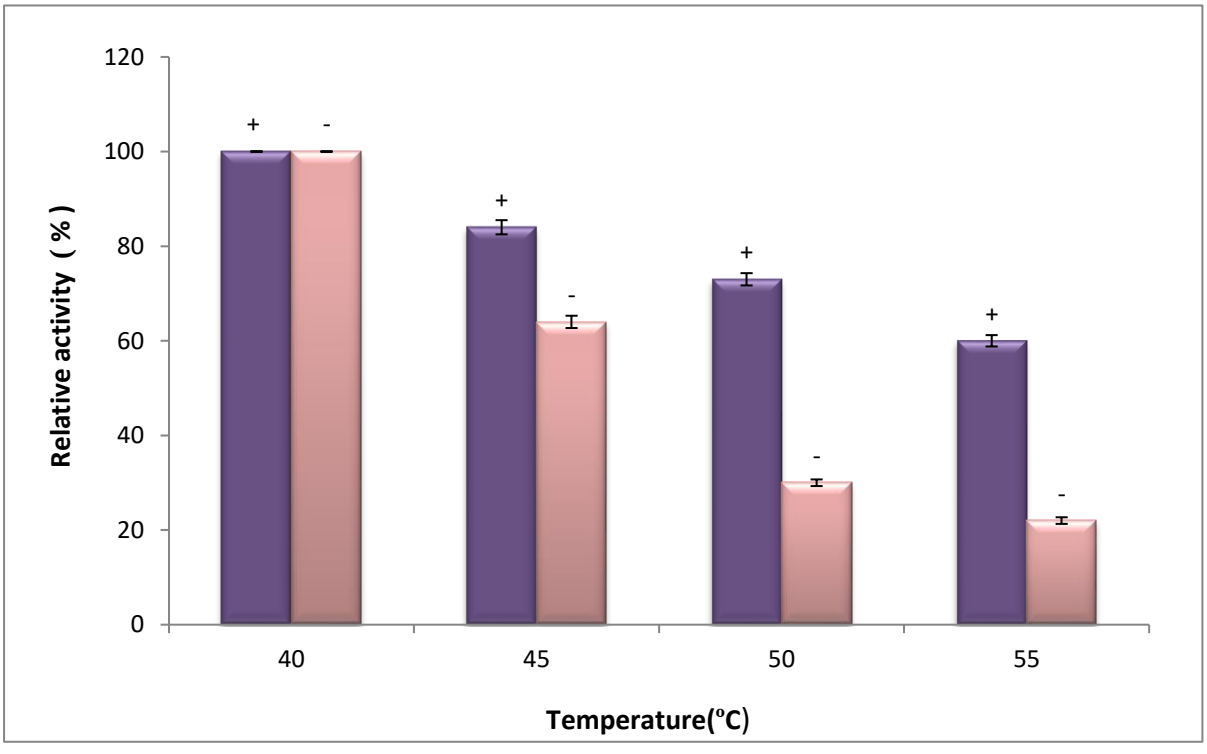


Fig. 35: Effect of 10 % (w/v) glycol chitosan on thermostability of L-methioninase.

Table 22: Effect of succinic and maleic anhydrides at 5 mM on the activity of L-methioninase.

Anhydrides (5 mM)	Enzyme activity (U mg^{-1} protein)	% of control
Control	28.0 ± 0.7	100
Succinic anhydride (SA)	21.0 ± 0.4	75
Maleic anhydride (MA)	14.0 ± 0.5	50

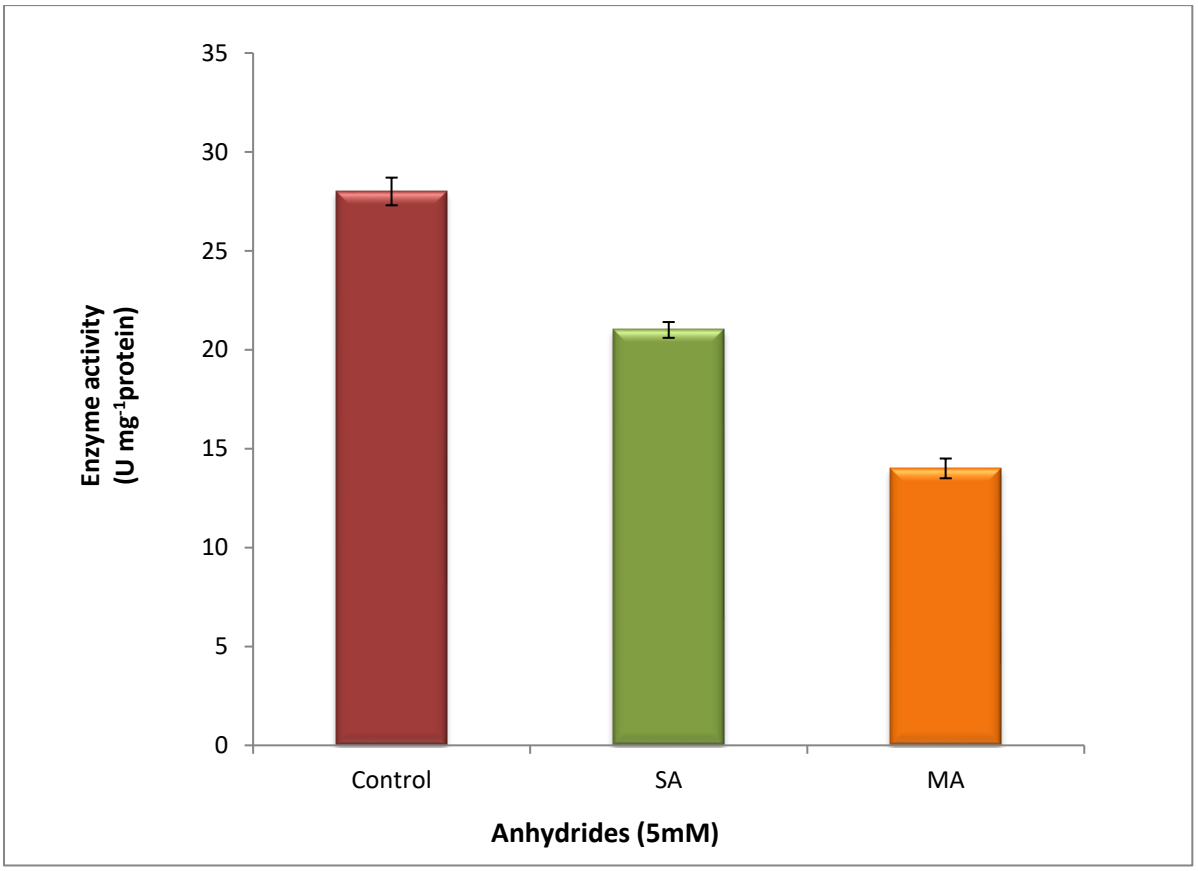


Fig. 36: Effect of succinic and maleic anhydrides at 5 mM on the activity of L-methioninase .

5.12. Effect of DL-homocysteic acid on L-methioninase activity

DL-homocysteic acid is an analog of L-methionine. The effect of DL-homocysteic acid on L-methioninase was tested at various concentrations (2, 4, 6, 8 and 10 mM). The results in **(Table 23 and Fig. 37)** show that this compound activated the enzyme at the lower concentrations until 6 mM (28.7 U mg^{-1} protein) then inhibited the enzyme activity at the higher concentrations (8 and 10 mM), where the activities were 26.4 and 21 U mg^{-1} protein, respectively.

5.13. Effect of hydroxylamine on L-methioninase activity

Hydroxylamine is an inhibitor for PLP- dependent enzymes. The effect of hydroxylamine on L-methioninase was tested at various concentrations (2, 4, 6, 8 and 10 mM). The results in **(Table 24 and Fig. 38)** show that this compound activated the enzyme at the lower concentrations until 4 mM where the activity was 19.6 U mg^{-1} protein and representing 127.2 % of the control values then inhibited the enzyme activity at the higher concentrations (6, 8 and 10 mM). At 10 mM the enzyme activity declined to 8.3 U mg^{-1} protein representing 53.8 % of the control values.

5.14. Effect of phenylmethylsulfonyl fluoride (PMSF) on L-methioninase activity

Phenylmethylsulfonyl fluoride (PMSF) is known to be inhibitor for different enzymes. The effect of PMSF on L-methioninase was tested at various concentrations (2, 4, 6, 8 and 10 mM). The results in **(Table 25 and Fig. 39)** show that this compound activated the enzyme at the lower concentrations until 8 mM where the activity was 15 U mg^{-1} protein and representing 182.9 % of the control values then inhibited the enzyme activity at the highest concentration 10 mM where the activity declined to 13.9 U mg^{-1} protein representing 169.5 % of the control values.

5.15. Effect of DL-propargylglycine on L-methioninase activity

DL-propargylglycine is found to inhibit other enzymes. The effect of DL-propargylglycine on L-methioninase was tested at various concentrations (2, 4, 6, 8 and 10 mM). The results in **(Table 26 and Fig. 40**) show that this compound inhibited the enzyme activity at all the tested concentrations till the activity declined to 1.9 U mg^{-1} protein representing 21.1 % of the control value at 10 mM.

Table 23: Effect of DL-homocysteic acid on the activity of L- methioninase.

Concentration (mM)	Enzyme activity (U mg^{-1} protein)
0	20.3 ± 0.6
2	22.5 ± 0.6
4	25.3 ± 0.6
6	28.7 ± 0.6
8	26.4 ± 0.5
10	21.0 ± 0.5

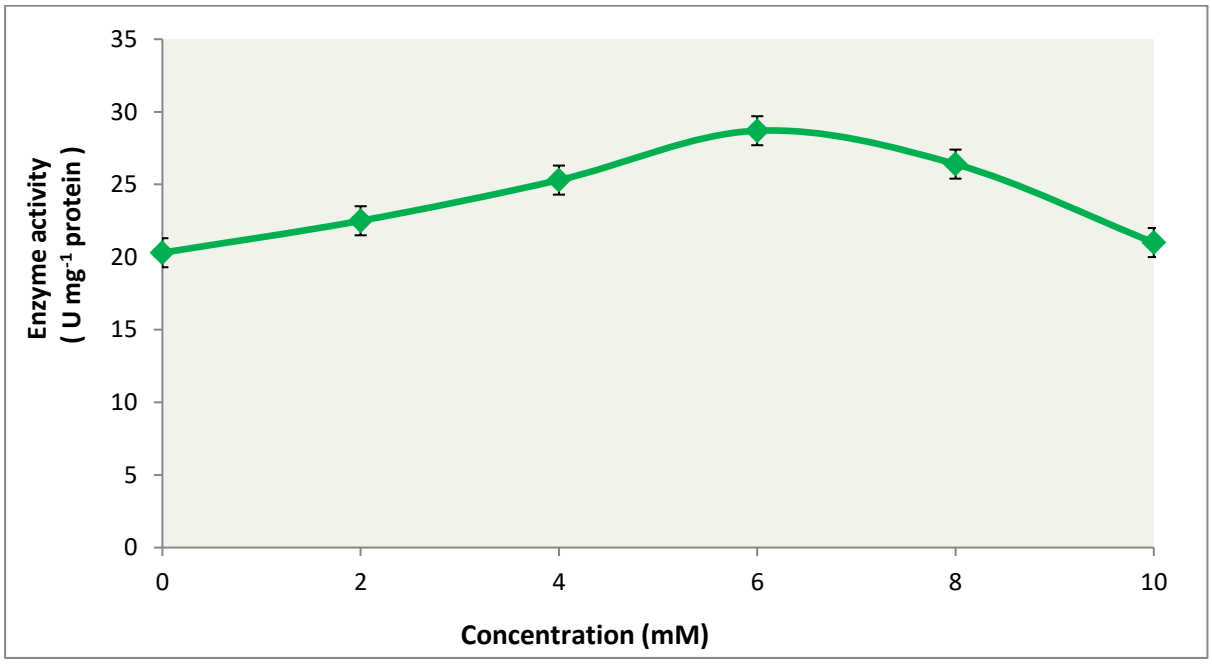


Fig. 37: Effect of DL-homocysteic acid on the activity of L- methioninase.

Table 24: Effect of hydroxylamine on L-methioninase activity.

Concentration (mM)	Enzyme activity (U mg^{-1} protein)	% of control
0	15.4 ± 0.6	100
2	16.9 ± 0.7	109.7
4	19.6 ± 0.5	127.2
6	14.8 ± 0.6	96.1
8	11.9 ± 0.4	77.2
10	8.3 ± 0.3	53.8

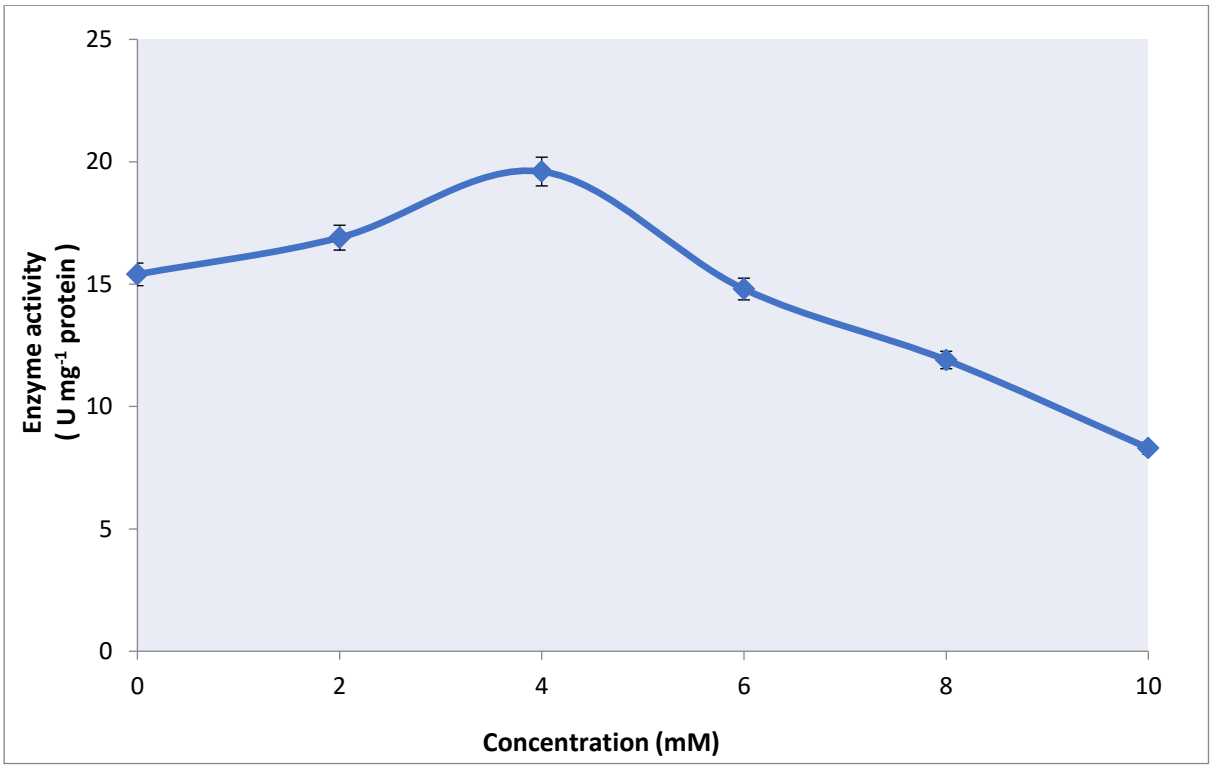


Fig. 38: Effect of hydroxylamine on L-methioninase activity.

Table 25 : Effect of phenylmethylsulfonyl fluoride (PMSF) on enzyme activity of L-methioninase.

Concentration (mM)	Enzyme activity (U mg^{-1} protein)	% of control
0	8.2 ± 0.2	100
2	9.2 ± 0.3	112.1
4	11.1 ± 0.3	135.3
6	13.8 ± 0.2	168.2
8	15.0 ± 0.3	182.9
10	13.9 ± 0.4	169.5

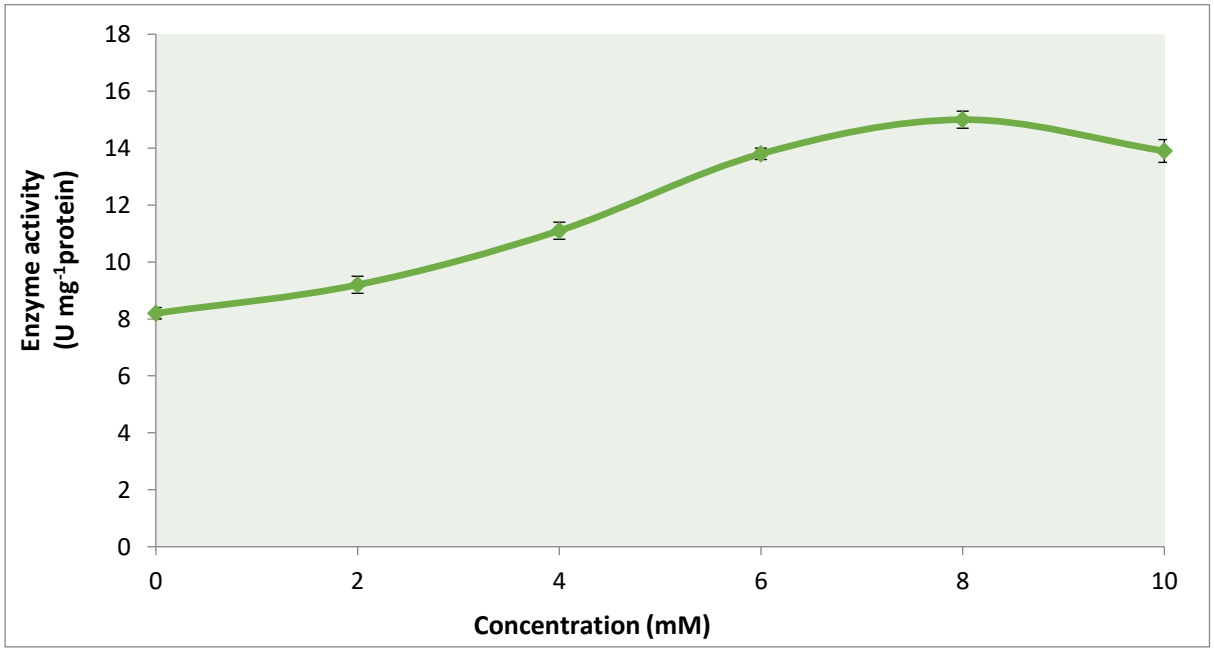


Fig. 39 : Effect of phenylmethylsulfonyl fluoride (PMSF) on enzyme activity of L-methioninase.

Table 26: Effect of DL-propargylglycine on enzyme activity of L-methioninase

Concentration (mM)	Enzyme activity (U mg^{-1} protein)	% of control
0	9.0 ± 0.3	100
2	7.8 ± 0.3	86.6
4	5.8 ± 0.4	64.4
6	3.8 ± 0.4	42.2
8	2.1 ± 0.2	23.3
10	1.9 ± 0.1	21.1

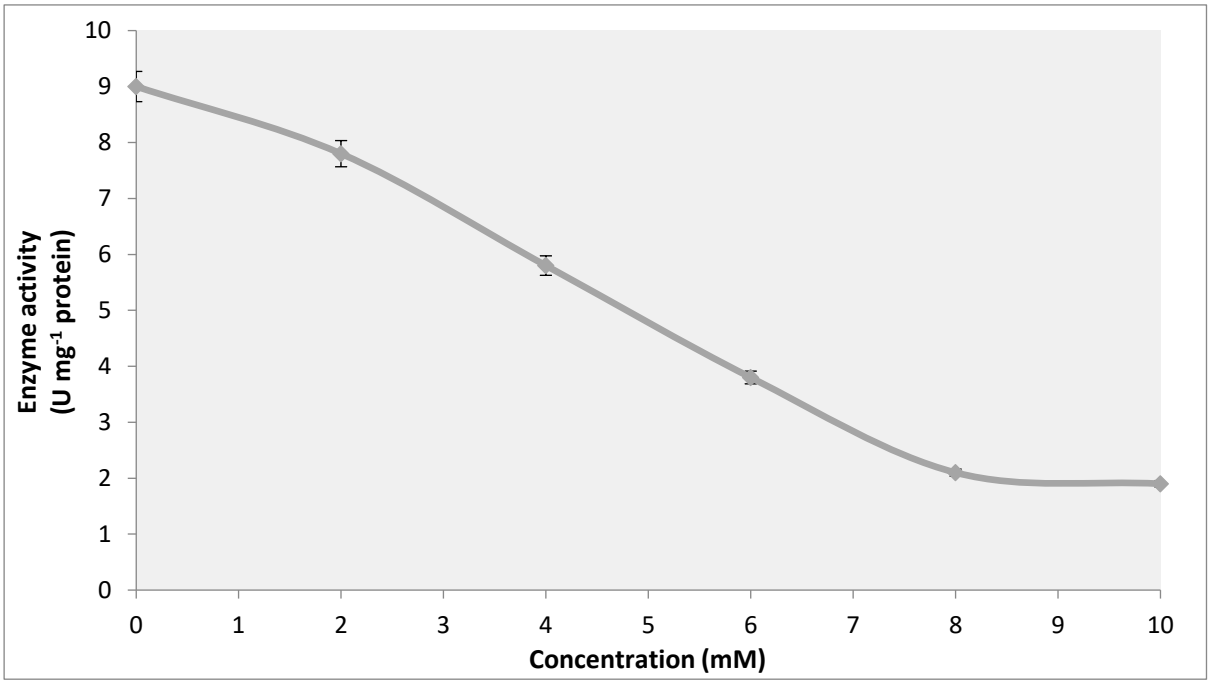


Fig. 40: Effect of DL-propargylglycine on enzyme activity of L-methioninase.

5.16. Immobilization of purified L-methioninase

The purified L-methioninase was immobilized on to supports namely chitin and carrageenan. The immobilization efficiency values were 71.4 % and 92 % for two supports **(Table 27 and Fig. 41)** on respectively.

5.17. Effect of L-methioninase on cancer cell lines

The effect of L-methioninase on cell inhibition and survival in three different cell lines: WI-38, HeLa and MCF-7 was examined. The data is presented through graphs that display the relationship between the concentration of L-methioninase (µg/ml) and the percentage of cell inhibition or survival.

The results in **Table 28 and Figs. 42 and 43** focus on the WI-38 cell line, depicting a dose-response curve that shows the percentage of cell survival at various concentrations of L-methioninase . The IC_{50} value, which is the concentration required to inhibit 50 % of the WI-38 cells, is 273.5 µg/ml. This high IC_{50} value indicates that WI-38 cells are relatively resistant to L-methioninase, necessitating a higher concentration of the enzyme to achieve significant cell inhibition.

The results in **(Table 29 and Figs. 44 and 45)** explore the impact of L-methioninase on the HeLa cell line, illustrating the percentage of cell inhibition at different concentrations of the enzyme. The IC_{50} value for HeLa cells is 8.96 µg/ml suggesting that a relatively low concentration of L-methioninase is sufficient to reduce the survival of 50% of the HeLa cells. This lower IC_{50} value compared to WI-38 cells implies that HeLa cells are more sensitive to L-methioninase, with a higher efficacy in reducing cell survival.

The results in **(Table 30 and Figs. 46 and 47**) explore the effects of L-methioninase on the MCF-7 cell line, showing the percentage of cell inhibition at various concentrations of the enzyme. The IC_{50} value for MCF-7 cells is 11.45 µg/ml indicating that MCF-7 cells are like HeLa cells in their sensitivity

to L-methioninase since they require a low concentration of the enzyme to achieve a 50% reduction in cell survival.

Overall, the three cell lines demonstrate different sensitivities to L-methioninase. HeLa and MCF-7 cells exhibit greater sensitivity, with low IC_{50} values, compared to WI-38 cells, which have a much higher IC_{50} value. The differential sensitivity of these cell lines to L-methioninase suggests its potential for selective targeting in therapeutic applications. Specifically, the enzyme could be used to preferentially inhibit certain cancer cells, while sparing normal cells. These findings highlight the therapeutic promise of L-methioninase and provide a foundation for further research and development in its application for cancer treatment.

Table 27: Immobilization of purified L-methioninase.

Beads of immobilization	Immobilization efficiency (%)
Chitin	71.4 ± 1.3
Carrageenan	92.0 ± 1.6

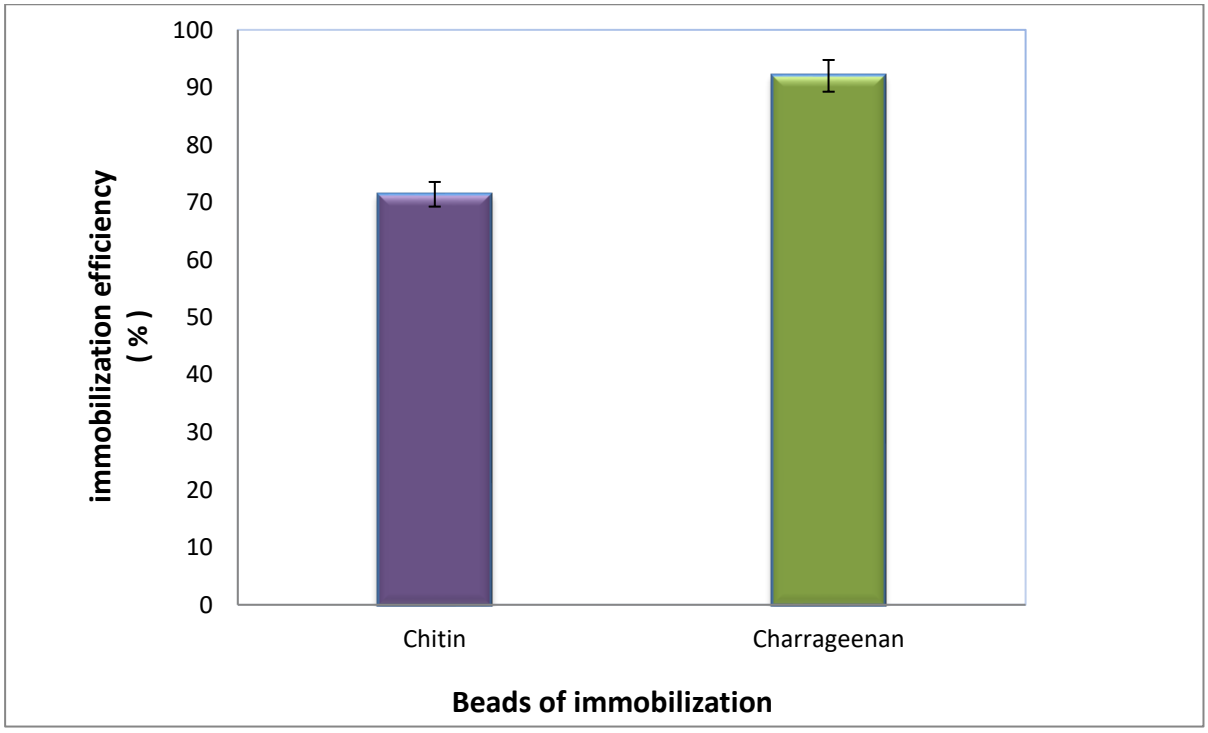


Fig. 41 : Immobilization of purified L-methioninase

Table 28: Effect of L-methioninase on the cell survival of WI-38 cell line.

Conc. (µg/ml)	Log conc.	% Cell survival WI-38			Mean ± SE
1.56	0.193	98.2	97.264	97.564	97.676 ± 1.5
3.123	0.494	95.35	94.256	95.09	94.898 ± 1.6
6.25	0.795	92.356	92.78	93.085	92.740 ± 1.4
12.5	1.096	89.56	89.221	89.36	89.380 ± 1.3
25	1.397	84.356	84.356	84.178	84.296 ± 1.2
50	1.698	80.247	80.789	80.789	80.608 ± 1.4
100	2.0	62.32	65.34	64.25	63.970 ± 1.1

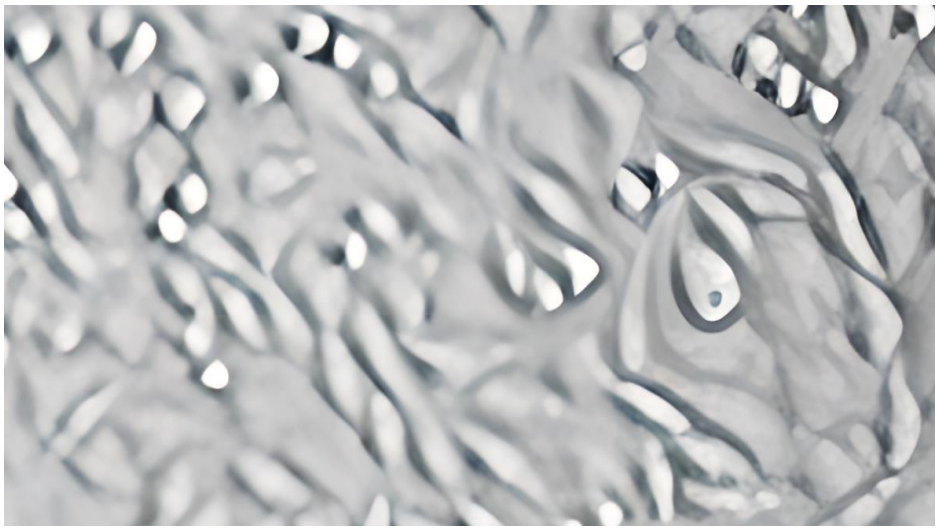


Fig. 42: Anticancer effect of L-methioninase on WI-38 cell line.

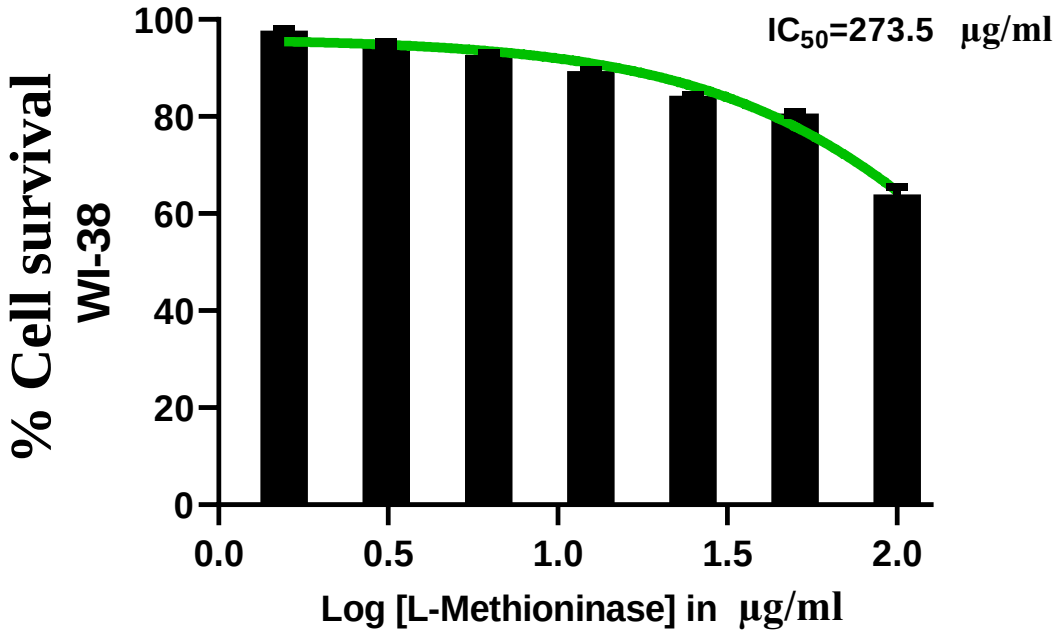


Fig. 43: Effect of L-methioninase on the cell survival of WI-38 cell line

Table 29: Effect of L-methioninase on the cell inhibition of HeLa cell line.

Conc. (µg/ml)	Log conc.	% Cell inhibition HeLa			Mean ± SE
100	2.0	91.23	93.78	92.56	92.52 ± 1.3
50	1.698	90.23	90.78	91.23	90.74 ± 1.2
25	1.397	80.25	81.25	80.789	80.76 ± 1.4
12.5	1.096	74.23	74.89	75.236	74.78 ± 1.2
6.25	0.795	55.69	55.612	54.78	55.36 ± 0.7
3.123	0.494	50.25	51.23	54.256	51.91 ± 0.9
1.56	0.193	42.356	42.56	41.89	42.26 ± 0.8

Fig. 44: Anticancer effect of L-methioninase on HeLa cell line.

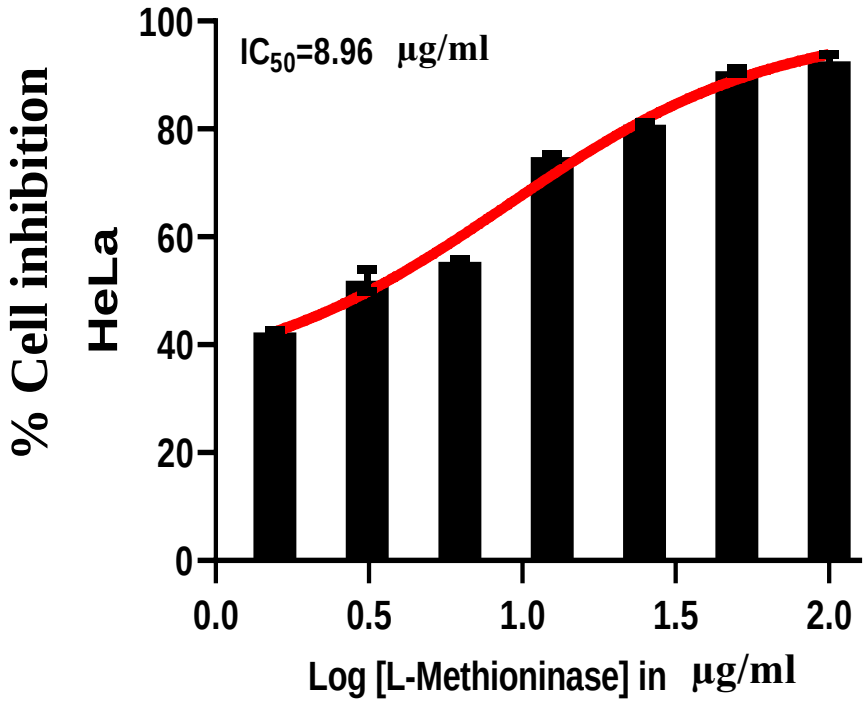


Fig. 45: Effect of L-methioninase on the cell inhibition of HeLa cell line.

Table 30: Effect of L-methioninase on the cell inhibition of breast cancer cell line (MCF-7).

Conc. (µg/ml)	Log conc.	% Cell inhibition MCF-7			Mean ± SE
100	2.0	92.899	92.3569	92.355	92.536 ± 1.2
50	1.698	84.56	84.589	83.65	84.266 ± 1.4
25	1.397	70.256	71.256	70.256	70.589 ± 1.0
12.5	1.096	63.25	64.255	64.254	63.916 ± 1.1
6.25	0.795	40.256	41.233	40.256	40.581 ± 0.7
3.123	0.494	32.56	33.56	34.256	33.458 ± 0.6
1.56	0.193	24.25	23.23	23.98	23.820 ± 0.7

Fig. 46: Anticancer effect of L-methioninase on breast cancer cell line (MCF-7).

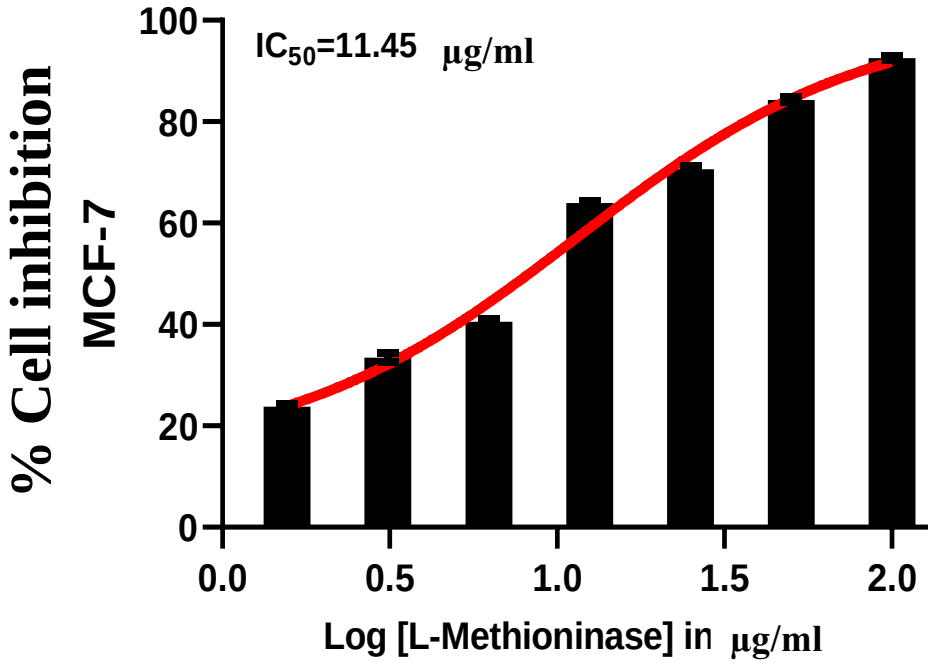


Fig. 47: Effect of L-methioninase on the cell inhibition of breast cancer cell line (MCF-7).

6. Discussion

Production of enzymes is a necessary event in the industrial sectors, due to the high and superior performances of enzymes from different microbes, which works well under a wide range of varied physical and chemical conditions. L-methioninase is one of few enzymes with high therapeutic values (**Selim *et al.*, 2015; El-Shora *et al.*, 2021 and Reda *et al.*, 2023**).

Therefore, our study gave much attention to L-methioninase from *Staphylococcus sciuri* for therapeutic application. L-methioninase secreted from some bacterial species have high therapeutic value because of association with high immunogenicity and low substrate specificity (**Reda *et al.*, 2023).**

L-methioninase was purified from *Staphylococcus sciuri* using ammonium sulphate (85%), DEAE cellulose, Sephadex G-200 and Q-Sepharose. After purification, the final specific activity of L-methioninase was 168.7 U mg^{-1} protein with purification fold of 177.5 and yield of 26.4 %.

The purification process gave a pure enzyme with a single band on SDS-PAGE with a molecular mass 48 kDa. This is in accordance with **Salim *et al.* (2020)** who reported the molecular weight of 48 kDa of purified L-methioninase from *Trichoderma harzianum*.

Moreover, L-methioninase purified from *Candida tropicalis* with 34.19-fold and 27.98% yield. The purified enzyme revealed a single band on SDS-PAGE with a molecular weight of 46 kDa **(Selim *et al.*, 2015)**. **Selim *et al.* (2016)** has purified L-methioninase from *Streptomyces* sp with 47 kDa.

The reaction of the enzyme with its substrate will continue to increase continuously as long as some of the active sites of the enzymes are still able to breakdown the substrate. However, when all the active sites of the enzyme are full occupied then the rate of the enzyme reaction will approach the maximum

rate (V_{max}) and not well be affected by further increase of substrate concentration (**El-Shora *et al.*, 2024a**).

Plotting V^{-1} versus S^{-1} gave a linear relationship from which K_m and V_{max} values for L-methioninase were calculated. K_m values was 1.7 mM and V_{max} value was 33.3 U mg^{-1} protein.

The optimal pH for L-methioninase was 8.0 in the present investigation. However, the optimal pH was 7.0 from *Bacillus licheniformis* **(Alrumman *et al.*, 2019).**

The optimal temperature for L-methioninase was 40 ºC. However, the optimal temperature for the enzyme from *Bacillus licheniformis* was 37 ºC **(Alrumman *et al.*, 2019).**

The present results revealed that the tested phytohormones: GA_3, JA, BAP and Kin activated L-methioninase activity. This is in agreement with the results of **Reda *et al.*, (2023)**. Also, GA_3 induced other enzymes such as α-glucosidase (**El-Shora *et al.*, 2009; El-Shora *et al.*, 2018**), phosphoenolpyruvate carboxylase (**Bihzad and El-Shora, 1996**), NADH-glutamate synthase (**El-Shora, 2001a**) and phenylalanine ammonia lyase (**El-Shora, 2002**).

In addition, GA_3 increased the activities of sucrose synthase and sucrose phosphate synthase (**Kaur *et al.*, 2000**). The increase in the enzyme activity by these phytohormones may be due their activation of the enzyme molecules and consequently increase the enzyme reaction **(El-Shora *et al.*, 2009).**

The four tested reagents of the active groups (N-bromosuccinimide, butanedione, N-ethymaleimide and trinitromethane) revealed their inhibitory effect on the enzyme with different rates indicating the necessity of tyrptophanyl, arginyl, sulfhydryl and tyrosyl groups for the enzyme catalysis (**El-Shora *et al.*, 2024b**).

The five sulfur compounds including thioglycolate, N-acetyl cysteine, cysteine, reduced glutathione and cystamine activated L-methioninase at the lower concentrations when they are included in the reaction medium, however at high concentrations they become inhibitors. Cysteine as amino acid activated other enzymes such as urease, acid phosphatase and asparaginase (**El-Shora, 2001b; El-Shora and Metwally**, **2009; Warangkar and Khobragade, 2010**).

The tested chelating agents including α-α-dipyridyl, phenanthroline, and EGTA inhibited the enzyme in a concentration-dependent manner. In support **Selim *et al* (2015)** reported the inhibition of L-methioninase by the chelating agent EDTA. The inhibition of L-methioninase by chelating agents seems likely to be mediated through chelation of the metal present in the enzyme molecule converting it to non-active enzyme. Thus, the inhibition of L-methioninase indicates that L-methioninase is a metalloenzyme.

Cysteine activated other enzymes such as protease from *Pterocladia capillacea* (**El-Shora *et al.*, 2016)** and fungal enzymes tyrosinase **(El-Shora and El-Sharkawy, 2020**). This activation by sulfur compounds may indicate protection of the sulfhydryl group (-SH) during the reaction time at low concentrations. Also, it is possible that these sulfur compounds at lower concentrations may lower the K_m of the enzyme by lowering its affinity to the substrate and thus enhancing its activity particularly. The sulfur compounds at higher concentration interfere between enzyme and its substrate preventing formation of ES complex and thus reducing the enzyme activity.

Xylitol protected L-methioninase from heat inactivation at the higher temperature over the optimum (40, 45, 50 and 55 ºC). Xylitol is one of polyol compounds. The polyol molecules are preferentially excluded from the surface layer of the protein molecule and the water shell around the protein molecules

of the enzyme is preserved, so that the conformation of the protein become more rigid (**Longo and Combes, 1999**).

In general, the structure and activity of water is changed after addition of polyols all such as xylitol (**Huang *et al.*, 2022**). This effect leads to increase thermal stabilization to enzyme protein structure in the presence of such kind of additive. In addition, it is expected that the formation of new hydrogen bonds between glycol chitosan chains and hydrophilic residues at the surface of L-methioninase molecules could increase conformational rigidity to this enzyme, and consequently improve its resistance to elevated temperatures. Both of these effects depend on the amount of additive in the enzyme protein solution, and could justify the thermal stability behavior of L-methioninase.

Trehalose followed the same trend of xylitol in protection of the enzyme from denaturation at 40, 45, 50 and 55 ºC which are higher than the optimum. In support, trehalose protected bacterial phytase activity from *E. coli* and *S. aureus* against thermal denaturation (**El-Shora *et al.*, 2019**). So, trehalose is reported as an effective protector for many other enzymes against thermal inactivation (**El-Shora *et al.*, 2014; El-Shora *et al.*, 2016; El-Shora *et al.*, 2018; El-Shora *et al.*, 2022**).

Also, trehalose participates in protecting proteins through stabilizing their most compact structure, thus it reduces the backbone movements away from the fully folded state (**Solapenna and Meyer-Fernandes 1998; Nirmal and Laxman, 2014**).

Glycol chitosan in the present work offered protection for L-methioninase at the higher temperatures over the optimum. In support, glycol chitosan was evaluated as thermoprotectant additive for other enzymes such as trypsin in aqueous solutions **(Fernández, *et al.*, 2005).**

Glycol chitosan is a derivative of chitosan which is partially deacetylated polysaccharide derived from chitin which has been used in a variety of

research fields, including biomedicine and environmental science. Because of its nontoxicity, bioavailability and low immunogenicity, chitosan has attracted attention among polymer materials in biomedical and pharmaceutical applications. Glycol chitosan as a derivative of chitosan offers the advantages of biocompatibility and biodegradability **(Huang *et al.*, 2022).**

The improved thermostabilization of L-methioninase after adding glycol chitosan is mainly mediated by electrostatic forces. In this sense, both the occurrence of direct electrostatic interactions between the positive charged polysaccharide and the protein structure as well as changes in the electrostatic potential at the microenvironment of the enzyme after adding the cationic polymer could be involved in this stabilizing mechanism (**Fernández *et al.*, 2005**).

Preservation of L-methioninase activity at high temperatures in the presence of glycol chitosan due to the formation of electrostatic interactions at the surface of the enzyme between the negative charged groups of the protein and the amino groups from the polysaccharide. By this mechanism, the polysaccharide chains could increase the rigidity of the enzyme structure through multipoint electrostatic cross-links, reducing the protein chain mobility and preserving the enzyme conformation at high temperatures (**Fernández *et al.*, 2005**).

In the present work the two anhydrides maleic and succinic anhydride inhibited L-methioninase at 5 mM. The two compounds inactivated other enzymes such as ribonuclease and this was attributed to the fact that anhydride considered blocking reagent for amino groups in proteins **(Takahashi, 1977**).

L-methioninase from *Staphylococcus sciuri* was inhibited by DL-homocysteic acid, hydroxylamine, PMSF and DL-propargylglycine in a concentration-dependent manner. DL-propargylglycine is a non- proteinogenic L-α-ainmo acid that is L-alanine in which one of the methyl hydrogens has

been replaced by an ethynyl group. DL- propargylglycine was found to inhibit other enzymes which are PLP-dependent such as alanine transaminase **(Echizen *et al.*, 2023)**. In support, **Lockwood and Coombs (1991)** reported the inhibition of L-methioninase activity by DL-homocysteic acid. Also, L-methioninase was inhibited by hydroxylamine and PMSF **(Salim *et al.*, 2020).**

Hydroxylamine can alter the reaction of the enzyme by interacting with the active site of the enzyme or the allosteric site of the enzyme, thereby affecting its function. This interaction can slow down the rate of enzyme reaction (**Dinos and Coutsogeorgopoulos, 1997**) .

L-methioninase from *Staphylococcus sciuri* was immobilized on chitin and carrageenan with immobilization efficiencies of 71.4 % and 92 %, respectively. L-methioninase from other sources such as fenugreek (**Red *et al.*, 2022**) was immobilized on Ca- alginate and chitosan. Also, L-methioninase from *Wickerhamomyces subpelliculosus* was immobilized on a zirconium based metal (**Hassabo *et al.*, 2019**). The immobilized enzyme can be used as antioxidant (**El-Hadedy *et al*., 2023**).

Cancer is defined by unregulated cell proliferation caused by changes in the expression of tumor-suppressing and tumor-promoting genes. Cancer is a major threat to world health because of its high rates of metastasis and the development of resistance to established anti-cancer therapy. Conventional therapies such as radiation, chemotherapy, surgery, and ultrasound are regularly utilized; they frequently fail to completely cure metastasis and can mistakenly harm healthy cells **(Javia *et al*., 2024**).

Using enzymes to limit the availability of essential nutrients required for the rapid growth and proliferation of tumor cells is a unique approach to the treatment of cancer. The Food and Drug Administration (FDA) has approved the use of enzymes for a variety of therapeutic uses, including anti-cancer, anti-cardiovascular, anticoagulants, antimicrobials, and antioxidants (**Baindara and**

Mandal, 2020). Remarkably, many tumor cells are auxotrophs for certain amino acids such as L-methionine, glutamine, asparagine and arginine, since there are no intrinsic enzyme systems that synthesize these amino acids.

In the present investigation L-methioninase from *Staphylococcus sciuri* proved to be anticancer enzyme. The effect of L-methioninase on cell inhibition and survival in three different cell lines: WI-38, HeLa and MCF-7.

The impact of L-methioninase on the WI-38 cells showing IC_{50} value which is the concentration required to inhibit 50 % of the WI-38 cells, is 273.5 µg/ml. This high IC_{50} value indicates that WI-38 cells are relatively resistant to L-methioninase, necessitating a higher concentration of the enzyme to achieve significant cell inhibition.

The impact of L-methioninase on the HeLa cell line, illustrating the percentage of cell inhibition at different concentrations of the enzyme. The IC_{50} value for HeLa cells is 8.96 µg/ml. This lower IC_{50} value compared to WI-38 cells implies that HeLa cells are more sensitive to L-methioninase, with a higher efficacy in reducing cell survival.

The effects of L-methioninase from *Staphylococcus sciuri* on the MCF-7 cell line, showing the percentage of cell inhibition at various concentrations of the enzyme. The IC_{50} value for MCF-7 cells is 11.45 µg/ml. The L-methioninase from *Trichoderma harzianum* inhibited the growth of breast cells (MCF-7 cell line) with IC_{50} values of 20.07 µg/ml **(Salim *et al.*, 2020).**

Overall, the three cell lines demonstrate different sensitivities to L-methioninase. HeLa and MCF-7 cells exhibit greater sensitivity, with low IC_{50} values, compared to WI-38 cells, which have a much higher IC_{50} value. The differential sensitivity of these cell lines to L-methioninase suggests its potential for selective targeting in therapeutic applications. Specifically, the enzyme could be used to preferentially inhibit certain cancer cells, while sparing normal cells. These findings highlight the therapeutic promise of L-

methioninase and provide a foundation for further research and development in its application for cancer treatment.

Cancer is an increasing cause of mortality and morbidity throughout the world. L-methioninase seems to be one of few microbial enzymes with high therapeutic value and has potential application against many types of cancers. L-methionine plays an important role in tumor cells. These cells become L-methionine dependent and eventually follow apoptosis due to L- methionine limitation in cancer cells.

Therefore, L-methioninase from *Staphylococcus sciuri* in this study represents promising candidate as anticancer enzyme for the two types of the studied cell lines (MCF-7 and HeLa). However, the application needs further investigation.

7. Summary

In the present work L-methioninase was isolated and purified from *Staphylococcus sciuri* and some biochemical characteristics were investigated. The obtained results are summarized in the following points:

1. The enzyme was isolated and purified using ammonium sulphate, DEAE-cellulose, Sephadex G-200 and Q-Sepharose with specific activity of 168.7 U mg^{-1} protein and purification fold of 177.5
2. The SDS-PAGE exhibited a single band at 48 kDa and this confirms purity of the L-methioninase.
3. Analysis of amino acids of the purified enzyme exhibited variation in the content of the amino acids. The most dominant amino acids were cysteine, aspartic acid, and methionine.
4. The values of V_{max} and K_m of the purified enzyme were 33.3 U mg^{-1} protein and 1.7 mM.
5. The optimal pH and the optimal temperature of the enzyme were 8.0 and 40 ºC.
6. The phytohormones acid (GA_3), jasmonic acid (JA), benzylaminopurine (BAP) and kinetin (Kin) activated the L-methioninase with different rates.
7. The four reagents of the active groups namely N-bromosuccinimide (NBS), butanedione (BD), N-ethylmaleimide (NEM) and trinitromethane (TNM) inhibited L-methioninase in a concentration-dependent manner confirming the necessity of tryptophanyl, arginyl, sulfhydryl and tyrosyl groups for L-methionine catalysis.
8. The three chelating agents α-α-dipyridyl, phenanthroline and EGTA inhibited the enzyme activity in a concentration-dependent manner with

different rates. This inhibition reveals and confirms that the enzyme is a metalloenzyme.

9. The five sulfur compounds thioglycolate, N- acetyl cysteine, cysteine, glutathione and cystamine activated L-methioninase at the lower concentrations; however the higher concentrations were inhibitors. These results confirm the necessity of sulfhydryl group for L-methioninase catalysis.
10. Xylitol, trehalose and glycol chitosan offered partial protection for the enzyme against heat inactivation at the higher temperatures (40, 45, 50 and 55 ºC) over the optimal and the protection was dependent on the protector compound.
11. The two anhydrides maleic and succinic inhibited the enzyme by different rates.
12. DL-homocysteic acid, hydroxylamine and phenylmethylsulfonyl fluoride increased L-methioninase activity at lower concentrations and inhibited the activity at higher concentrations. However, DL-propargylglycine inhibited the enzyme activity at all the tested concentrations.
13. L-methioninase was immobilized successfully on chitin and carrageenan with immobilization efficiencies of 71.4 % and 92 %, respectively.
14. L-methioninase exhibited appreciable anticancer effect against HeLa and MCF-7 cell lines.

8. CONCLUSION

L-methioninase is an inducible enzyme that is formed when L-methionine is added to the culture medium of the tested organism. The tested thiol compounds can be used to induce activity of the purified L-methioninase from *Staphylococcus sciuri* particularly at the lower concentrations which is important phenomenon if the purified enzyme will be applied in the industry of some products.

L-methionine plays an important role in tumor cells. These cells become L- methionine dependent and eventually follow apoptosis due to L- methionine limitation in cancer cells.

Therefore, L-methioninase from *Staphylococcus sciuri* in this study represents promising candidate as anticancer enzyme for the two types of the studied cell lines (MCF-7 and HeLa). However, the application needs further and more investigation.

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جامعة بنها
كلية العلوم
قسم النبات والميكروبيولوجى

إنزيم الميثيونينيز البكتيرى وتطبيقاته المحتمله كمضاد للسرطان

رسالة مقدمة من

سماح على محمود السيد إسماعيل
بكالوريوس العلوم (ميكروبيولوجي وكيمياء، 2016)

إلى

قسم النبات والميكروبيولوجي - كليه العلوم - جامعه بنها
كجزء متمم لمتطلبات الحصول علي درجه الماجستير في العلوم (الميكروبيولوجي)

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جامعة بنها
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قسم النبات والميكروبيولوجى

إنزيم الميثيونينيز البكتيرى وتطبيقاته المحتمله كمضاد للسرطان

رسالة مقدمة من

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بكالوريوس العلوم (ميكروبيولوجي وكيمياء ،٢٠١٦)

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