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Short Review on Microbial L- methioninase and it's Applications

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ABSTRACT : This review is an attempt to briefly describe L-methioninase sources, three dimensional structure, biochemical characterization, potential catalytic activity and its therapeutic applications for treatment of ageing, obesity, Parkinson's, cardiovascular disease and cancer.
KEYWORDS L-methioninase, sources, structure, treatment.

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INTRODUCTION

L- Methioninase (methionine γ-lyase) has important biotechnological applications because of exhibiting hydrolytic property to catalyze α-γ-elimination of L-methionine, an essential amino acid, to α- ketobutyrate, methanethiol and ammonia. Methionine deprivation inhibits tumor growth and metastasis. The catalytic activity of L-methioninase could be used as enzyme supplementation therapy for these diseases. This enzyme has been extensively studied from a wide range of organisms, including plants, terrestrial and marine microbes Methioninase secreted from some bacterial species have high therapeutic value because of association with high immunogenicity and low substrate specificity. The enzyme is used for cancer treatment by depleting supply of methionine from exogenous source to cancer cells. The microbial production of L-methioninase enzyme is cost effective and easily produced. Several researches aimed to explore new strains for producing L-methioninase having therapeutic values. Therefore, in this review, we briefly describe the historical aspects of microbial L-methioninase, their biochemical characterization and their industrial application

1. L-Methioninase:

This enzyme belongs to the family of lyases, especially the class of Carbon-sulfur layers. Other names in common use include L-methionine gamma-lyase (MGL), and L-methionine methanethiol-lyase (deaminating). This enzyme participates in seven amino acids metabolism. It employs one cofactor, pyridoxal phosphate **(Brown, 2019** and **Richts *et al.*, 2019).** PLP reduces the energy for conversion of amino acids to a zwitterionic carbonion (**Richard and Amyes, 2004**) and substantially the apoenzyme catalyzes the cleavage of substrate bond yielding the product (**Wolfenden, 2011).** MGL is a cytosolic enzyme inducibly formed by addition of *L*-methionine to the culture medium (**Lockwood and Coombs, 1991).**

The abnormality behavior of methionine based metabolic process result ageing, obesity, Parkinson's, cardiovascular and cancer diseases among human being. The catalytic activity of L-methioninase could be used as enzyme supplementation therapy for these diseases. This enzyme is present in wide range of organisms including, plant, bacteria, and fungi. Methioninase secreted from some bacterial species have high therapeutic value because of association with high immunogenicity and low substrate specificity. These enzymes are also used for cancer treatment by depleting supply of methionine from exogenous source to cancer cells (**Kharayat, and Singh, 2018).**

2. L-methioninase mechanism of action

L-Methioninase (METase, EC.4.4.1.11) is a pyridoxal phosphate dependent enzyme catalyzing elimination reactions of L-methionine to methanethiol, ketobutyrate and ammonia(fig. 1) (**Inoue *et al.*, 1995 and Kharayat and Singh, 2018)**. It is absent in mammalian system and intracellularly present in bacteria and extracellularly in fungi (**Sharma *et al.*, 2014)**.

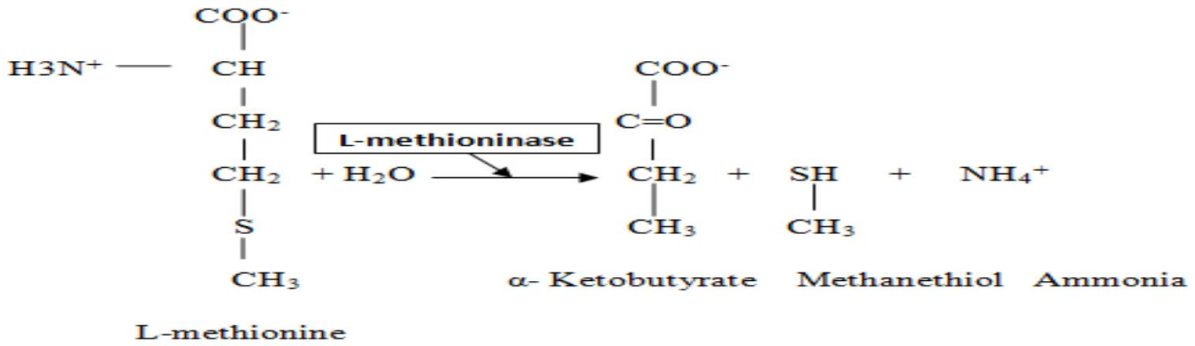

Fig. 1. Catalytic pathway for catalysis of L-methioine by L-methioninase (Inoue *et al.*, 1995).

L -methioninase is a homotetrameric, multifunctional enzyme belongs to the $_{\gamma}$ family of PLP (pyridoxal 5-phosphate) dependent enzymes and the active methioninase tetramer consists of two sets of strongly associated catalytic dimers (**Motoshima *et al.*, 2000; Nikulin *et al.*, 2008a and 2008b**). It catalyses α- and $_{\gamma}$- removal with α $_{\gamma}$ replacement of L-methionine with the equimolar yield of α-keto acids (2-oxobutyrate and pyruvate), methanethiol, and ammonia (figure 1) (**Inoue *et al.*, 1995**). Methionine is undergoing oxidative deamination with the presence of L-methioninase produces α-ketomethionine and further dethiomethylated with the production of methanethiol part of which oxidized to dimethyl disulfide and dimethyltrisulfide. α-ketobutyrate which is the key intermediate of methionine degradation can be converted to propionyl-CoA and subsequently methylmalonylCoA, which can be converted to succinyl CoA, a citric acid cycle intermediate, and thus enter the citric acid cycle (**Tokoro *et al.*, 2003**). The sulfur or oxygen atom at the β- or $_{\gamma}$-position of the substrate is substituted with the thiol during the β- or $_{\gamma}$- replacement reactions. The comprehensive reaction mechanism catalyzed by L-methioninase comprise of the 6 following steps (Fig. 2) (**Tanaka *et al.*, 1985; Chin and Lindsay, 1994; Faleev *et al.*, 1996**):

i) Amine group of the methionine attacks the internal aldimine structure.
ii) Formation of external aldimine by the Schiff's base transformation
and the lysine group of MGL released.
iii) Formation of ketimine, tyrosine moiety of MGL attacks and removes
the hydrogen group from α- position of methionine.
iv) Formation of Quinonoid, the hydroxyl group of tyrosine moiety from MGL donates its hydrogen group to the β- position which leads to the release of thiol group.
v) Water moiety attacks the imine bond and releases the α-ketoacid.
vi) Formation of internal aldimine, amine group lysine moiety from MGL attacks the amine bond which forms aldimine structure and releases ammonia.

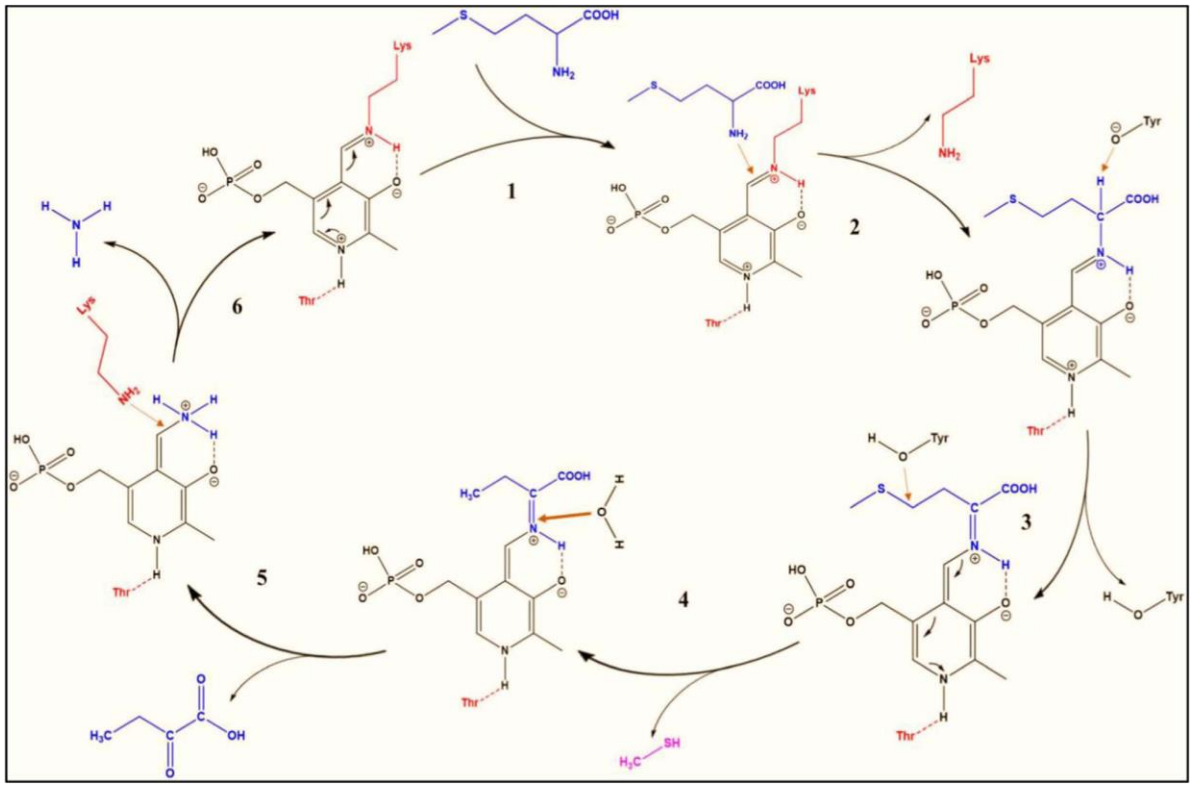

Fig. 2. Proposed reaction process of α, ɤ-elimination of methionine by methioninase [Substrates, intermediates, and products are shown in blue, while PLP is shown in black. Amino group reactions shown in red]. Structures (1) to (6) correspond to the reaction intermediates explained above. (**Tanaka *et al.*, 1985; Chin and Lindsay, 1994; Faleev *et al.*, 1996**)

3. Crystal structure and pyridoxal phosphate dependence of L-methioninases:

MGL is homotetrameric protein, consists of 389-441 amino acids and forms a homotetrameric protein of about 172–188 kDa, and 43–47 kDa under non-denaturing and denaturing gel, each subunit binds one molecule of cofactor-PLP (**Soda *et al.*, 1983; El-Sayed 2011; Georgiou *et al.*, 2015**). Amino acids residues involved in catalysis have been identified from the crystal structure of MGL. Lys240, Asp241, and Arg61 of one subunit and Tyr114 and Cys116 of an adjacent subunit form hydrogen-bond network in the L-methioninase active site that confers specificity to the enzyme (**Georgiou *et al.*, 2015**).

The three dimensional structure of L-methioninase emphasizes its existence as tetramer in solution (Figure 4 a, b) (**Inoue *et al.*, 1995; Motoshima *et al.*, 2000; Kudou *et al.*, 2007**). Spatially, each monomer is constructed from three functionally defined domains (Figure 4C) (**Motoshima *et al.*, 2000**): one N-terminal domain (residues from one to 63), which includes two α- helices and three β-strands, two pyridoxal phosphate binding domain (moiety from 64 to 262), which comprise seven parallel β-sheets sandwiched between eight α- helices, and three C-terminal domains (moiety 263 to 398) (**Kudou *et al.* 2007**). Functionally, the N-terminal domain is involved in the stabilization of the dimer–dimer interaction (residues 1–39), active conformational catalytic sites (residues 34–63), and stabilization of the subunit interaction (residues 42–63). The active dimer is formed by the tight association of the two monomers and packing of the pyridoxal 5'-phosphate against the C-terminal (Figure 4,d) as described by **Kudou *et al.* (2007**). The spatial dimer association is usually preserved by hydrogen bonding and hydrophilic and intermolecular active site interaction. The spatial association of the four subunits stabilizes four active sites per active dimer–dimer structure.

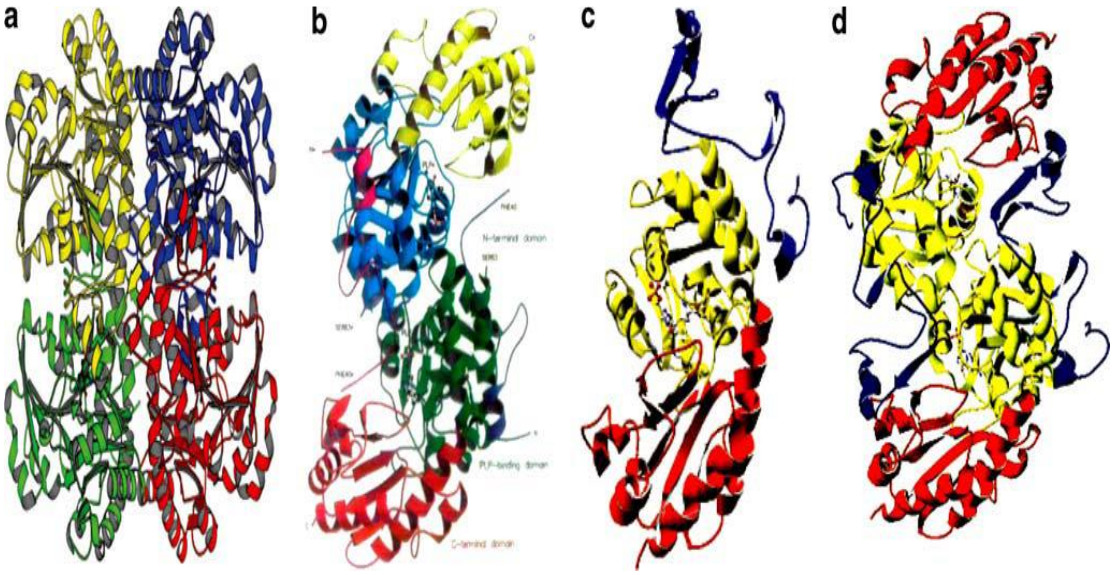

Figure 4: Three-dimensional structure of *P. putida* L-methioninase. A) Overall tetramers of the enzyme; the four subunits are colored-coded. B) Stereo view of the active dimer. C) Stereo ribbon presentation of the active subunit in which the N-terminal is blue, pyridoxal-binding domain is yellow, and C-terminal domain is red. D) Active dimer, N terminals (blue) forming dimer–dimer interface (Kudou *et al.* 2007).

C. sporogenes MGL is a homotetramer with a total molecular weight of about 170 kDa (**Morozova *et al.*, 2013).** Structures of the enzyme from P. putida **(Motoshima *et al.*, 2000) and** *C. freundii* (**Mamaeva *et al.*, 2005**) identify that it belongs to the cystathionine-lyase structural subclass (**Ka¨ck *et al.*, 1999**). The tetrameric assembly can be subdivided in to two so-called catalytic dimers in which two active sites contain residues from both subunits (Fig. 5a, green/blue or A/B and red/orange or C/D subunits). The monomer consists of three domains: N-terminal, central PLP-binding and C-terminal. The N-terminal domain (residues 1–62) is composed of one short 310-helix and two _-helices, which are connected by a long loop containing 27 residues (Fig.5&6). The position of the loop is fixed in the tetramer by contacts with the neighboring subunit (**Revtovich *et al.*; 2016**).

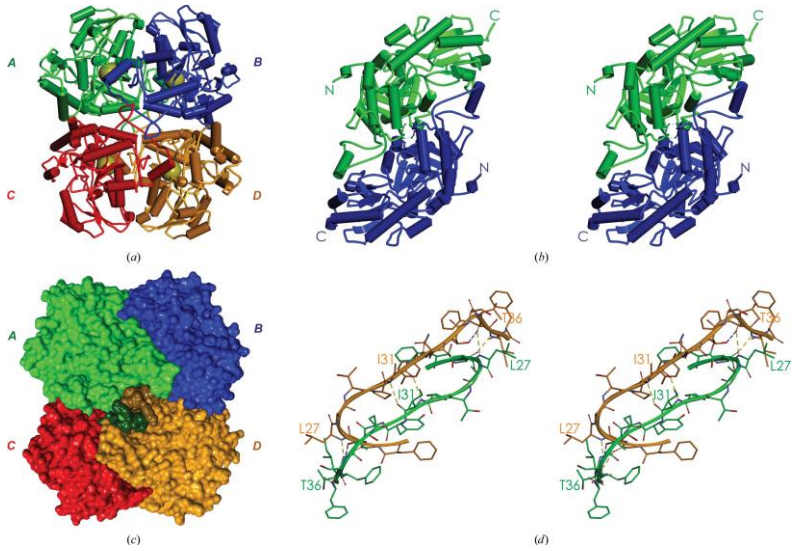

Fig5: Overall structure of *C. sporogenes* MGL. Catalytic dimers are shown in green/blue and red/orange. (a) Schematic model of a tetramer. PLP-binding sites are shown as yellow balls. (b) Stereo-view of a catalytic dimer. (c) Solvent-accessible surface of the enzyme tetramer; 'paws' are shown in darker colors. (d) Antiparallel _-sheet-like structure organized by N-terminal _-strands of the two adjacent monomers (orange and green) (**Revtovich *et al.*; 2016**).

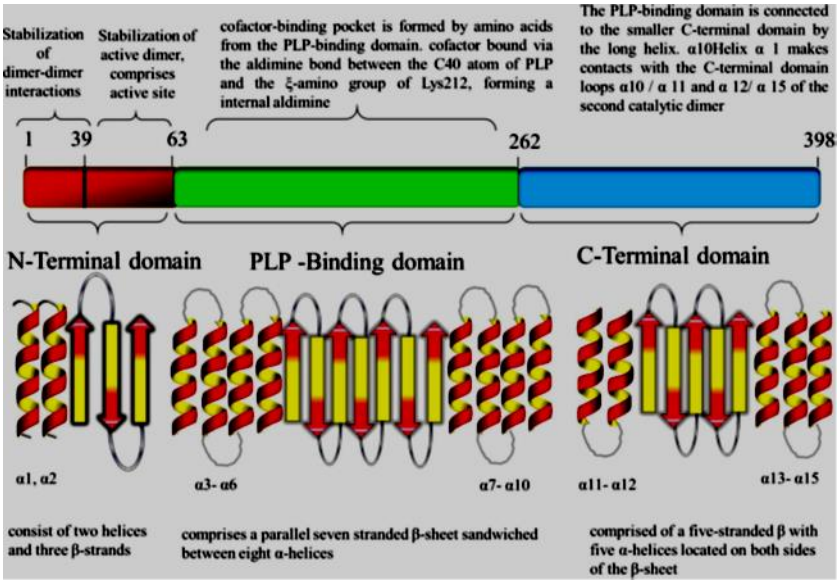

Fig. 6: Schematic representation of domain structure of L-methioninase.

Regarding *Clostridium sporogenes*, the crystal structures of MGL dimer of (PDB ID: 5DX5, 2.37) and the MGL monomer of *Citrobacter freundii* in complex with glycine (PDBID: 4HF8, 2.45) have been determined (**Revtovich *et al*; 2016 and Revtovich *et al*; 2014**).

4. **Biological sources of L-methioninase**

The presence of L-methioninase has been reported in several organisms including plants, bacteria, fungi, actinomycetes, algae protozoa, and plants etc. except humans (**El-Sayed, 2010; Bhupender *et al*., 2014; Unissa *et al.*, 2015**). Large scale production of the enzyme from microbial sources is much easier due to their simplistic production methods (**Khalaf and El-Sayed, 2009a, 2009b**).

L-methioninase was comprehensively characterized from many bacterial species as intracellular enzyme (**Tanaka *et al*.; 1976**) and from fungal species as intracellular and extracellular enzyme and absent in mammals (**Ruiz-Herrera and Starkey, 1969a,b; Abu-Seidah and Youssef, 2000; El-Sayed, 2009; Kharayat, and Singh, 2018**).

Therapeutic efficiency of bacterial L-methioninase has rarely occurred without some evidence of toxicity and immunogenic reactions, especially with regard to multiple doses (**Tan *et al*., 1997**). Therapeutic efficiency of fungal L-methioninase occurs with fewer immunogenic and allergic reactions, which may be attributed to the higher specificity of their substrates compared with the substrate analogues, displaying fewer problems during the course of tumor therapy (**Hawkins *et al*., 2004**).

4.1. Microbial sources of L-methioninase enzyme :

A. Bacteria

MGL is produced as intracellular enzymes in most of the bacterial species from various sources. Both Gram-positive and Gram-negative bacteria have been reported to produce MGL (**Bhawana and Priyanka, 2018 ; Rodionov *et al*., 2004**). Gram negative bacteria have gained more consideration as compared to Gram-positive. **Takakura *et al*. (2006)** reported that MGL is considered as a key enzyme in the bacterial metabolism of L-methionine. L-methioninase had been isolated, purified, and characterized from several bacterial species such as *P. putida* (**El-Sayed, 2016; Esaki and Soda, 1987; Esaki *et al*., 1979; Ito *et al*., 1976; Lishko *et al*., 1993b; Nakayama *et al*., 1984; Tanaka *et al*., 1977; Tanaka *et al*., 1976**), *Clostridium sporogenes* **(Tanaka *et al*., 1977),** *Aeromonas sp.* **(Nakayma et al., 1984),** *Citrobacter intermedius* **(Faleev *et al*., 1996),** *Bacillus linens* **(Dias and Weimer, 1998 and Pinnamaneni *et al*., 2012) and** *Trichomonas vaginalis* **(Lockwood and Coombs, 1991),** *Alcaligenes* sp. MT-B (**Mohkam *et al*., 2020**), *Bacillus haynesii* JUB2 (**Bopaiah *et al*., 2020),** *Hafnia alvei* **(Alshehri W.A., 2020),** *Enterobacter Cloacae* (**Prihanto *et al*., 2018**) *Brevibacterium linens* (**Dias and Weimer, 1998; Suwabe *et al*., 2011**).

Also, some actinomycetes as *Streptomyces variabilis* 3MA2016, *Streptomyces* sp. and *Streptomyces diastaticu* were reported as producers of L-methioninase (**El Awady *et al*., 2017; Khalaf and El-Sayed, 2009; Nwachukwu and Ekwealor, 2009 and Kavya and Nadumane;2020).**

Filamentous Fungi and yeast:

Due to the frequent distribution of L-methioninase as extracellular enzyme in the fungal extract, fungi could be used as potent producers of this enzyme. As it was reported for other fungal enzymes, the extracellular yield is about four times higher than the intracellular one (**Pandey *et al.* 1999; El-Sayed, 2008).**

Several studies were reported on production, optimization and characterization of L-methioninase from fungi including *Geotrichum candidum* (**Bonnarme *et al*., 2001**), *Penicillium notatum* (**Khalaf and El-Sayed, 2009**), *Penicillium sp., Aspergillus sp., Humicola fuscoatra and A. flavipes* (**Swathi, 2015**), *Aspergillus flavipes* MTCC 6337 **(Swathi, 2015),** *Aspergillus ustus* (**Abu-Tahon and Isaac 2016**). Other reports observed presence of L-methioninase in culture filtrates of yeasts such as *Geotrichum candidum, Debaromyces hansenii and Saccharomyces cerevisiae* (**Bonnarme *et al*., 2001 and Sharma *et al*., 2014**).

4.2. Protozoan sources

In contrast to other organisms possessing L-methioninase, anaerobic parasitic protists, namely *Entamoeba histolytica and Trichomonas vaginalis*, harbor a pair of methioninase isozymes such as MGL1 and MGL2 (**Sato *et al*., 2006, 2008; Anderson and Loftus, 2005; Lockwood and Coombs, 1991**). Protozoans produce L-methininase extracellularly such as *Entamoeba histolytica* (**Tokoro, *et al*., 2003),** *Porphyromonas gingivalis* (**Yoshimura *et al*., 2000**) and *Treponema denticola* (**Fukamachi *et al*., 2005**.).

4.3. Plant sources

Reports are available on the production of L- methioninase from *Arabidopsis* spp. Constituent expression of *Arabidopsis thaliana* methioninase in aerial organs and roots showed the housekeeping function in plants (**Pratelli and Pilot, 2014; Amir, 2010; Goyer *et al*., 2007; Rébeillé et al., 2006).** Dissimilation of methionine and production of methanethiol was noticed also in plant cell suspension cultures from *Catharanthus roseus* and *Cucumis melo* (**Schwenn *et al*., 1983; Gonda *et al*., 2013**). **Huang *et al*. (2014**) identified a functional L-methioninase in potato, and proposed a more universal role of methioninase in plant methionine catabolism.

5. Microbial L-methioninase fermentation

L-methioninase can be produced by various organisms by submerged fermentation (SMF) (**Abu-Tahon and Isaac, 2016; Khalaf and El-Sayed, 2009**). L-methioninase production by fungi under submerged fermentation (SMF) by *Aspergillus* sp. (**Ruiz-Herrera and Starkey, 1969b**), *Debaromycs hanseni* (**Bonnarme *et al*., 2001**), *A. flavipes* (**Khalaf and El-Sayed, 2009**) has been reported. SSF has also been reported for its production by *A. flavus* (**El-Sayed, 2009**). Physiologically L-methionine can be rapidly oxidized through Millard reactions forming Amador compounds that consequently reduce their bioavailability as carbon and nitrogen for the organism (**Delgado-Andrade *et al*., 2007**). Thus, using L-methionine as a substrate for enzyme induction could be, at least technically, not the superior substrate, in addition to the economic expense of these medium components (**El-Sayed, 2010**).

The contents of synthetic media used for L- methioninase fermentation are very expensive and might be replaced with more economically available agro-industrial wastes such as corn, tea waste, soya bean, palm oil, sesame oil and wheat bran (**Ikram-ul-Haq *et al*., 2003; Ramachandran *et al*., 2007; Queiroz *et al*., 2016**).

Consequently, the search for novel producers and new forms of the growth medium for large scale production for this enzyme had been a challenge for many researchers. SSF on agro-industrial residues promises a cost-effective bioprocess as it requires small vessels and gives a higher yield (**El-Sayed, 2009**). SSF for the enzyme production employing methionine-containing solid substrates displayed a more stable physical form of bounded methionine that could be a new strategy from the technical and economical points of views (**El-Sayed, 2009**). **Abu-Tahon and Isaac (2016**) reported that maximum L-methioninase yield was obtained when wheat bran, followed by rice bran and soya bean meal were screened as substrates for L-methioninase production under solid-state fermentation (SSF). Production of high levels of L-methioninase under optimized culture conditions using several agro-industrial residues by *Aspergillus flavipes* MTCC 6337 was also reported by **Swathi (2015**).

6. **Methionine and its metabolism**

Methionine, an essential amino acid, is required for normal development and cell growth. In all living cells, the fate of methionine was implicated with various pathways (**Suganya *et al*., 2017**).In eukaryotes, L-methionine can be metabolized via two main catabolic pathways; firstly, deamination pathway where it is oxidized by L-methioninase forming methanethiol, α-ketobutyrate, and ammonia. Methanethiol readily oxidized to dimethyl disulfide and dimethyl trisulfide (**Chin and Lindsay 1994; El-Sayed, 2010**), while α-ketobutyrate may convert to propionate via pyruvate dehydrogenase or to hydroxybutyrate by reaction with pyruvate under catalysis of acetolactate synthase (Fig.7) (**Epelbaum *et al*., 1996; Kharayat** and **Singh, 2018**). Secondly, trans-sulfuration pathway, in which methionine is converted to S-adenosylmethionine (SAM) active form, principal methyl donor **(Bolander-Gouaille and Bottiglieri, 2007)** by methionine adenosyltransferases, methyladenosyltransferase I/III (MATI/III) and methyladenosyltransferase II (MATII) (fig. 8).

S-adenosylmethionine (SAM) is the key intermediate for the main three sulfur-metabolic pathways: trans-methylation, trans-sulfuration pathways and polyamine synthesis **(El-Sayed, 2010 & Cavuoto and Fenech, 2012 and Suganya *et al.*, 2017)**. Transmethylation reaction, catalyzed by different methyltransferases or glycine methyltransferase that transforms glycine to sarcosine (methyl-glycine), the final product of which is S-adenosylhomocysteine (SAH) (**Luka *et al.*, 2006 ; Cavuoto and Fenech, 2012; Suganya, 2017 and Pascale *et al.*, 2019**) during methylation of DNA and a large range of proteins and other molecules **(Zingg and Jones, 1997**). SAH is then hydrolyzed to homocysteine (Hcy) and adenosine in a reversible reaction by the action of S-adenosylhomocysteine hydrolase (E.C 3.3.1.1), Hcy is metabolized through two major pathways: methylation and trans-sulfuration **(Bolander-Gouaille and Bottiglieri, 2007)**. Under normal conditions, approximately 50% of Hcy is re-methylated to form methionine which, in most tissues, occurs via methioninesynthase (5-methyl tetrahydrofolate-homocysteine methyl transferase, MTR) which requires folate, cobalamine as coenzymes. Hcy may also be converted to methionine via betaine–homocysteine S-methyltransferase which is predominantly present in the liver, which convert betaine, a metabolite of choline, to dimethylglycine donating the methyl group to homocysteine-forming methionine (**Sunden *et al.*, 1997** ; **Martinez-Lopez *et al.*, 2008 ; El-Sayed, 2010 and Cavuoto and Fenech, 2012).**

In the liver, trans-sulfuration pathway is a very active mechanism in producing glutathione as the main antioxidant that reduces reactive oxygen species (ROS), thereby protecting cells from oxidative stress (**Anderson, 1998).** Homocysteine can react with serine by the action of cystathionine β-synthetase (E.C 4.2.1.22) forming cystathionine (**Hullo *et al.*, 2007**), which undergoes subsequent γ-lysis by the action of cystathionine γ-lyase (E.C4.4.1.1) releasing cysteine, α-ketobutyrate and ammonia or by β-lysis-forming homocysteine and pyruvate (**Irmler *et al.*, 2008, El-Sayed, 2010** and **Cavuoto and Fenech, 2012**). Subsequently, cysteine can react with glutamate by the action of γ-glutamylcysteine synthetase (E.C 6.3.2.2) forming γ-glutamylcysteine, which reacts with glycine by the action of glutathione synthetase (E.C 6.3.2.3) releasing glutathione (**Cellarier *et al.*, 2003** and **Pascale *et al.*, 2019) (**Fig.8).

In polyamine synthesis, SAM and ornithine decarboxylated by specific decarboxylases produce decarboxylated SAM (dSAM or propylamine moiety) and putrescine, respectively then dSAM was accepted by putrescine forming spermidine by the action of spermidine synthase, which subsequently react with S-adenosylmethionine forming spermine via spermine synthase (**Thomas and Thomas 2001; Martinez-Lopez *et al.*, 2008 ; El-Sayed, 2010 ; Cavuoto and Fenech, 2012 ; & Suganya, 2017 and Pascale, *et al.*; 2019**).

A by-product of polyamine synthesis is methyl thioadenosine (MTA). MTA is catabolized by methyl thioadenosine phosphorylase (MTAP) as the first in a series of steps in the salvage of methionine **(Pirkov *et al*; 2008 and Sekowska, *et al.*, 2018)**. The immediate precursor to methionine in the salvage pathway is methylthiooxobutyrate (MTOB) which can be converted to methional, a potent inducer of apoptosis (**Quash *et al.*, 1995**)(Fig. 8).

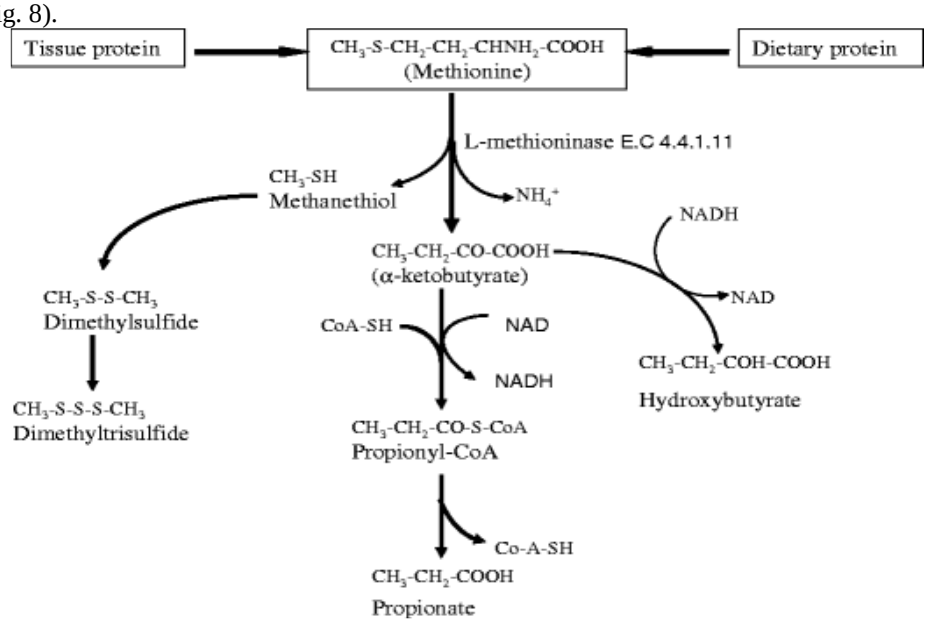

Fig 7: Deamination pathway of L- methionine by L-methioninase

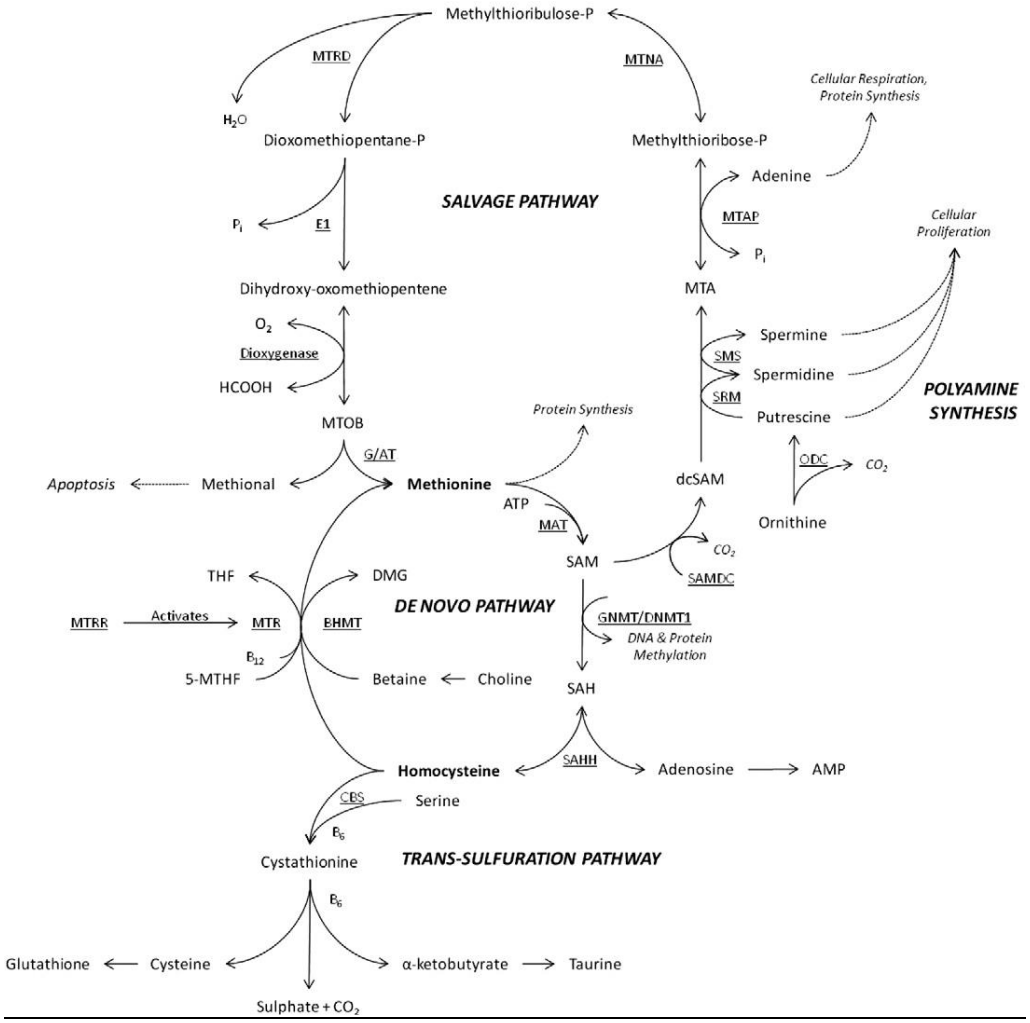

Fig.8. Methionine cycle and trans-sulfuration pathway. Enzymes are underlined. 5-MTHF, 5-methyltetrahydrofolate; B12, vitamin B12; B6, vitamin B6; BHMT, betaine– homocysteine S-methyltransferase; CBS, cystathionine b-synthase; dcSAM, decarboxylated SAM; DMG, dimethylglycine; E1, enolase-phosphatase 1; G/AT, glutamine or asparagine transaminase; GNMT/DNMT1, glycine N-methyltransferase or DNA methyltransferase 1; MTA, methylthioadenosine; MAT, methionine adenosine transferase; MTAP, methylthioadenosine phosphorylase; MTOB, methylthiooxobutyrate; MTR, methionine synthase; MTRR, methionine synthase reductase; MTRD, methylthioribulose dehydratase; MTNA, methylthioribose isomerase; ODC, ornithine decarboxylase; SAH, S-adenosylhomocysteine; SAHH, SAH hydroxylase; SAM, S-adenosylmethionine; SAMDC, SAM decarboxylase; SMS, spermine synthase; SRM, spermidine synthase and THF, tetrahydrofolate.

The folate cycle may also provide methyl groups (**Pascale *et al*; 1982 and Pascale *et al*; 2019**) transformation of tetrahydrofolate (THF) to 5,10-methylenetetrahydrofolate (MeTHF) catalyzed by methyltetrahydrofolate reductase, which is coupled with the resynthesis of glycinefrom sarcosine. This is followed by the synthesis of 5 methyltetrahydrofolate (MTHF) catalyzed by 5,10-methylene tetrahydrofolate reductase. MTHF is converted to THF by methionine synthetase, and the recovered methyl group is used to convert homocysteine to methionine (Fig 9). MeTHF can also be transformed to dihydrofolate (DHF) in a reaction catalyzed by thymidylate synthetase. Through this reaction, the folate cycle impacts deoxythymidyl triphosphate (dTTP) synthesis, while THF, after transformation to 10-formyl-THF by a specific synthetase, may be involved in purine synthesis (**Wagner *et al*; 1985 & James *et al*; 1993& Reed *et al*; 2015 and Sekowska *et al*; 2018).**

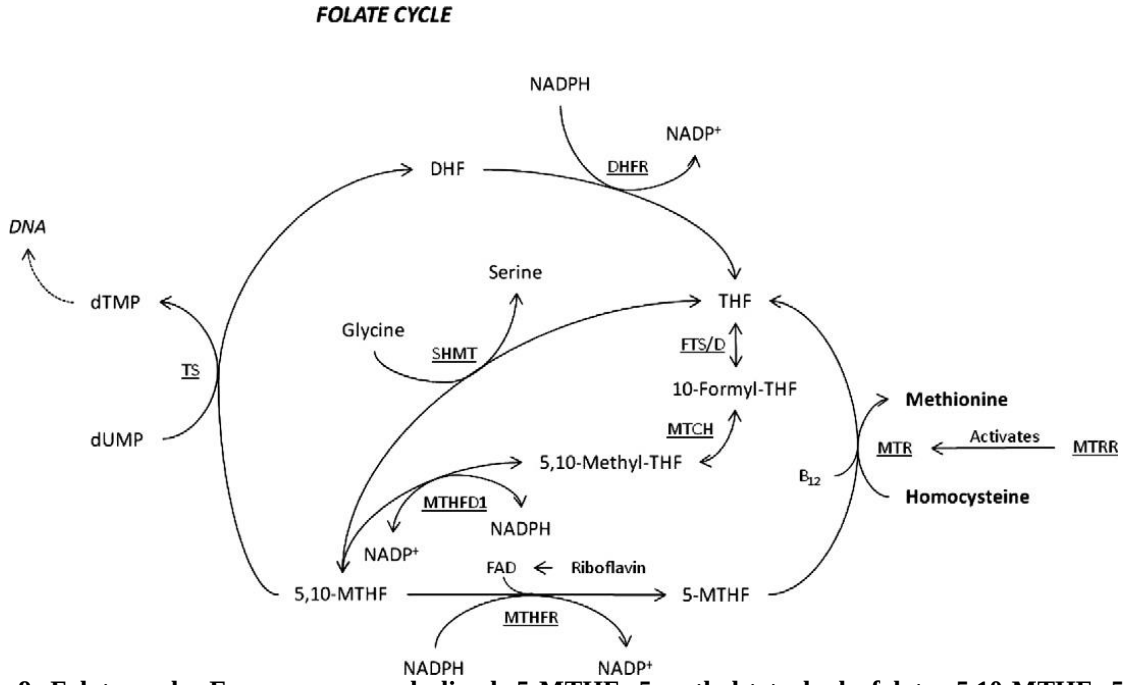

Fig. 9. Folate cycle. Enzymes are underlined. 5-MTHF, 5-methyl-tetrahydrofolate; 5,10-MTHF, 5,10-methenyl-tetrahydrofolate; B12, vitamin B12; DHF, dihydrofolate; DHFR, dihydrofolate reductase, dTMP, deoxy-thymidine-monophosphate; dUMP, deoxy-uracil-monophosphate; FAD, flavin adenine dinucleotide; FTS/D, 10-formyl-tetrahydrofolate synthase or 10-formyl-tetrahyrofolate dehydrogenase; MTCH, 5,10-methenyl-tetrahydrofolate cyclohydrolase; MTHFD1, 5,10-methenylenetetrahydrofolate dehydrogenase; MTHFR, 5,10-methylene-tetrahydrofolate reductase; MTR, methionine synthase; MTRR, 5-methyl-tetrahydrofolate-homocysteine methyl-transferase reductase; SHMT, serine-hydroxy-methyl transferase; THF, tetrahydrofolate and TS, thymidine synthase (Pascale *et al*; 1982 and Pascale *et al*; 2019).

7. Different Assay Method for Estimation of Activity of L-Methioninase

One international unit of L-methioninase has been defined as the amount of enzyme required to liberate 1micromolar of methanethiol product under standard condition. Methanethiol is an organosulfur compound which is spectrophotometrically estimated by using Ellman's reagent (DTNB) (5, 5′-dithio-bis(2-nitrobenzoic acid) (**Riddles *et al*., 1979**). TNB (2-nitro-5-thiobenzoic acid) as colored species was released after reaction of DTNB with a sulfhydryl group of the methanethiol which was spectrophotometrically measured in visible range at 420 nm (fig. 10).

Other product of α-ketobutyrate was measured spectrophotometrically with oxidation followed by coupling reaction using MBTH (3-Methyl-2-Benzothiazoline Hydrazone) (**Frenzel *et* al., 1992 and Soda, 1967**). The released product of α-Keto acids after α, ɣ-elimination of methioninase was estimated as 2,4 dinitrophenylhydrazone derivatives by thin-layer chromatography (TLC) with 1-butanol saturated with 3% ammonia, 1-butanol: water: ethanol (5:1:1, v/v/v) as solvent systems using silica gel plates. The Rf values of phenylhydrazone derivatives of α-keto acids produced from L-methionine were in the range (Rf = 0.62–0.69) (**Takakura *et al*., 2004 and Johnston *et al*., 1981**).

The deamination rate was calculated with the help of nessler's reagent by forming a precipitate of mercury (II) amido-iodide with end product ammonia (**Krug *et al*., 1979**). The color of the precipitate varies from yellow to brown, depending on the quantity of ammonium ion, which could be measured at 500 nm (**Sun, *et al*., 2005 and Kominami *et al*., 2002**).

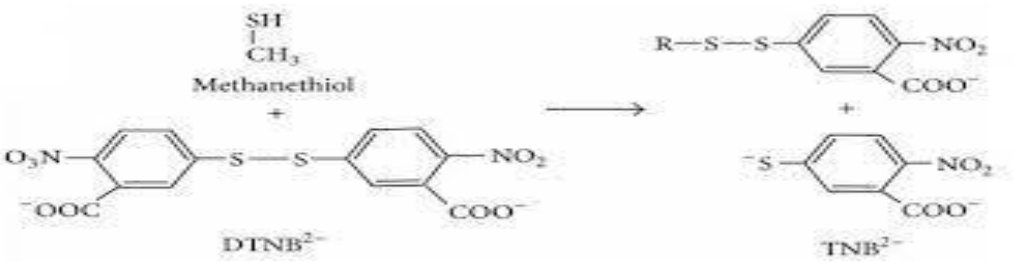

Fig. (10): Methioninase quantitative detection by DTNB

8. **Culture conditions and biochemical Characterization of L-methioninase**

Different methods of L-methioninase for the purification and production from various organism have been reported which include submerged fermentation (SmF) and solid state fermentation (SSF) (**Bonnarme *et al.*, 2000; Amarita *et al.*, 2004; Martinez-Cuesta *et al.*, 2006 and Khalaf and El-Sayed, 2009a, 2009b).** L-methioninase is used as an anticancer drug, which requires high level of purity. Some L-methioninase enzyme has reported intracellular in nature, which is difficult to handle as compared to extracellular enzymes. L-methioninase production from microbial sources is mostly methionine dependent. L-methionine present in aqueous medium and can be immediately oxidized via Maillard reaction in the presence of reducing sugars and ions and forming amadori compounds (**Delgado-Andrade *et al.*, 2007; El-Sayed, 2010 and Bhawana and Priyanka, 2018)** and that subsequently reduce their bioavailability as carbon and nitrogen for the organism.

Many microorganisms, including fungi, degrade methionine but do not grow on it, possibly because of their inability to metabolize the deaminated (α -ketomethionine) and demethiolated (α –ketobutyric acid and methanthiol) residues of L-methionine. The inability of filamentous fungi to grow on L-methionine may be partly overcome by the usage of a codissimilator such as glucose and other carbohydrates. Remarkably, with the use of carbon-free medium, the three parameters of enzyme yield, methionine uptake, and biomass by fungus were considerably reduced by about 97.4%, 68.61%, and 85%, respectively, compared with glucose containing medium **(Suganya, 2017)**. The highest yield of L-methioninase and amino acid uptake can be attained by using 1% glucose as a codissimilator and it plays an important role in the activation of plasma membrane H+ adenosine triphosphatase (ATPase) (**El-Sayed, 2011**) (i.e., cyclic adenosine monophosphate [cAMP] signaling and protein phosphorylation). The induction of L-methioninase is nitrogen regulated and L-methionine dependent (inducible enzyme) (**Ruiz-Herrera and Starkey, 1969**).

Alshehri (2020) introduced a bacterial strain of *H. alvei* harboring methionase gene, mdeA (1194 bp), that is previously unreported to secrete L-methioninase enzyme and showed that a carbon source is a mandatory supplement whereas L-methionine is not a mandatory supplement for L-methioninase enzyme production of *H. alvei.* The highest yield of L-methioninase enzyme was reached after 48 h of incubation when the acidity of the growing medium was adjusted to pH 7.5 and the temperature was 35˚**C** in nutrient medium containing: galactose (2.0 g L^{-1}), MgSO4 (0.25 g L^{-1}), L-methionine as an inducer (2.0 g L^{-1}), and L-asparagine as an additional N source (1.5 g L^{-1}). **Mohkam *et al.* (2020),** reported production of L-methioninase from bacterium *Alcaligenes* spp. via submerged fermentation. The Alcaligenes sp. MT-B under optimized conditions demonstrated the highest activity at 35˚C and had a pH optima of 6.0 with yeast extract 0.2%; lactose 0.75%. **Bopaiah *et al.* (2020)** found that highest activity among MGL was obtained in tris-HCl buffer of pH 8.0–9.0, yielding 1.42 µmoles/minutes/ml. **Abu-Tahon and Isaac (2016)** approved that the productivity of MGL from *Aspergillus ustus* increased by increasing the alkalinity of the medium to reach maximum yield at pH 8.5.

L-methionine dependency of enzyme production was reported from the microorganisms such as *Aspergillus flavipes, Achromobacter starkeyi, Pseudomonas ovalis, and Yarrwia lipolytica* (**Bondar *et al.*, 2005**). 0.8% L-methionine was found to be the optimal concentration for higher methioninase production and concentration more than 3.2% suppresses the enzyme production. In contrast, L-methioninase biosynthesis by *Pseudomonas putida*, and *Geotrichum candidum* were found to be L-methionine independent (**Bonnarme *et al.*, 2001**). Addition of phosphorus in the medium shows the significant effect on L-methioninase productivity and methionine uptake by certain fungi. 0.32% KH_2PO_4 as the sole phosphorus source with 0.24% K_2HPO_4 up rises the enzyme production. The supreme fungal productivity for L-methioninase at a neutral pH associated to the balance of the ionic strength of plasma membrane, the maximum activity of H^+ pumping ATPase, and the optimum fluxing of ions that affect the activities of calmodulin and adenylate cyclase.

Salim *et al.* (2020) purified l-methioninase from *Trichoderma harzianum* (7.15-fold) with a recovery of 47.9% and the specific activity of 74.4 U/mg of protein. The purified enzyme has an apparent molecular mass of

48 kDa on SDS-PAGE and exhibited maximum activity at pH 8 and 35 °C. The enzyme was catalytically stable below 50 °C and at a pH range of 6.0–8.5. The thermal inactivation of l-methioninase exhibited first-order kinetics with the *k* value between 5.71 × 10–4 min−1 and 1.83 × 10–2 min−1.The studies on thermodynamic parameters of l-methioninase indicated the compaction and aggregation of the enzyme molecule during denaturation. This is the first report of thermodynamic analysis of thermal inactivation in l-methioninase. The purified enzyme showed *Km*, *V*max and *k*cat value of 1.19 mM, 21.27 U/mg/min and 16.11 s−1, respectively. The l-methioninase inhibited the growth of human cell lines hepatocellular carcinoma (Hep-G2) and breast carcinoma (MCF-7) with IC50 values of 14.12 µg/ml and 20.07 µg/ml, respectively. The in vivo antitumor activity of l-methioninase was evaluated against DAL cell lines bearing in Swiss albino mice.

El-Sayed (2011) purified L-Methioninase to electrophoretic homogeneity from cultures of *Aspergillus flavipes* using anion-exchange and gel filtration chromatography by 12.1 fold compared to the crude enzyme preparation. The purified enzyme had a molecular mass of 47 kDa under denaturing conditions and an isoelectric point of 5.8 with no structural glycosyl residues. The enzyme had optimum activity at pH 7.8 and pH stability from 6.8-8.0 at 35°C. The enzyme appeared to be catalytically stable below 40°C. L-methioninase has a higher catalytic affinity towards L-methionine (Km, 6.5 mM and Kcat, 14.1 S(-1)) followed by a relative demethiolating activity to L-homo-cysteine (Km, 12 mM and Kcat, 9.3 S(-1)).

The biochemical and catalytic properties of L-methioninase from various microbes have been summarized in (Table1). As it was observed, the physical properties for L-methioninase from different microbes are relatively similar. For example, pH optima ranged from 7.0 to 8.0, pH stability from 6.0 to 9.0, and optimum temperature around 30°C that seems identical in all enzymes, independent to the source.

The tetrameric identity of microbial L-methioninase was reported as a conformational structure by several reports. **Nakayama *et al.* (1984)** reported that L-methioninase from *Aeromon*as sp. is constructed of four identical subunits, each one of 41.0 kDa, which appeared as single band on the denaturizing PAGE. Consistently, L-methioninase was detected as one fraction of 159 kDa by the low-angle light-scattering method (**Takagai (1981**) and 149 kDa using TSK G3000SW gel permeation/HPLC (**Kato *et al.*, 1980**). **Inou *et al.* (1995)** reported that the polypeptide of L-methioninase cloned from *Pseudomonas putida* is composed of 398 amino acid residues with a calculated molecular weight of 42,626 Da, corresponding to the subunit of the homotetrameric enzyme. However, in slight variations, the enzyme open reading frame from *Brevibacterium linens* was found to be1,275 bp, encoding 425 amino acid with a calculated molecular weight 44.66 kDa per subunit, which coincide with that observed by SDS-PAGE (**Dias and Weimer 1998; Amarita *et al.*, 2004)**. Also, the molecular weight was found to range from 43.0 to 45.0 kDa per subunit for *Pseudomonas ovalis* (**Tanaka *et al.*, 1976)**, *Clostridium sporogenes* (**Kreis and Hession 1973a, b**), *Trichomonas vaginalis* (**Lockwood and Coombs 1991**), *Citrobacter freundii* (**Manukhov *et al.*, 2005**).

Regardless of the specific activity, the Km value for the *Brevibacteria* sp. L-methioninase is approx. 6-fold higher than that reported for *P. putida* and *Citrobacter intermedius* and 8.7-fold for *C. freundii*, revealing the highest specificity of the latter to L-methionine compared to others. Kinetically, the maximum catalytic efficiency was observed for *P. putida* L-methioninase for L-methionine as substrate (**Takakura *et al.*, 2004**). Nevertheless, the strict conformational specificity to the L-methionine was neglected for L-methioninase of different source, as observed from the various catalytic affinities toward various substrate analogues including Lethionine, L-homocysteine, and cysteine. Interestingly, the specificity of fungal **(Ruiz-Herrera and Starkey, 1969a, b)** L-methioninase was higher than that reported for bacterial enzymes as clearly observed from the values of Km for L-methionine. For example, Km value of the *Aspergillus* RS-1a L-methioninase was lower than that of *P. putida* by 1,000-fold, revealing the conformational variations of the enzyme catalytic centers from the two organisms. However, **Ferchichi *et al.* (1985**) reported that the Km value of *B. linens*, L-methioninase is a growth phase dependent, and it ranged from 14 to 46 mM. Unlike bacterial L-methioninases, *C. sporogenes* appear to have very low specificity to L-methionine as substrate (**Kreis and Hession, 1973a, b**) with a significant higher activity toward DLhomocysteine and L-ethionine and inability to attack D-methionine, suggesting their very low efficiency as antitumor agent. Interestingly, L-methioninase from *Aspergillus* sp. (**Ruiz-Herrera and Starkey 1969a, b)** displayed a higher affinity to L-methionine with the lowest activity against DL-homocysteine compared to the enzyme from the different organisms, ensuring their higher therapeutic efficiency. However, all the purified bacterial L-methioninases have higher affinity toward L-ethionine and L-cysteine compared to L-methionine (**Kreis and Hession 1973a; Lockwood and Coombs 1991; Inoue *et al.*, 1995; Manukhov *et al.*, 2005**). **El-Sayed, (2010)** concluded that the enzyme, independent to the source, has the ability to attack C–S, C–O, and C–F with no ability to hydrolyze C–C bond.

Table (2.1): Purification methods and properties of L-methioninase from various organisms.

Microbes	Mol. wt. (kDa/ subunit)	optimum pH	Optimum temp(˚C)	Km (mM)	Catalytic Efficiency Kcat/s	References
Pseudomonas putida	42.6	8.0	37	0.8		**Lishko *et al.* , 1993**
				1.7	48.6	**Takakura *et al.*, 2004**
				0.2	33.4	
				30	1.4	
				0.92	25.3	**Nakayama *et al.*, 1988a, b**
				0.53	2.27	
				1.8	44.8	
				0.8	-	**Manukhov *et al.*, (2006)**
Aeromonas sp.	41.0	8.0	25-30	-	-	**Nakayama *et al.*, 1984**
Pseudomonas ovalis	45.0	7.2	37	1.33	-	**Tanaka *et al.*, 1976**
Citrobacter freundii	43.0	8.0	30	0.7	6.5	**Manukhov *et al.*, 2005**
Citrobacter intermedius	-	-	30	1.13	-	**Faleev, *et al.*, 1996**
Lactococcus lactis	55	-	-	.87	-	**Martinez-Cuesta *et al.*, 2006**
Brevibacterium linens BL2	43.0	7.5	25	6.12	-	**Dias and Weimer, 1998**
Brevibacteium linens	43	6.8-8	25	6.12	-	**Suwabe, *et al.*, 2011**
Clostridium sporogenes	37.5	7.5-8.5	-	90	-	**Kreis and Hession, 1973a**
Methylobacterium aminovorans	-		37			**Kavya, and Nadumane, 2020**
Hafnia alvei	-	7.5	35	-	-	**Alshehri, 2020**
Bacillus haynesii JUB2	-	7-8	37			**Bopaiah, *et al.*, 2020**
Aspergillus Rs-1a	-	8.0	35	0.0013	-	**Ruiz-Herrera and Starkey, 1969b**
Aspergillus flavipes	-	8.0	30	-	-	**El-Sayed, 2009**
Aspergillus flavipes	47	7.8	35	6.5	14.1	**El-Sayed, 2009**
Cladosporium cladosporioides	-	9.0	30	11.6	-	**Abu-Seidah and Youssef, 2000**
Trichomonas vaginalis	44.0	7.0-8.0	37	4.3	-	**Lockwood and Coombs 1991**
Trichoderma harzianum	48	8	35	1.19	21.27	**Salim *etal.*, 2020**
Entamoeba histolytica	43.0	7.0	37	0.61	1.82	**Sato *et al.*. 2008**
Treponema denticola	43.5	-	-	0.55	-	**Fukamachi *et al.*, 2005**

Aspergillus flavipes	47	7	37	6.5	39.6	**El-Sayed, 2011**
Candida trophicolis	46	4-6	45-55	0.5	-	**Dias and Weimer, 1998a, b**
Citrobactor freundii	43.0	-	-	0.7	6.5	**Ruiz-Herrera and Starkey, 1969**
Fusobacterium nucleatum ATCC 25586	43.0	-	25-	0.32	-	**Nakayama *et al.*, (1984)**
Idiomarina	-	7.4	30	-	-	**Song *et al.*, 2015**
Streptomyces sp	47	-	-	0.7	441	**Huang *et al.*, 2014**
Streptomyces variabilis 3MA2016	-	7-8	30-40	0.43		**El-Awady *et al.*, 2017**
Streptomyces diastaticus	-	8	30	-	-	**Kavya and Nadumane, 2020**
Arabidopsis thaliana	48	8	30	72	9.5	**Goyer *et al.*, 2007**
Cucumis melo (melon)	48.7	8	30	-	-	**Gonda *et al.*, 2013**

9. Inhibitors of L-methioninases:

Inhibitors of L-methioninase can be used as a reliable indicator for the catalytic and structural identity of the enzyme corresponding to their source. L-methioninases produced by *T. vaginalis* (**Lockwood and Coombs, 1991**) and *B. linens* (**Dias and Weimer, 1998**) were found to be completely inhibited by the carbonyl reagents as L-cycloserine, hydroxylamine, DL-penicillamine, and DL-propargylglycine, which are strongly irreversible inhibitors to the pyridoxal 5-phosphate, ensuring the pyridoxal dependence of this enzyme. Also, the enzyme was significantly inhibited by thiol reagents as iodoacetamide, p-chloromercuri-benzoic acid, and 5-5′-dithiobis-(2-nitrobenzoic acid), which react with their sulfur amino acids (**Lockwood and Coombs, 1991**).

Similarly, the activity of *Lactococcus lactis* enzyme was found to be completely inhibited by the carbonyl reagents as hydroxylamine and 3- methyl-2-benzothiazolinone hydrazone (**Martinez-Cuesta *et al.*, 2006**) but not affected by EDTA, suggesting the absence of metals as cofactors for this enzyme.

Otherwise, **Ruiz- Herrera and Starkey (1970)** reported reduction in demethiolation, but not deamination, of L-methionine by iodoacetate, p-chloromercuri-benzoic acid (80%), sodium arsenate (69%), and semicarbazide (31%) by *Achromobacter starkeyi* enzyme as detected from the high accumulation of α-ketomethionine during the bacterial growth, assuming the sulfur amino acids' identity of the demethiolating catalytic sites. The sequential analysis of L-methionine reported by **Ruiz-Herrera and Starkey (1970**) proposed that, L-methionine firstly undergoes oxidative deamination forming α-keto methionine, which subsequently demethiolate to methanethiol and α-ketobutyrate. This assumption was in coincidence to those suggested by **Canellakis and Tarver (1953)** and **Segal and Starkey (1969)**.

In contrast, the activity of L-methioninase seems to be not affected by the pyridoxal 5-phosphate coenzyme inhibitors as hydroxylamine, sodium cyanide, and sodium azide as evidenced from the analysis of L-methionine byproducts of *Lipomycs starkeyi* L-methioninase (**Ruiz-Herrera and Starkey, 1970).** Furthermore, the activity of *Aspergillus flavipes* (**El-Sayed, 2009**) and *L. lactis* L-methioninase (**Martinez-Cuesta *et al.*, 2006**) was significantly reduced by sulfhydryl reagents including iodoacetic acid, 2-mercaptoethanol, and DMSO.

In addition, **El-Sayed (2009)** observed the significant inhibition of *A. flavipes* L-methioninase activity by Cd^{2+}, EDTA, Li^{2+}, sodium azide, and Na^{+}, with slightly stimulatory effect of Co^{2+}, Mn^{2+}, Cu^{2+}, Mg^{2+}, and Ni^{2+} on the enzyme activity. These results are in consistent with that reported for fungal L-methioninase by **Ruiz-Herrera and Starkey, (1969a, b) and Abu- Seidah and Youssef (2000**). The stimulatory effect of metal ions as Ca, Mg, Mn, and Cu to the enzyme activity may be ascribed to their stabilizing effect to the conformational structure, protection of the enzyme from autoproteolysis, and thermal denaturation (**Secades and Guijarro, 2001**).

On the other hand, the irreversible inactivation of L-methioninase by the suicide inhibitors as aminoethyloxyvinylglycine and propargylglycine was extensively exploited for bacterial (**Nakayama *et al.*, 1988a, b; Kudou *et al.*, 2007**) and protozoal enzymes (**Tokoro *et al.*, 2003; Sato *et al.*, 2008**) via reaction of its

hydroxyl groups forming two hydrogen bonds with the guanidinium group of Arg373 moiety of the enzyme. Consequently, arginine residue is assumed to be greatly involved in the binding with the α-carboxyl group of L-methionine. Kinetically, the inactivation of L-methioninase elaborated from the binding of DL-propargylglycine with one α and one β subunit of the tetramers (**Johnston *et al*., 1979; Nakayama *et al*., 1988a, b**).

The competitive inhibitors of L-methioninase by various substrate analogues were investigated by **Lockwood and Coombs (1991)**, and the denantiomers of methionine, cysteine, and L-forms of threonine and serine have apparently no effect on the enzyme activity, with a marked inhibition by DL-homocysteic acid. The enzyme activity was enhanced by Li^{+} and inhibited by Cu^{2+}, Co^{2+}, Fe^{2+}, Hydroxylamine and PMSF **(Salim *et al*., 2020)**. **El-Sayed, (2011)** reported that *A flavipes* L-Methioninase activity was strongly inhibited by DL-propargylglycine, hydroxylamine, PMSF, 2-mercaptoethanol, $Hg^{(+)}$, $Cu^{(2+)}$, and $Fe^{(2+)}$, with slight inhibition by Triton X-(100).

10. Biotechnological Applications of Microbial l- methioninase

10.1. Role of L-methioninase as an antimicrobial drug

The limited distribution of L-methioninase as intracellular enzyme among all microbial pathogens, but not in humans, makes this enzyme a promising drug target for antibacterial, antifungal, and antiprotozoal therapies (**Sato *et al*., 2008**). Trifluoromethionine (Strifluoromethyl- L-homocysteine) is a halogenated methionine analogue, which shows antimicrobial activity against some anaerobic bacteria, *Mycobacterium smegmatis*, *M. phlei*, *Candida lipolytica*, *P. gingivalis*, *F. nucleatum* and parasitic protozoa such as *E. histolytica* and *Trichomonas vaginalis* (**Ferchichi *et al*., 1985; Dias and Weimer, 1998a, 1998b**).

Antimicrobial action of L-methioninase inside the parasitic protozoa was represented in (Fig.11). Inside the cell, trifluoromethionine is catabolized by methioninase and releases trifluoromethanethiol which is unstable under physiological conditions and non-enzymatically converted to carbonothionic difluoride, an effective cross-linker of primary amine groups, by which trifluoromethionine exerts its toxic effects. In addition, trifluoromethionine and its derivatives are found to be effective against 5′- nitroimidazole-resistant *E. histolytica*, *T. vaginalis*, periodontal bacteria and *Citrobacter freundii*.

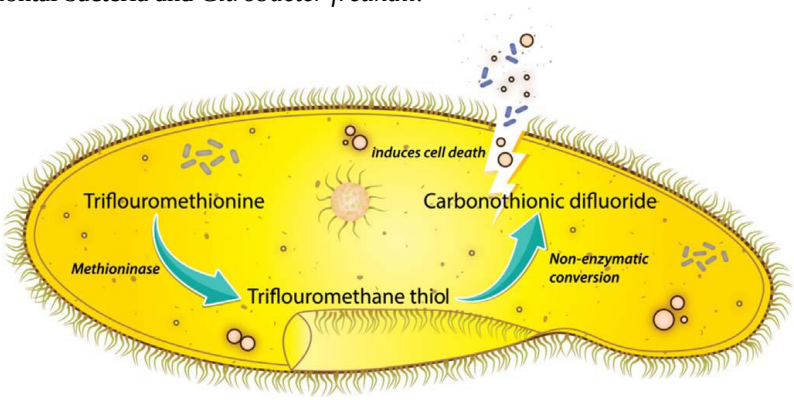

Fig. (11): Antimicrobial action of L-methioninase inside the parasitic protozoa

Methanethiol trapped by mercuric acetate precipitated as mercuric methanethiolate displays a significant antifungal activity against *Aspergillus niger* (**Yang *et al*., 2004a, 2004b,2004c**).

Absence of MGL in mammals makes the enzyme a potential target for antimicrobial therapy. Suppression of growth of *T. vaginalis* (**Coombs & Mottram, 2001**), *P. gingivalis* (**Yoshimura *et al*., 2002**) and *E. histolytica* (**Sato *et al*., 2010**) cells by a suicide substrate of MGL, l-trifluoromethionine, has been demonstrated in vitro and in vivo. Also, **Anufrieva *et al*. (2015),** revealed that, the complex of MGL with another suicide substrate, S-allyll-cysteine sulfoxide, revealed antimicrobial activity against *Staphylococcus aureus* and *Citrobacter freundii*.

10.2. Role of L-Methioninase in Cancer therapy:

Cancer is an increasing cause of mortality and morbidity throughout the world. L-methionase is 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 methionine dependent and eventually follow apoptosis due to methionine limitation in cancer cells. L-Methionine also plays an indispensable role in gene activation and inactivation due to hypermethylation and/or hypomethylation. Membrane transporters such as GLUT1 and ion channels like Na^{2+} , Ca^{2+}, K^{+}, and Cl^{-} become overexpressed. Further, the α-subunit of ATP synthase plays a role in cancer cells growth and development by providing them enhanced nutritional requirements. Currently, selenomethionine is also used as a prodrug in cancer therapy along with enzyme methionase that converts prodrug into active toxic chemical(s) that causes death of cancerous cells/tissue. More recently, fusion protein (FP) consisting of L-methionase linked to annexin-V has been used in cancer therapy. The

fusion proteins have advantage that they have specificity only for cancer cells and do not harm the normal cells **(Sharma *et al*., 2014).**

L-Methioninase had been reported as a potent anticancer agent against various types of tumor cell lines: breast, lung, colon, kidney, and glioblastoma. Methioninase (METase), a powerful enzyme drug causing methionine depletion, has been widely used as a therapeutic strategy for gastric carcinoma in clinic (**Xin *et al*., 2013**). Moreover, methionine starvation therapy using a methioninase-free diet or total parenteral nutrition (TPN) prolongs the survival time of high-stage gastric carcinoma patients (**Song and Wen-Ping, 2004**).

Mahsen *et al*. (2015) purified L-methioninase from *Candida tropicalis* and studied chemical and physical properties of the pure enzyme. Moreover, the anti-tumor activity of the purified enzyme against different cancer cell lines was evaluated. Previously, MGL was tested as a potent anti-proliferative enzyme towards Lewis lung and human colon carcinoma (**Tan *et al.*, 1998**) glioblastoma (**Kokkinakis *et al.*, 2001**) and neuroblastoma (**Hu *et al*., 2009**).

10.2.1. Methionine restriction and cancer

Cancer is the uncontrolled cellular growth causing change in expression of tumor suppressing genes and tumor promoting genes. Majority of cancers results from alterations in DNA integrity caused by environmental genotoxic factors and endogenous metabolism (**Stratton *et al.*, 2009**). Link between metabolic regulation and cancer progression is tightly regulated by availability of nutrients (**El-Sayed *et al*., 2014**). Metabolic analysis is therefore a promising approach to understand the nutritional dependency of cancer cells (**Cairns *et al*., 2011**). It has been known for almost a century that metabolism of cancer is unique because cancer cells have a much higher demand for and are often starved of nutrients and oxygen which are needed in abundance for rapid growth. Alanine is an important metabolic factor for hepatoma and brain tumors (**Vander Heiden *et al*., 2009**). Glycine acts as an essential precursor for denovo purine synthesis (**Fu *et al*., 2003**). Nitrogen for nucleotide and amino acid synthesis mainly depend on glutamine (**Griffin and Shockcor, 2004; Amelio *et al*., 2014**). Asparagine plays an important role in cellular proliferation **(Krall *et al*., 2016)**. Likewise L-Methionine plays an essential role in methylation of DNA, polyamine synthesis and mammalian protein synthesis (**Halpern *et al*., 1974**). Methionine also plays a central role in the metabolism of all macromolecules, control of gene expression, cytoprotection and membrane integrity. Thus Methionine dependence is the only known general metabolic defect in cancers (**Lu and Epner, 2000**). 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 MGL seems to be a promising therapeutic approach to treat cancer (**Tan *et al.* 2010; Cavuoto and Fenech 2012; El-Sayed *et al.*, 2012; Hoffman RM, 2015; Bopaiah *et al.*, 2020).** Therapeutically, the efficient enzyme should have a low Km value for methionine and actually has no activity toward homocysteine to approve their potentiality against tumor cells and no effect on the normal cells. The antitumor efficiency of L-methioninase was firstly reported for *C. sporogenes* enzyme against carcinoma cell culture of ascites and Walker carcinoma in rats (**Kreis and Hession, 1973a, b).** Following these studies, several studies focused on the purification and characterization of this enzyme from different microbes. Most of the researcher found that in vivo therapeutic effects of the enzyme L-methioninase usually have some minimal complications like vomiting, weight loss, mild anemia **(Yang *et al*., 2004a, 2004b**). Beside the potential antitumor efficacy of L-methioninase, it has quick plasma clearance, short biological half-life time and high immunogenicity (**Sun *et al.* 2003 and Vellard, 2003**).

Therefore, several strategies were assumed in an attempt to improve the pharmacological potentiality and therapeutical efficiency of this enzyme. Firstly, immobilization of L-methioninase on polyethylene glycol is one of the most effective trials for reducing the enzyme immunogenicity, reduction of hyper-ammoniemia, lengthening the plasma half-life time and resistant to the proteolytic cleavage in vivo (**Tan *et al*., 1998; Sun *et al.* 2004; Yang *et al*., 2004; Takakura *et al*., 2006**). **Sun *et al*.** (**2003**) observed the remarkable prolongation of the enzyme life time from 2 to 38 h by using 120:1 molar ratio of PEG/L-methioninase with a significant reduction in their immunogenicity. PEGylation significantly increases the serum half-life in mice from 2 h for the native protein to 12, 18, and 30 h for PEGylated forms having, respectively, 4, 6, and 8 PEG molecules attached to each subunit (**Kominami *et al*., 2002**).The poly(d,l-lactide-co-glycolide acid) (PLGA) coated polyethylene glycol possesses diverse advantages including high biocompatibility, biodegradability, and low toxicity (**Siegel and Steven, 2011**). Some antibodies against PEG-METase were produced upon frequent challenges in the primate model. Still, the level of such antibodies was 100–1000 fold less than those stimulated by the native enzyme and the antibodies were incapable of neutralizing the activity of the enzyme so that each challenging dose was effective in exhausting serum methionine levels (**El-Sayed *et al*., 2012**). Conjugation or encapsulation of therapeutic proteins with PEG or PEG modified NPs has significant advantages, especially in the aspect of reducing antigenicity (**Chang *et al.*, 2011)**. Moreover, block copolymers formed by PLGA combination with PEG have been proven to promote drug loading capacity, and have been widely used as drug carrier materials (**Zhao and Lin, 2008**).

El-Sayed *et al.* (2017) showed that immobilization of L-methioninase on dextran increase activity of AfMGL(Aspergillus flavipes Methionine –Lyase) towards the tested tumor cells(MCF-7, HEPG-2, HCT, PC3, HEP-2) suggesting the dramatic increasing of MGL hydrophilicity and catalytic efficiency**.** The thermal stability of dextran AfMGL was increased by two folds over the free enzyme. The dextran AfMGL had a higher resistance to proteinase K, retaining about 70 % of its initial activity comparing to 20 % to the native enzyme after 30 min of proteolysis at 37°C. The in vivo half-life time of dextran modified AfMGL in New Zealand rabbits was increased by 2.3 folds comparing to free enzyme. Dextran also used as a carrier for methotrexate (**Dang *et al.*, 1994; Varshosaz *et al.*, 2010**), mitomycin C (**Nomura *et al.*, 1998**), 5- fluorouracil (**Hao *et al.*, 2006**), cisplatin (**Nakashima *et al.*, 1999**), INF-β (**Yasukawa *et al.*, 2002**), and L-arginine deiminase (**El-Sayed *et al.*, 2015b**). Dextran had been also used as a carrier for anti-cancer drugs as Daunorubicin and Doxorubicin. Conjugates of these drugs with dextrans were found to reduce the toxicity in mice (**Mitra *et al.* 2001; Zhang *et al.* 2008).**

Secondly, in combinational therapy, MGL in combination with chemotherapeutic agents such as cisplatin, 5-fluorouracil (5-FU), 1-3-bis(2-chloroethyl)-1-nitrosourea (BCNU), and vincristine has shown efficacy and synergy, respectively, in mouse models of colon cancer, lung cancer, and brain cancer (**Yoshioka *et al.*, 1998; Hu *et al.*, 2009; Tan *et al.*, 1999**).

L-Methioninase acts as a biochemical modulator for 5-fluorouracil, through methionine starvation that accelerates the conversion of methyltetrahydrofolate to tetrahydrofolate, which catalyze the binding of 5-fluorouracil to thymidylate synthetase as a competitive inhibitor to thiamine (**Poirson-Bichat *et al.*, 1997**), causing a significant suppression on the DNA synthesis of tumor cells. Similarly, combination of L-methioninase with intercalants drugs, as doxorubicin, displayed a significant effect against human lung carcinoma H460, with no activity using the enzyme or doxorubicin, separately (**Gupta *et al.*, 2003**). It has been observed during methionine restriction that the percentage of cells in S and G2 phase increased, making the cells more sensitive to the DNA-affecting drugs as doxorubicin with a reversible blockage to the cell cycle during supplementation by methionine (**Cellarier *et al.*, 2003**). In contrast to normal tissue, most tumors cannot sustain as an active metabolic cycle to support their propagation without exogenous methionine. Supplementation of homocystein in the place of methionine or disintegration of methionine with methioninase in methionine dependent (MET- dp) tumor cells growing exponentially in culture, results in cell cycle arrest, strict inhibition of mitosis and eventual death (**Hu and Cheung, 2009**). Methionine deprivation affects tumor cells with the propensity to divide and causes them to arrest predominantly in the late S/G2 phase of the cell cycle and to eventually undergo apoptosis (**Ali and Nozaki, 2007**). Cells that arrest in late-S/G2 phase are susceptible to spontaneous death and are hypersensitive to chemotherapeutic agents. Reduction of plasma methionine to a level below 5 µmol/L arrests human xenograft growth in athymic mice. The pleiotropic effect of methionine stress on tumors resulted in alterations in the expression of a number of genes related to cell cycle and cell cycle check point controls, cytokines, interleukins and the TNF-α and TGF-β pathways (**Alston and Bright, 1983**). In nude mice, intraperitoneal injection of methioninase arrested the progression of Yoshida sarcoma and decelerated growth of H460 human non-small-cell lung carcinoma (**Vellard, 2003**). Therefore 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 (Fig. 12).

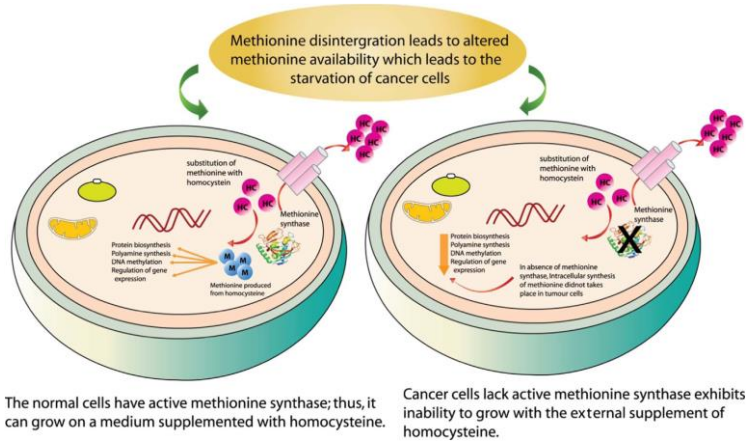

Fig. 12: Mechanism of action of L-methioninase in normal and cancer cells with the presence and absence of methionine synthase.

Thirdly, retroviral vectors gene therapy by transduction of *Pseudomonas putida* (PpMGL) methioninase gene into the cancer cells is done, one of the most developed strategies for treating many types of cancers recently (**Miki *et al.*, 2001; Gupta *et al.*, 2003 and Yamamoto *et al.*, 2003**). Compared to the different gene therapies for cancers, the combination between methioninase gene and active methioninase potentially suppressed the growth of human lung cells H460 (**Miki *et al.*, 2000a**). Recombinant PpMGL has been developed as effective anticancer drug to deplete intracellular and extracellular methionine in cancer cells levels, has been attempted (**Miki *et al.*, 2000b and Miki *et al.*, 2001**). **Miki *et al.* (2000b)** was developed a recombinant adenovirus having the methioninase gene, in addition to the exogenous L-methioninase,showed a strong antitumor activity against fibrosarcoma and human ovarian cancer. Some tumor cells may release antiapoptic mitochondrial protein bcl-2 that counteract the introduced metase gene (**Yamamoto *et al.*, 2003**).

The antibody-directed L-methioninase-selenomethionine therapy against various types of methionine-dependent tumors represents a novel approach in the evolution of antitumors (**Napier *et al.*, 2000 and Zhao *et al.*, 2006).** Selenomethionine (SeMET) acts as suicide prodrug substrate for L-methioninase and effectively inhibit the tumor growth in rodents and prolonged their survivals (**Miki *et al.*, 2000a ; Miki *et al.*, 2001**).

$$\text{Selenomethionine} + H_2O \xrightarrow{\text{L- Methioninase}} \alpha\text{-ketobutyrate} + \text{ammonia} + \text{methylselenol}$$

The nontoxic prodrug, selenomethionine, is converted to a powerful toxic methylselenol, as shown above, which readily diffused to the surrounding non-transduced tumor cells, destroying the mitochondrial membrane by the oxidative stress (Fig. 12) (**Li *et al.*, 1997**). Methylselenol catalyzes the oxidation of thiols, generating toxic reactive oxygen species as superoxides causing mitochondrial swelling, releasing cytochrome c, activating the caspase cascade, and inducing the cell apoptosis and death (**Green and Reed 1998**). Selenomethionine is relatively nontoxic to the mammalian cells due to their lack of L-methioninase (**Li *et al.*, 1997**). **Yamamoto *et al.* (2003)** observed the significant bystander effect of selenomethionine during the combination methioninase gene and methioninase treatment against antiapoptic-producing lung cancer cells. Also, the maximum antiprostate cancers activity was observed by selenomethionine–methioninase treatment (**Li *et al.*, 1997**). The sensitivity of tumor cells to selenomethionine was increased by 1,000-fold via transduction by adenovirus-mediated methioninase gene (**Miki *et al.*, 2000a**).

The methylselenol is required in very low concentration to induce cell cycle arrest and apoptosis (**Zeng *et al.*, 2009**). **Zu *et al.*, (2006)** revealed that Methylselenol promotes the expression of matrix metalloproteinase (MMP) and tissue inhibitor of metalloproteinase (TIMP) that inhibits the migration of tumor cells. Methylselenol induced apoptosis reported in many cancer cells such as murine melanoma B16F10 (**Kim *et al.*, 2007**) fibrosarcoma cells HT1080 (**Zeng *et al.*, 2009**; **Zu *et al.*, 2006; Zeng *et al.*, 2006)**, colon cancer derivedHCT-116, inhibits cell proliferation in the cancerous HCT116 cells as compared to normal cellsNCM460 (**Zeng *et al.*, 2012**), and human prostate cancer cells LNCaP (**Cho *et al.*, 2004**) decreased cellular prostate-specific antigen (PSA) level in LNCaP cells . The combination of methioninase gene and selenomethionine are effective against all methionine-dependent tumors (**Miki *et al.*, 2001; Yamamoto *et al.*, 2003; Sun *et al.*, 2004; Zeng *et al.* 2006**) **(Fig.13)**.

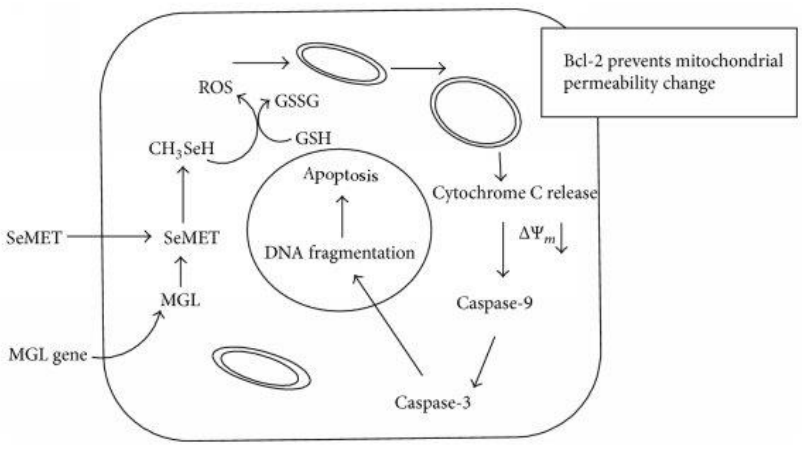

Fig. (13): A proposed mechanism of MGL/SeMET-induced apoptosis by combinational therapy method. MGL gene (PpMGL) along with selenomethionine as prodrug was inserted inside the tumor cells. MGL

gene produces *L*-methionase that catalyzes the breakdown of methionine from prodrug and synthesizes a toxic molecule "methylselenol" that produces reactive oxygen species (ROS). The ROS thereby causes mitochondrial apoptosis by caspase activation.

10.2.2. Use of Fusion Proteins in Cancer Cell Targeting.

The oxidative stress in tumor cells caused exposure of phosphatidyl serine on the surface of the vascular endothelium of blood vessels in tumors but not on normal cells (**Stafford and Thorpe, 2011**). The fusion protein (FP) consisting of *L*-methionase linked to human annexin-V injected into the bloodstream will bind to the marker on vascular endothelial cells of the tumor only. The FP catalyzed the conversion of nontoxic prodrug selenomethionine into toxic methylselenol and also prevented the methionine supplementation to the tumor cells, thereby killing the tumor and/or inhibiting its growth due to methionine restriction (**Harrison and Norman, 2011; Rite *et al*., 2013; Rite *et al*., 2011**). The great advantage of FP is that it does not require to be delivered directly to the tumor cells but only to the bloodstream. ATF methionase FP (amino-terminal fragment of urokinase) was used to inhibit cancer cell proliferation and migration, which supports targeting *L*-methionase to the surface of the cancer cells. The FP has potential as a selective therapeutic agent for the treatment of various methionine-dependent cancers (**Palwai *et al*., 2009**).

10.2.3. L-methioninase side effects

Many researchers reported that the in vivo therapeutic effect of L-methioninase usually was accompanied by many complications (**Yang *et al*., 2004a, 2004b, 2004c**). Despite its potential anticancer activity, administration of L-Methioninase leads to lethality to normal cells. Generally it has quick plasma clearance, short biological half-life time and high immunogenicity (**Vellard, 2003**). L - Methioninase administration produces broad range gastrointestinal reactions such as vomiting, decreased food consumption, mild anemia and weight loss. Autopsy examinations from the primates had shown that the anaphylaxis with ascites and scattered foci of necrosis in the liver. Also, plasma antibody during methioninase treatment period demonstrated that anti-METase antibodies were induced significantly and the detection of antibody subtypes specified that IgG was the dominant, these antibodies may quicken methioninase clearance and subsequently reduces its therapeutic efficacy. In some cases, the release of excessive ammonia by the action of methioninase leads to hyper-ammoniemia, which causes the detrimental effect to kidney and liver.

To overcome this problem, polyethylene glycol-conjugated MGL (PEG-MGL) was prepared. Simultaneous co-administration of pyridoxal 5′-phosphate and oleic acid or dithiothreitol treatment also strengthened effectiveness of PEG-MGL. To improve the MGL therapeutic potential, MGL was coupled to methoxy polyethylene glycol succinimidyl glutarate-5000 (MEGC-PEG-5000). The half-life due to pegylation increased 6 to 19 times while plasma methionine depletion efficacy decreased 8 to 48 times. Protective effect of high-level of pegylation helps to remove PLP dependence. PEG-rMGL demonstrated a significant decrease in antigenicity **(Sun *et al*., 2003).** The specific activity of PEG-MGL increased with DTT **(Takakura *et al*., 2006)**. Although *L*-methionase from bacterial (prokaryotic) origin has immunogenic issues that can be overcome by PEGylation and by other methods such as deimmunization by combinational T-cell epitope removal using neutral drift **(Cantor *et al.*, 2011).**

11. Other therapeutic applications of L-methioninase

Da Prada *et al*. (1995) showed that methioninase regulates obesity by dietary methionine deprivation in the rats by targeting melanocyt -stimulating hormone (MSH) emelanocortin- 4 receptor and its peptide ligand. Methioninase used to reduce circulating methionine such that catechol-O-methyl transferase is optimally inhibited. Lifelong reduction of methionine from 0.86% to 0.17% of the diet results in a 30% longer lifespan in male Fischer 344 rats (**Fan *et al*., 1997; Ruckenstuhl *et al*., 2014**). The r-Methioninase could manage serum methionine level to possibly increase lifespan (**Huang *et al*., 2015; Jia *et al*., 2016**). **Erickson *et al.* (1996)** revealed that L-methioninase lowered the level of homocysteine (HCY) in plasma of rat suffered from Parkinson's disease.

Allain *et al.* (1995) reported that methioninase produced by *Trichomonas vaginalis* has high substrate specificity and high catalytic efficiency towards HCY-induced cardiovascular disease.

S-adenosyl methionine and its precursor methionine play an important role in Parkinson's disease (**Miller *et al*., 2005**). S-adenosyl methionine is considered as rate limiting as a methyl donor by catechol- O-methyl transferase (COMT) in the O-methylation of levodopa, dopamine, 4-dihydroxy phenyl acetic acid and L-dopa decarboxylase, used in the clinical treatment of Parkinson's disease and dopamine-responsive dystonia (**Troen *et al*., 2007**). Interruption of o-methylation of levodopa and dopamine can efficiently increase its bioavailability and therapeutic potential. S-adenosylmethionine together with folate concurs with the metabolism of the one-carbon units (**Pascale *et al*., 2019; Zheng *et al*., 2019**).

Liver lesions induced by different xenobiotic compounds, including preneoplastic and neoplastic liver lesions, are associated with profound modifications of the methionine metabolism, whose pathogenic role has been well proved **(Cavuoto and Fenech, 2012**).

12. Role of L- methioninase in food industry

L-methioninase from Coryneform bacteria found on the surface of smear cheeses, and give the typical sulfur notes, has important role in food industry by imparting a specific aroma in variety of cheese like limburger, camembert, blue cheeses and cheddar tilsiter, livarot, epoisses, and munster (**Arfi *et al.*, 2003 and Rouseff *et al.*, 2008**). Methanethiol, produced from enzymatic catalysis of L-methionine in presence of L- methioninase from yeast strains, with acyl-coenzyme A (acyl-CoA) releases a wide variety of sulfur-bearing compounds such as dimethyldisulfide (DMDS), dimethyl trisulfide (DMTS), and S-methylthioesters (**Yvon *et al.*, 1997**). These volatile sulfur compounds (VSCs) are of vital importance in the overall flavor of cheese and make a significant influence to the characteristic aromas of various cheeses (**Selhub *et al.*, 1995**). Several cheese microorganisms are capable of liberating VSCs from L- methionine. Some of them are *Brevibacterium linens, Corynebacteria, Micrococcus luteus, Arthrobacter sp., G.candidum and Staphylococcusequorum* (**Weimer *et al.*, 1999**; **Bonnarme *et al.*, 2000a; Rébeillé *et al.*, 2006**). Some yeast strains *Kluyveromyces lactis, Saccharomyces cerevisiae) Kluyveromyces marxianus* and some bacterial strains (Lactobacilli, Lactococci) are considered as safe organisms and used commercially as food additive (**Selhub *et al.*, 1995).**

13. Role of L- methioninase in plants

Methioninase is expressed in many plant tissues as Arabidopsis thaliana with an atypical regulation of expression in seeds. Methioninase mRNA was highly accumulated but not translated in dry seeds, the protein was highly expressed in imbibed seeds. This distinct regulation of methioninase expression proposes that immediate production of methioninase is indispensable in the initial germination process. Methioninase plays an important role in the resumption process during methionine homeostasis in various tissues (**Orentreich *et al.*, 1993**).
Methionine degradation products such as methanethiol and 2-oxobutyrate are used for the synthesis of S-methyl-L-cysteine and isoleucine, respectively. Functional analysis of a methioninase defective mutant of *A. thaliana* showed that methioninase is involved in the alternative reverse-transsulfuration pathway, in which methionine is metabolized to cysteine, not via cystathionine (**Hoffman, 1997**). **Crowell *et al.* (1993)** detected the volatile sulfur compounds including methanethiol in the wounded leaves of guava as defense molecules against herbivorous insects. The productions of sulfur compounds with anti-insect activity might be the major physiological function of methioninase in plants.

Conclusion

Importance of microbial L-methioninase in different fields including pharmaceutical and food industries had been extensively studied during the last few decades. Exploring new potent strains capable of producing L-methioninase is an important goal of many scientists as the microbial production of L-methioninase is cost effective and easily produced. Biochemical and molecular aspects of microbial L-methioninase should be also investigated in order to improve its potential catalytic activity.

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