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Original article

Isolation and characterization of *Trichoderma harzianum* L-methioninase with promising a powerful anticancer

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ABSTRACT

Bioactive components derived from medicinal herbs have recently acquired popularity due to their efficacy in treating various ailments, including cancer and infectious diseases. In this study, the anticancer enzyme, L-methioninase isolated from medicinal plants endophytic fungi, *then* evaluated its promising therapeutic agents against different types of human cancers. L methionine was purified using column chromatography with the stationary phase of Sephadex G-200 with 6.6-fold purification, which increased the specific activity of 71.3 U/mg of protein with a recovery rate of 48.2 %. On the SDS-PAGE chromatogram, the apparent molecular mass of the isolated enzyme was 48 kDa, and its highest activity was observed at pH 8 and 35 °C. The enzyme was catalytically stable within the pH range of 6.0–9.0 and below 40 °C. This study demonstrates that isolated L-methioninase is particularly efficient against tumour cell lines in vitro. The crude and purified L-methioninase inhibited 60 and 80 % of the growth of the breast cancer cell line (MCF-7), respectively, with an estimated IC_{50} = 12.6 μg/ml (crude) and IC_{50} = 5.0 μg/ml for purified L-methioninase from isolate 8 with accession no MZ675362. Because of this, pure L-methioninase has better catalytic characteristics and significant thermal stability, which could be used as a cancer-fighting substance than the enzyme purified from other sources.

1. Introduction

Bioactive constituents developed from herbal medicines have recently gained popularity because they are beneficial in treating various conditions like cancer and microbial disorders (Treasure et al., 2020). A significant microbial community known as endophytes is a collection of extraordinarily diverse microorganisms, such as fungi and bacteria, that reside inside plant tissues but are typically asymptomatic; it is present inside the majority of these plants, according to previous studies, the link between these microbes and their hosts is quite complex (Du et al., 2020). Numerous fungal endophyte strains have been found to induce a variety of novel biologically active metabolites with significant antimicrobial, antiviral, anticancer, anti-inflammatory, and antioxidant features (Kubota et al., 2022). As a result, these organisms are regarded as valuable resources for creating pharmaceutical products (Talukdar et al., 2020).

Current anticancer medications are either low-weight compounds or monoclonal antibodies that block a critical phase in cancer growth, hindering its propagation (Lim et al., 2021). Cancer prevention and treatment are complex challenges. There is no guaranteed way to prevent cancer; existing treatments can have harmful side effects (Tangri et al., 2001). This article highlights current efforts to create L-Methioninase, an enzyme utilized by some fungi, such as *Trichoderma harzianum*, as a natural defense mechanism that helps the body fight off infections, such as breast cancer (Yamamoto et al., 2022).

As more manufacturers focus on the benefits of enzyme therapies in treating disease, the use of these treatments is growing in medicine. Compared to other drug types, enzymes stand out for two key reasons (Wiesendanger & Nisman, 1953). First, enzymes frequently bind and work with high specificity and affinity on their intended targets. Second, they are catalytic so enzymes can change a range of target molecules into the required end products. Enzymes are potent medications that, unlike small molecules, can affect the body's therapeutic biochemistry. Enzymes are particular and powerful because of these two essential

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properties. Therefore, various enzyme-based medications have been created to treat various illnesses. (Adrio and Demain, 2005).

Methionine is a sulfur-containing amino acid that functions in many biological processes, including protein synthesis, detoxification, and energy production. It is also essential for the development and function of the nervous system (Wise and Thompson 2010). The increased requirement of plasma methionine for protein synthesis and regulation of DNA expression was observed in cancer cells. Under methionine deficiency, the cancer cells were arrested in the late S-G2 cell cycle phase and underwent apoptosis (Cellarier et al., 2003).

Research indicates that bacterial enzymes are highly immunogenic, have poor substrate selectivity, and can harm the liver and kidneys. Fungal-derived L-methioninase is a powerful enzyme source for anti-cancer therapy since it has a high substrate selectivity and less immunogenic and allergic responses (Hawkins et al., 2004).

L-Methioninase is present in practically all living organisms, including bacteria (Amarita et al., 2004; Hendy et al., 2022), moulds, protozoa, and plants except mammals (El-Sayed, 2009). L-methioninase (EC 4.4.1.11) is a pyridoxal phosphate (PLP) dependent hydrolytic enzyme and also known as L-Methionine- γ-lyase, L-methionine-γ-demethiolase, methionase, and L-methionine-methanethiol-lyase (Tanaka et al., 1985). This enzyme directly converts L-methionine ($C_5H_{11}NO_2S$) into -ketobutyrate, ammonia, and methanethiol (Sharma et al., 2014).

L-Methioninase is one of few microbial enzymes with high therapeutic value (İpek et al., 2023), which is a potent antitumor agent versus different cancer cell lines involving those from the breast, colon, pulmonary, renal, and glioblastoma (Tan et al., 1998). The essential amino acid L-methionine is required to propagate and develop different primary tumours and human carcinoma cells. Conversely, due to active methioninase synthase, normal cells can grow on homocysteine rather than methioninase (Miki et al., 2000).

It has been shown that the L-methionine-depleting enzyme recombinant L-methioninase α, γ-lyase (rMETase), which was cloned from *Pseudomonas putida*, is efficient against a range of cancer cell types (Sugisawa et al., 2021). The methioninase-cleaving enzyme would lower L-methioninase concentrations more than L-methioninase starvation, which would have a more substantial treatment impact (Tan et al.,2010).

Consequently, this work aims to determine the ideal conditions for production, purification, characterization, optimization, and anticancer properties of L-methioninase from the endophytic fungus *Trichoderma harzianum* that harbour two significant medicinal plants.

2. Material and methods

2.1. Plant samples harvesting

Two healthy and mature medicinal plants (Astragalusannularis and Calotropisprocera) were selected from the Delta region, Egypt, and used as sources of fungal endophytes. Disease-free plant parts stem, root, and leaves were separated with a sterile scalpel and stored in sterile plastic bags at 4 °C until transported to the laboratory to isolate the endophytic fungi. The Plants were identified taxonomically, and laboratory procedures were carried out at the Plant Taxonomists and Microbial Molecular Biology Laboratories in the Botany Department, Faculty of Science, Mansoura University.

2.2. Isolation and characterization of endophytic fungi

Modified methods described by Hallmann et al. (2006) were used for isolating the fungal endophytes; the plant samples were washed for 10 min with tap water to remove dust. Each plant sample's leaves and small branches were divided into 1 cm long pieces using a sterile scalpel before being immersed in 75 % ethanol for one minute, treated with 5 % sodium hypochlorite solution for three minutes, added 75 % ethanol for 30 s, rinsed using sterile distilled water for three to five sec, and dried with sterilized filter papers below-controlled conditions. About 6–8 sterilized segments of each plant species were inoculated on potato dextrose agar (PDA, Oxiod, UK) supplemented with antibacterial to inhibit bacterial growth and incubated at 28 °C until the growth of endophytic fungi appeared. The developed fungal isolates were purified on PDA plates free of antibiotics and incubated for 14 d at 28 °C. After this incubation period, these pure cultures were preserved under 20 % glycerol (Sigma, Egypt) at −70 °C used as a stock culture for further studies.

The isolated fungal species were morphologically identified by Domsch et al. (1980). Calculations were made to determine each endophytic fungus's colonization frequency (CF%) and the % of dominant endophytic fungi (Petrini and Fisher, 1988; Kumar and Hyde, 2004).

$$CF\,\% = \left(\frac{\text{No segments colonized by single endophytes}}{\text{Total count of segments}}\right) \times 100$$

2.3. Isolation of L-Methioninase-producing fungi

L-methioninase was isolated, as described by Ruiz-Herrera and Starkey (1969). The modified medium contains 5 g of methionine, glucose (10 g/l), 1 g of each K_2HPO_4 and KH_2PO_4, 0.1 g of $CaCl_2$, 0.5 g of $MgCl_2$, 0.02 g of each $FeCl_3$ and $ZnCl_2$, and 20 g of agar, all components were homogenized in 1L of sterilized water, and the pH was accustomed to being neutral. The flask was incubated at 28 °C for seven days.

2.4. Screening and selection of L-methioninase producers

2.4.1. Qualitative assay test

2.4.1.1. The rapid assay plate technique. The above medium was applied to test the L-methioninase productivities of the fungal isolates. The medium was supplemented with phenol red (0.07 %) as a pH indicator before pouring the plate. The plate was then kept at 28 °C for 168 h. According to William and Hariharan (2013), the pink colour around the growth area, caused by ammonia produced when L-methioninase acts on L-methioninase, allowed researchers to identify isolates with L-methioninase.

2.4.2. Quantitative test

2.4.2.1. The activity of L-Methioninase. L-Methioninase activity was evaluated following Laakso and Nurmikko (1976), utilizing L-methioninase as a substrate. Methanethiol (MTL), a product from the substrate, reacted with 5,5-dithio-bis-2-nitrobenzoic acid (DTNB; Sigma–Aldrich, Egypt) to form thio-nitrobenzoic acid, which was noticed at 420 nm using a colourimeter.

The reaction mix consisted of 20 mM of L-methioninase, 0.05 M of phosphate buffer (pH 7.0), 0.01 mM pyridoxal phosphate (PLP; Sigma–Aldrich, Egypt), 0.25 mM of DTNB, and the supernatant in end volume of 1 ml. After thirty minutes of keeping it at 37 °C, the absorbance of the yellow colour was read at 420 nm. Separately, prepared controls without heat-denatured filtrate (at 95 °C for 30 min). The amount of Methanethiol (MTL) released was calculated according to a standard curve obtained with sodium methane thiolate. One unit (U) of L-methioninase was expressed as the amount of enzyme that releases 1 mol of MTL per minute under optimal assay conditions.

According to Thompson and Morrison (1951), Nesslerization was also used to measure the regular L-methioninase activity. 100 μL of pyridoxal phosphate and 1000 μL of the crude L-methioninase were added to 1000 μL of L-methioninase in citrate buffer (pH 7.2), homogenized and incubated at 37 °C for 30 min. After that, 0.5 ml of TCA (1.5 M, Sigma–Aldrich, Egypt) was put in to inhibit the enzyme; then, it was collected by centrifugation at 4500 × g for 5 min. The NH_3 was

estimated by adding 0.5 ml of Nessler reagent and measuring the resulting colour at 480 nm. Under optimal assay conditions, one unit of L-methioninase is the volume of the enzyme that catalyzes the release of one mole of ammonia per hour. The specific activity of L-methioninase was expressed as the enzyme activity in terms of units per milligram of protein.

2.4.2.2. Methioninase uptake measurement. The procedure of Hess and Sullivan (1943) was used to calculate the residual methioninase in the culture filtrate. 500 µL of glycine (3 %), 500 µL of sodium hydroxide (1 N), and 1000 µL sodium nitroprusside (2 %) were mixed with 1 ml of the supernatant, then heated to 40 °C for 10 min and then cooled for 5 min in ice bath; then added 1 ml of an HCl: H_3PO_4 mixture 1:9 (v/v), the absorbance was recorded at a wavelength of 530 nm. The methioninase content in endophyte supernatant was measured using the standard curve of methioninase generated under identical circumstances.

$$\text{Methioninase uptake} = \text{Consumed methioninase} / \text{initial methioninase concentration} * 100$$

2.4.2.3. Determination of biomass. After fermentation, the culture was centrifuged for 10 min at 4500 rpm at 4 °C. The endophyte pellets were rinsed with water several times and dried at 70 °C for two hours. The dried pellets were determined as g/L of fermentation medium.

2.5. Molecular characterization of selected Methioninlytic isolates

The Qiagen kit (Germany) extracts the chromosomal DNA following the producer's guidelines. Universal primers were used to amplify the 16S rRNA gene ITS1/ITS4(5′TCCGTAGGGTGAACCTGCGG3′)/(5′TCCTCC GCTTATTGATATGC3/) White et al. (1990). The PCR reaction was performed in a 50 µL volume using the following components:

- 1x green Taq PCR Buffer; * 200 mM of each dNTP; * 100 mg BSA.
- 10 pmol of each primer; * 2.5 U of Taq DNA polymerase (Sigma, Egypt).
- 10 ng of DNA.

The following conditions reached PCR: 5 min at 95 °C subsequent by 35 cycles, each cycle consisted of denaturation at 95 °C for 1 min, annealing at 55 °C for 30 sec, and extension at 72 °C for 1 min, terminal extension step at 72 °C for 7 min. The PCR product was analyzed by agarose gel electrophoresis. The PCR products were purified by Quick gel extraction and PCR purification combo kit. The PCR products were sequenced using an automated DNA sequence (ABIPRISM 3700). Then, the sequences were submitted to the NCBI GenBank database, a public repository of genetic sequences (https://www.ncbi.nlm.nih.gov/). The sequences from this investigation were then compared to the GenBank database applying the BLAST program, also available on the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/).

2.6. Purification of L-methioninase

The selected Methioninlytic isolates were used to purify L-Methioninase. *Trichoderma harzianum* strains were cultivated in specific broth media and incubated at 28 °C for seven days to produce the L-Methioninase. Before dialyzing, crude L-Methioninase was precipitated with ammonium sulfate (Sigma, Egypt) 70 % from culture supernatants; the mixture was stirred overnight under cooling. Using column chromatography, total homogeneity was obtained for purification on Sephadex G-200 homogenized in sodium citrate buffer (0.05 M, pH 6.5) and left to swell. The separation column was filled with swollen beads and allowed to settle to a constant height of 45 cm, lacking applying pressure.

The crude enzyme was loaded onto a Sephadex G-200 column equilibrated with sodium citrate buffer. The enzyme was then eluted from the column with the same buffer, 0.05 M (pH 6.5). Twenty fractions were collected at a 2 ml/15-minute flow rate. The collected fractions were assayed for protein at 280 nm. The fractions containing L-Methioninase were concentrated and dialyzed against 0.05 M sodium citrate pH 6.5, between the sample and another column must be washed with 1 M sodium chloride John (1969). Biuret's reagent evaluated the protein concentration in crude and pure L-methioninase. The standard curve of bovine serum albumin was used following Smith et al. (1985).

2.7. Determination of pure L-methioninase molecular weight by SDS-PAGE

The homogeneity of purified L-methioninase was checked using dissociating sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). It was carried out according to a protocol proposed by Laemmli, 1970, with a separating gel of 10 % and a stacking gel of 5 % containing 0.1 % SDS. The protein bands were detected by Coomassie blue staining and then de-stained using a mixture of methanol, glacial acetic acid, and distilled water. The molecular weight of L-methioninase was quantified compared with the mobility of protein standard marker (Tris-Glycine 4–20 %) with a wide range of molecular weights: 5–245 kDa and the gel was photographed under white light illumination.

2.8. Determination of the optimum pH and temperature for L-methioninase activity (crude-pure)

L-methioninase enzyme was incubated at several pH levels (3.5–12.0) for 10 min to determine the best pH for enzymatic activity. Various buffers (0.2 M) were used to determine the pH range: phosphate sodium hydroxide buffer to achieve a pH (of 11.0–12.0), glycine sodium hydroxide buffer to determine a pH range of 9.5–10.5, Tris-HCl buffer was used to assess pH 8.5–9.0, phosphate buffer for pH (6.0 to 8.0) and acetate buffer was used for the range from (3.5 to 5.5). To determine the optimum temperature, the reaction mixture was kept for 10 min in 50 mM potassium phosphate buffer (pH 8.0) at various temperatures ranging from 20 to 100 °C (at regular intervals of 10 °C). All the assays were carried out in triplicates; the maximum enzyme was defined as 100 %, and the relative activity was determined as a fraction of this value.

2.9. Determination of the optimum pH and temperature for L-methioninase stability (crude-pure)

The enzymatic stability was tested at various pH levels from 3.5 to 12.0 for 12 h before conducting the assay. All the assays were carried out in triplicates with appropriate controls, and the residual activity was estimated at standard assay conditions. To determine the heat stability, the test sample was kept at different temperatures between 20 and 70 °C for varying periods: 30, 60, 90, 120, and 150 min, followed by an estimation of enzyme activity at 37 °C. The residual activity was measured as the percentage of observed activity for the unheated enzyme.

2.10. Effect of inhibitors on crude and purified L-methioninase

The crude and purified L-methioninase were pre-incubated with the selected reagents in phosphate buffer (50 mM) at 35 °C for 30 min to determine the impact of the inhibitors on the enzyme activities; Tween 20, Tween 80, Triton X-100, EDITA, SDS, DMSO, 2-Mercaptoethanol, Sodium azide, Ethanol, Acetone, Methanol, and Cysteine. The enzyme activity was measured under the standard assay conditions as described earlier. Residual activity was determined by taking the activity of the control sample without inhibitor as 100 %.

2.11. Evaluation of cytotoxicity effects of L-methioninase (crude-pure) against MCF-7 cell line

These experiments occurred in the Regional Center for Mycology and

Biotechnology, Al-Azhar University, Egypt. The potential cytotoxicity and antitumor activity of crude and purified L-methioninase from the selected Methioninlytic isolates were tested against breast cancer cell line MCF-7. A dose-dependent curve was constructed for each isolate, and the 50 % inhibitory concentration (IC50) was calculated (Vijayan et al., 2004).

2.12. Statistical analysis

Statistical analysis of the data was done using the software IBM SPSS (ver Statistic 21). All the values are expressed as mean ± SD. The data were statistically analyzed by one-way ANOVA. Comparison of the data means was performed using Tukey's HSD test at $p \leq 0.05$. Followed by Dunnett comparison using GraphPad Prism 7 software.

3. Results

3.1. Identification and isolation of endophytic fungi

Two mature medicinal plants were found to have 78 endophytic fungi, representing 11 species and eight genera. *Aspergillus* (23), *Trichoderma* (15), *Penicillium* (11), *Alternaria* (9), *Rhizopus* (6), *Curvularia* (6), *Fusarium* (5), and *Mucor* (3) were the species and number classifications for the chosen isolates (Table 1 and Fig. 1).

3.2. Methioninlytic fungi (qualitative and quantitative assay)

Using L-methioninase as the sole carbon and nitrogen source, nine fungal isolates belonging to one genus and one species (*Trichoderma harzianum*) were screened. Pink colour surrounded the isolated fungi colonies as an indicator for the extracellular L-methioninase production of these isolates. The pink colour intensity rate around colonial growth depended on the rate of L-methioninase formation by each isolate tested.

The physicochemical properties of fungi isolate, i.e., growth rate, colour density, protein estimate, specific enzyme activity, and L-methioninase uptake, are shown in Table 2. From the data, we can conclude that the growth rate was varied, and enzyme formation was highly different and not correlated with each growth or methionine uptake. Isolates no. (4) and (8) had the highest levels of L-methioninase production (See Table 3).

The Molecular identification of two methioninlytic fungal isolates, no. 4 and 8, operated utilizing ITS1 and ITS4 primers, then deposited in GenBank with accession numbers MZ675363 and MZ675362.

3.3. Purification of L-methioninase from two isolates of Trichoderma harzianum

This experiment was carried out on isolates numbers 4 and 8. The extracellular L-methioninase of *Trichoderma harzianum* was precipitated by ammonium sulphate $(NH_4)_2SO_4$ (70 %), then dialysis of the crude and determined concentration by Biuret's reagent, then complete purification of by Column chromatography (sepadex 200). Collect at least 17 fractions from each sample after running on Column (2 ml/ 15 min). Optical density (OD) was separately measured at 280 nm for each fraction. A relationship between O.D. and fractions (Fig. 2), mixed between fraction no.4 (peak start) to fraction no. 14 (peak end), protein was precipitated by $(NH_4)_2SO_4$ (70 %), dialyzed and pure protein concentration was determined by Biuret's reagent Table 2.

Table 1
Endophytic fungi colonization frequency percentage (CF%).

Endophytic fungi	Number of isolates	CF %	Dominant fungi%
Aspergillus fumigatus	10 ± 0.9	62.5 ± 1.2	12.82 ± 0.2
Aspergillus terricola	7 ± 0.1	25 ± 0.9	8.97 ± 0.4
Aspergillus terreus	6 ± 0.9	75 ± 1.1	7.69 ± 0.6
Alternaria alternata	9 ± 0.8	50 ± 2.1	11.54 ± 0.1
Rhizopus oryzae	6 ± 0.2	62.5 ± 1.1	7.69 ± 0.5
Trichoderma harzianum	9 ± 0.4	75 ± 1.3	11.54 ± 0.8
Trichoderma atroviride	6 ± 0.6	25 ± 2.1	7.69 ± 1.1
Mucor mucedo	3 ± 0.2	50 ± 0.3	3.84 ± 0.2
Curvularia tuberculata	6 ± 0.5	12.5 ± 0.5	7.69 ± 0.3
Penicillium chrysogenum	11 ± 0.6	25 ± 0.5	14.1 ± 0.8
Fusarium oxysporum	5 ± 0.7	50 ± 0.7	6.41 ± 0.3

N = 3, the data are presented mean ± SE.

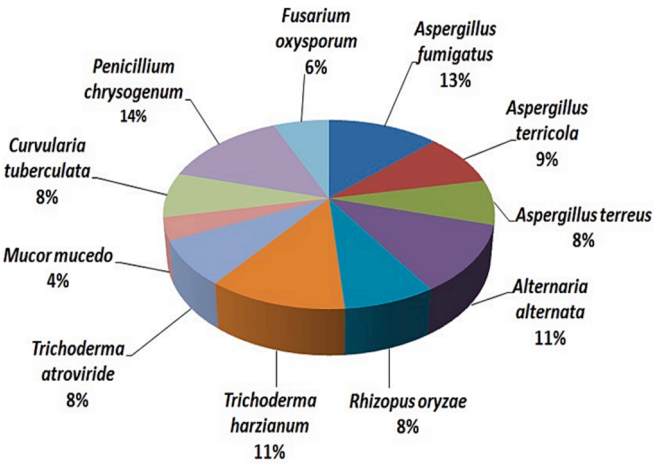

Fig. 1. Relative frequency of Dominant endophytic fungi isolated from six wild-type plants.

Table 2
Qualitative and quantitative assay screening of different *Trichoderma harzianum* isolates for L-methioninase production.

Qualitative assay		Quantitative assay			
Isolates no	Pink color intensity	Dry wt. (mg/ml)	Enzyme activity (unit/ml)	Specific activity (unit/mg)	MethionineUptake (%)
1	++	3.91 ± 0.11^{f}	299.6 ± 6.7^{b}	9.96 ± 0.09^{b}	88.4 ± 0.17^{d}
2	++	4.10 ± 0.14^{e}	405.1 ± 3.3^{a}	10.00 ± 0.08^{b}	90.8 ± 0.40^{c}
3	+	3.65 ± 0.92 ^{g}	216.1 ± 7.4^{e}	7.57 ± 0.02^{c}	82.4 ± 0.17^{f}
4	+++	4.7 ± 0.28^{b}	280.1 ± 3.8^{c}	10.10 ± 0.10^{b}	93.0 ± 0.15^{b}
5	+	4.10 ± 0.42^{e}	188.4 ± 1.4 ^{g}	5.20 ± 0.07^{e}	85.1 ± 0.14^{e}
6	+	3.35 ± 0.49 ^{h}	169.1 ± 3.2 ^{h}	6.09 ± 0.09 ^{d}	77.1 ± 0.23 ^{h}
7	+	4.25 ± 0.35^{d}	202.3 ± 3.2^{f}	6.20 ± 0.06^{d}	72.2 ± 0.17^{i}
8	+++	5.30 ± 0.34^{a}	282.9 ± 4.1^{c}	10.78 ± 0.25^{a}	93.9 ± 0.10^{a}
9	++	4.45 ± 0.78^{c}	224.6 ± 1.9^{d}	7.25 ± 0.03^{c}	80.2 ± 0.05 ^{g}

* In each row, values followed by the same letter are not significantly different according to Tukey's HSD test ($P \leq 0.05$), each value is the mean of three replicates ± SD.

Table 3
Estimation of crude and pure L-methioninase concentration in isolates no. 4 and 8 by Biuret's reagent.

Isolates no.	Crude L-methioninase		Pure L-methioninase	
	Optical Density (540 nm)	Concentration (ug/ml)	Optical Density (540 nm)	Concentration (ug/ml)
4	0.420a	13.36a	0.345a	12.23a
8	0.401b	12.75b	0.323b	12.13b

* In each row, values followed by the same letter are not significantly different according to Tukey's HSD test ($P \leq 0.05$), each value is the mean of three replicates ± SD.

3.4. Characterization of the purified L-methioninase by SDS-PAGE

The extracellular L-methioninase from isolates (8) was purified by ammonium sulfate precipitation (70 %) and showed specific activity 14.1 U/mg with 1.3-fold purification, followed by gel filtration chromatography (sephadex 200). The specific activity of the enzyme increased to 71.3 U/mg with 6.6-fold purification (Table 4). SDS-polyacrylamide electrophoresis was utilized to estimate the molecular weight of the purified L-methioninase (Lane 2), and the results exhibited a single band with an apparent molecular weight of around 48 kDa (using Bio-Rad software) (Fig. 3).

3.5. pH and temperature for the stability of crude and purified L–methioninase

This experiment was carried out on crude and pure L-methioninase extracted from isolates numbers 4 and 8 and gave the same results.

In the pH range of 5 to 9, *Trichoderma harzianum* L-methioninase was active. The crude L-methioninase showed its highest activity at a pH of 8.5, while the purified L-methioninase showed its most increased activity at 8 (Fig. 4A). Below pH 4.5, the enzyme activity was completely inhibited, while above pH 9.5, it significantly decreased. L-methioninase research on pH stability in the 4.5–10.5 range. After being incubated with different PH buffers for 24 h, The enzyme was stable at 6.0 to 9.0. (Fig. 4B). The L-methioninase produced was functional in a temperature range of 20 to 70 °C, with the purified and crude enzymes having optimal activities at 35 and 40 °C, respectively (Fig. 5A).

The isolated pure L-methioninase was incubated at degrading temperatures of 20–70 °C and at different times to determine the thermal resistance of the isolated enzyme. The highest stable of pure L-methioninase showed below 40 °C (Fig. 5B) with an enzymatic activity of 80 % for 120 min. However, the activity substantially reduced with raising the temperature above 50 °C and lost completely after incubation at 70 °C for 1 h (Fig. 5B).

3.6. Inhibitor effects on crude and purified L-methioninase

The primary significance of inhibitors in the enzyme industry is that they decrease the enzyme reaction's efficiency. It is possible to identify an enzyme's catalytic identity by looking at its inhibitors. Table 5 summarizes how inhibitors affect the activities of L-methioninase. A relative activity of 95 % was displayed by Pure L-methioninase in the existence of EDTA, confirming the non-metallic character of the enzyme. A disulfide bond is necessary to maintain the enzyme's molecular integrity, given that 2-mercaptoethanol and cysteine inhibit pure L-methioninase, resulting in relative activities of 56 % and 57 %, respectively.

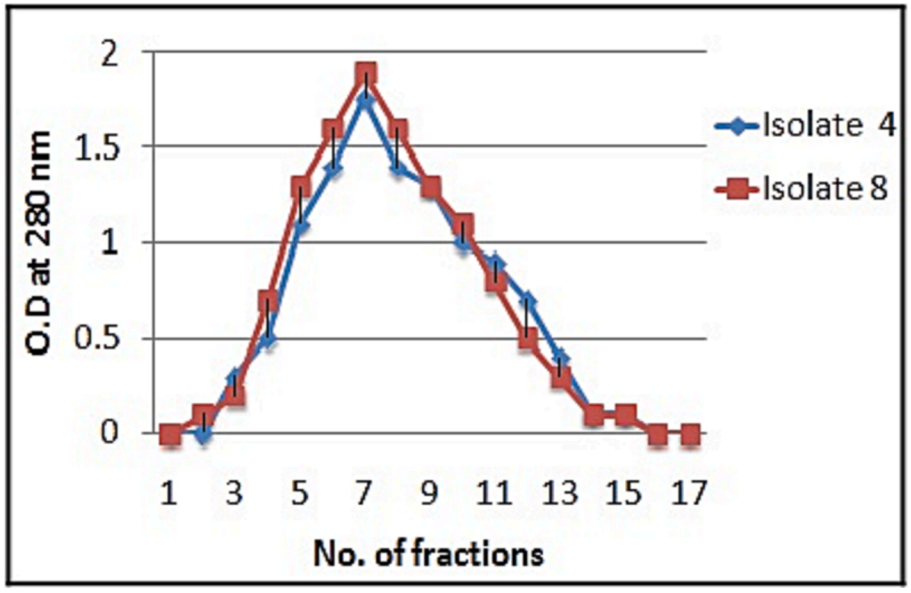

Fig. 2. Relationship between O.D. at 280 nm and fractions for isolates no 4 and 8 after crude L-methioninase purified on Colum chromatography.

Table 4
Purification of L-methioninase from isolates (4,8) (*Trichoderma harzianum*).

Purification step	Total Enzyme (U)		Specific activity(U /mg)		Purification fold		Enzyme yield(%)	
	Isolate 4	Isolate 8	Isolate 4	Isolate 8	Isolate 4	Isolate 8	Isolate 4	Isolate 8
Crude enzyme	3233 ± 3.6a	3303 ± 2.9a	10.1 ± 2.1c	10.78 ± 0.5c	1 ± 0.1b	1 ± 0.5b	100 ± 0.0a	100 ± 0.0a
Ammonium sulphate	3100 ± 4.1b	3163 ± 3.1b	13.5 ± 0.2b	14.1 ± 0.2b	1.33 ± 0.2b	1.30 ± 0.1b	95.8 ± 2.6b	95.7 ± 0.6b
Sephadex G-200	1551 ± 2.1c	1594 ± 1.9c	70.6 ± 0.3a	71.3 ± 0.9a	6.9 ± 0.6a	6.6 ± 0.3a	47.9 ± 0.9c	48.2 ± 0.9c

* In each row, values followed by the same letter are not significantly different according to Tukey's HSD test ($P \leq 0.05$), each value is the mean of three replicates ± SD.

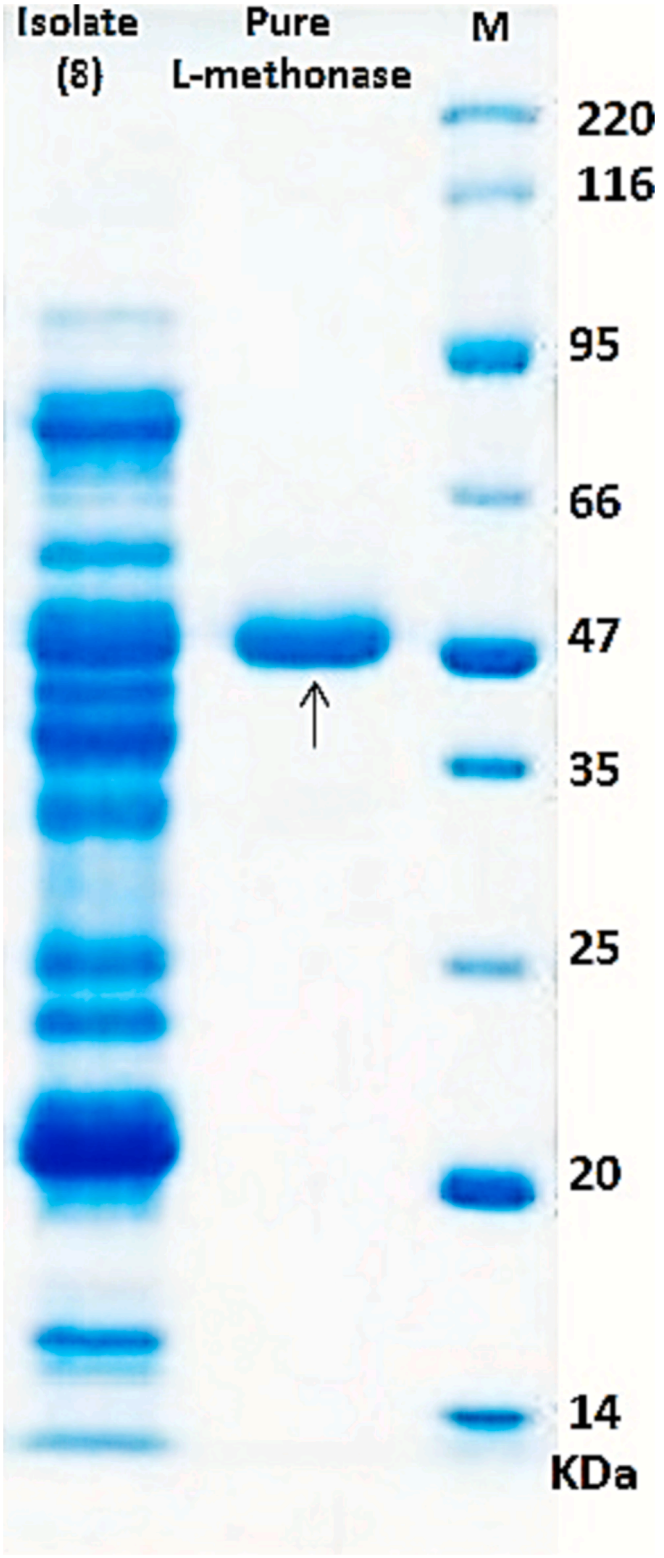

Fig. 3. SDS–PAGE protein profiles of *Trichoderma harzianum* Lans:1. Isolate 8, 2 pure L-methioninase by Colum chromatography, and 3.M protein molecular weight marker.

3.7. The cytotoxicity of crude and purified L-methioninase against the MCF-7 cell line

L-methioninase from isolates 4 and 8 of *Trichoderma harzianum* has a visible apoptotic effect against breast cancerous cell lines. All L-methioninase preparations inhibited cell line development, and the cells in the experimental group underwent morphological changes, becoming rounded and lifted off the bottom of the plates; however, control cell lines remained spindle-shaped; cell density depends on the efficiency of the L-methioninase from various isolates (Fig. 6). The highest inhibition of MCF-7 in cells treated with pure L-methioninase from isolate no. 8 (80 %), while crude L-methioninase reduced 60 % of cancerous cells. Compared to other treatments (Fig. 7). The IC_{50} of crude L-methioninase was 12.6 and 17.4 µg/ml for isolates 8 and 4, respectively, while the IC_{50} of pure methioninase was 5.0 and 15.2 µg/ml.

The IC_{50} of the *Trichoderma harzianum* isolate no 8 increased 3-fold due to purification; 5 µg/ml. L-methioninase use in the pharmaceutical industry as next-generation therapeutics will give new hope in treating cancers.

4. Discussion

Herbal plant myco-endopytes are a prospective source of novel bioactive compounds that may be employed in various applications (Treasure et al., 2020). Endophytes have been found in a broad spectrum of medicinal plant spp. *Astragalus annularis* and *Calotropis procera*, two significant medicinal plants, were used for endophytic fungi isolation. The findings revealed the diversity of myco-endophytes in these plants, which agreed with El-Maghraby et al. (2013) and Osman et al. (2020). Among the isolates, the species most abundant were *Penicillium chrysogenum*, *Aspergillus fumigatus*, *Alternaria alternata,* and *Trichoderma harzianum,* and these findings are in correspondence with the previous studies (Aharwal et al., 2014; Elazab, 2019).

Due to its hydrolytic capabilities, which enable it to catalyze the conversion of L-methioninase to ketobutyrate, methanethiol and ammonia. L-methioninase is a member of the lyase family, specifically the class of Carbon-sulfur layers. (Mohkam et al.,2020). It is reported in microorganisms like bacteria, actinomycetes, fungi, protozoa, and yeast (Huang et al., 2014). It is generated as intracellular enzymes in many microbial species, such as *Pseudomonas* (Sato & Nozak, 2009) and some fungal strains (Hendy et al., 2023). In addition, it is produced as extracellular in some fungal strains (El-Sayed, 2009), yeast, e.g., *Saccharomyces cerevisiae* (Sharma et al., 2014), and some protozoans which include *Entamoeba histolytica* (Tokoro et al., 2003).

Many microorganisms can break down methionine, but they do not grow on it because they can't metabolize the deaminated product (α-ketomethionine) or the demethylated product (α-keto-butyric acid and methanethiol) residues of L-methioninase (Selim et al., 2015). It is possible to partially overcome filamentous fungi's inability to develop on L-methioninase by using a growth-supporting organic substance like glucose or another comparable carbohydrate referred to as a co-dissimulator. (Ruiz-Herrera and Starkey, 1969).

Submerged fermentation (SmF) and solid-state fermentation (SSF) are two distinct techniques for cultivating microorganisms to purify and produce L-methioninase from various organisms (Khalaf and El-Sayed, 2009). Methanethiol is an organosulfure compound that is spectrophotometrically estimated by using DTNB (5, 5′dithio-bis (2-nitrobenzoic acid) (Riddles et al., 1979). TNB (2-nitro-5-thiobenzoic acid) was detected spectrophotometrically in the visible spectrum when DTNB interacted with a sulfhydryl group of methanethiol to form a colorful product. Another -ketobutyrate byproduct was detected spectrophotometrically using "Oxidation followed by condensation reactions with MBTH (3-Methyl-2-Benzothiazoline Hydrazone) (Soda, 1967). Using Nessler's reagent, the deamination rate was determined by precipitating mercury (II) amido-iodide with ammonia as the final product (Krug et al., 1979). Depending on the amount of ammonium ion, which can be detected at 480 nm, the residue can range in colour from yellow to brown. (Kominami et al., 2002).

The L-methioninase from *Trichoderma harzianum* used in this study had the highest specific activity compared to other studies. *Aspergillus flavipes* was purified 12.1-fold with a 39.8 % yield and had a specific

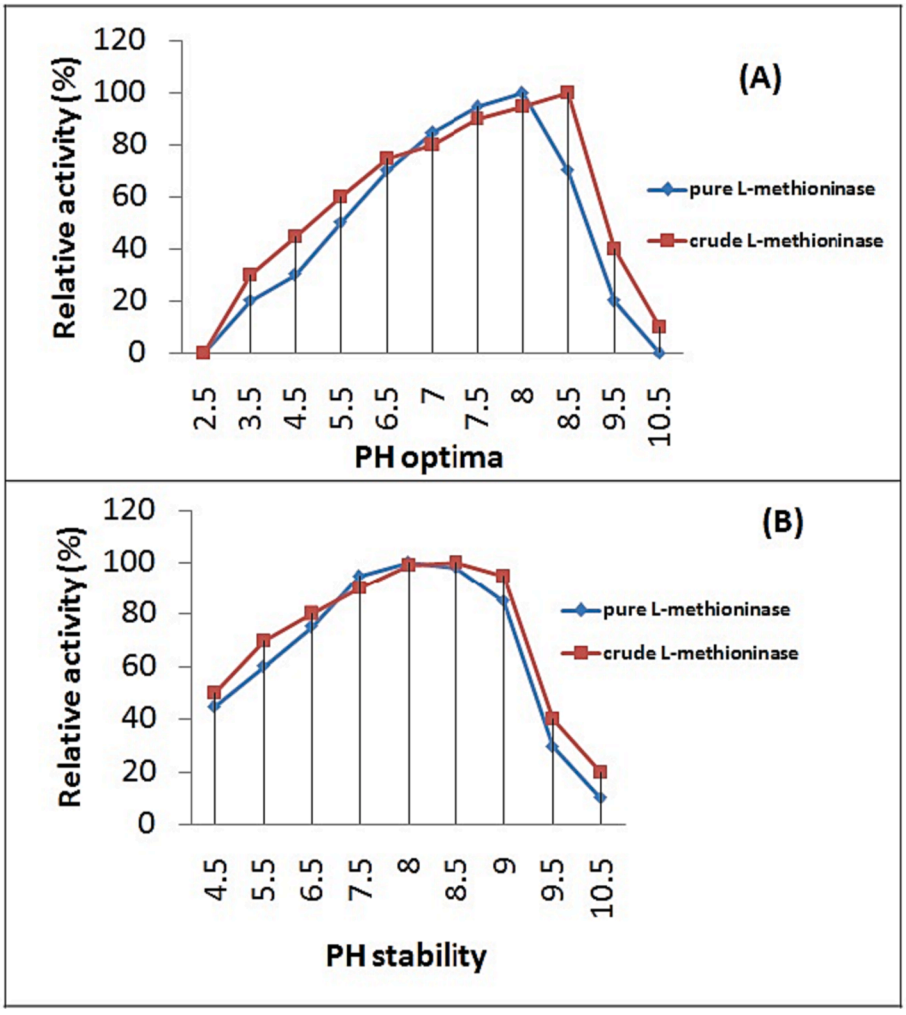

Fig. 4. (A)PH optima of pure and crude L-methioninase. (B) PH stability of pure L-methioninase from *Trichoderma harzianum.*

activity of 14.6 U/mg, which means that it can catalyze the conversion of 14.6 mol of L-methionine per minute per milligram of enzyme (El-Sayed, 2011). The enzyme purification in *Candida tropicalis* was carried out in three steps with a purification fold of 43.19 and 27.985 % enzyme recovery (Selim et al., 2015). A purification fold 270 was achieved with an overall yield of 8.4 % for l-methioninase from *Pseudomonas putida* (Ito et al., 1976). The lowest enzyme productivity and methionine uptake were detected in the culture filtrates of *Aspergillus ochraceous*, *Cladosporium oxysporum* (El-Sayed, 2009).

According to reports, L-methioninase is a multi-subunit enzyme with four equal subunits (El-Sayed, 2010), and L-methioninase purified from various resources has a molecular weight per subunit that is between 43 and 48 kDa (Ito et al., 1976), which agreement with our results. A 47 kDa L-methioninase from *Aspergillus flavipes* (El-Sayed, 2010), 46 kDa from *Candida tropicalis* (Selim et al. 2015), and 43–45 kDa from - *Streptomyces* species (Selim et al., 2015). have been previously reported. The molecular weight of the purified enzyme was within the range of L-methioninase isolated from different fungi (Hendy et al., 2022).

The type of amino acids at the active site, which are protonated and deprotonated, and the conformational alterations brought on by the ionization of the amino acids define how pH affects the enzyme activity. Since there is a specific pH optimum for enzymes, they only work best within a minimal pH range (Sabu et al., 2005).

El Sayed (2011) and Nakayama et al. (1984) have recorded that the optimum pH for L-methioninase from *Aspergillus flavipes* and *Aeromonas* species was 8, which agrees with the obtained findings in this study. According to (Pavani and Saradhi 2014) and (El-Sayed 2011) from *Brevibacterium linens* and *Aspergillus flavipes*, the enzyme was highly stable in the pH from 6.0 to 9.0. Selim et al. (2015) reported the optimum pH value for *Candida tropicalis* L-methioninase to be 6.5. *Trichoderma harzianum* L-methioninase displayed broad activity in the pH range of 5 to 10, with maximum activity at pH 8.

Purified *Trichoderma harzianum* L-methioninase showed the highest activity as results reported from *Aspergillus flavipes* at 35–40 °C (El-Sayed, 2011) and *Aspergillus* species (Hendy et al. 2023), however L-methioninase of *Brevibacterium linens* and *Streptomyces* species showed optimum activity at 25 °C and 45 °C, respectively (Dias and Weimer, 1998). Temperatures above the optimum value also affect the protein ionization state and species solubility in solution, thus reducing enzyme activity (Mukherjee and Banerjee, 2006).

The thermal stability of L-methioninase was seen below 40 °C in *Aspergillus flavipes* (El-Sayed, 2011) and *Brevibacterium linens* (Diaz and Weimer, 1998). The activity of *Aspergillus ustus* L-methioninase (İpek et al., 2023) peaked at 35 °C and then gradually decreased, retaining just 55 % of its activity at 50 °C. On the other hand, L-methioninase isolated from *Candida tropicalis* (Selim et al., 2015) was temperature-sensitive. Denaturation of the enzyme may cause its deactivation at a higher temperature.

Enzyme inhibitors are employed as indicators to determine the enzyme's catalytic identity. El-Sayed (2011 and 2017) reported similar findings on *Aspergillus flavipes* for L-methioninase production.

Antitumor medicines depend on L-methioninase and have been

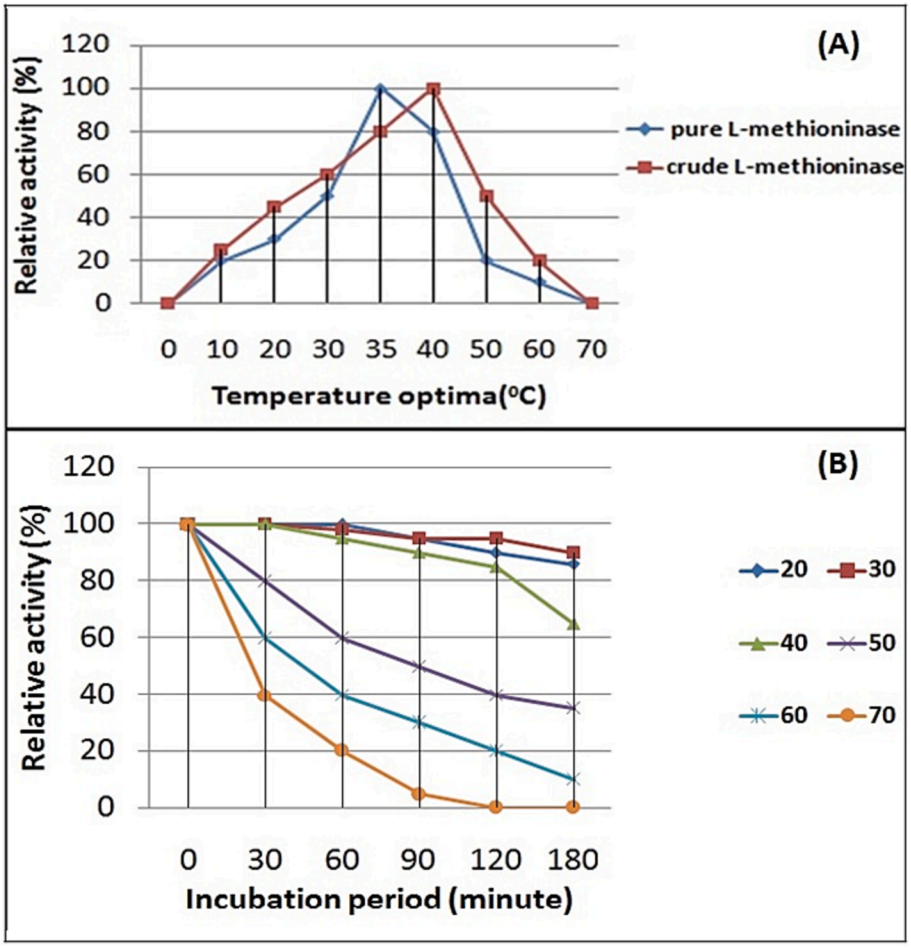

Fig. 5. (A) Temperature optima of pure and crude L-methioninase(B) Thermal stability of pure L-methioninase from *Trichoderma harzianum.*

Table 5
Estimation Effect of inhibitors, detergents, chelator and solvents on crude and pure L-methioninase from *Trichoderma harzianum.*

Compound	Concentration((V/V) %)	Relative activity (%)	
		Crude L-methioninase	Pure L-methioninase
Tween 20	0.5	75 ± 1.9^{e}	78 ± 1.5^{de}
Tween 20	1	61 ± 1.1^{h}	66 ± 0.6^{g}
Tween 80	0.5	72 ± 1.5^{f}	77 ± 1.2^{e}
Tween 80	1	70 ± 1.8^{fg}	71 ± 2.1^{f}
Triton × 100	0.5	91 ± 1.3^{b}	93 ± 1.9^{a}
Triton × 100	1	78 ± 1.3^{d}	80 ± 1.6^{d}
EDTA	1 mM	93 ± 2.1^{a}	95 ± 1.8^{a}
SDS	1	75 ± 1.8^{e}	77 ± 2.2^{e}
DMSO	1	78 ± 2.7^{d}	83 ± 1.9^{c}
2-Mercaptoethanol	1 mM	53 ± 1.7^{i}	56 ± 1.3^{h}
Sodium azide	1 mM	68 ± 1.9^{g}	72 ± 2.1^{f}
Ethanol	1	79 ± 2.6^{d}	83 ± 1.6^{c}
Acetone	1	68 ± 1.5^{g}	70 ± 1.7^{f}
Methanol	1	83 ± 2.2^{c}	87 ± 2.1^{b}
Cysteine	1 mM	52 ± 2.9^{i}	57 ± 2.2^{h}

* In each row, values followed by the same letter are not significantly different according to Tukey's HSD test ($P \leq 0.05$), each value is the mean of three replicates $\pm$ SD.

produced by inhibiting the exogenous addition of L-methionine for tumour cell cultures. Thus, due to the reinjection of homocysteine into the plasma, chemotherapeutic treatment for malignancies depends on methioninase depletion (Breillout et al., 1990). Several researchers reported on the effectiveness of L-methioninase against different cell lines (Tan et al., 2010; El-Sayed et al., 2012; Sundar and Nellaiah, 2013) as L-methioninase from *Streptomyces* species (Selim et al. 2015) was reported to possess remarkable L-methioninase activity against liver and breast cancer cell lines.

Hendy et al. (2023) showed the plausible antiproliferative activity of purified L-methioninase from *Aspergillus fumigates* towards the five experimented cell lines, and this effect was concentration-dependent. The enzyme is highly efficient against the prostate, liver, and lungs. Yamamoto et al. (2022) proposed that cancer cells require significantly

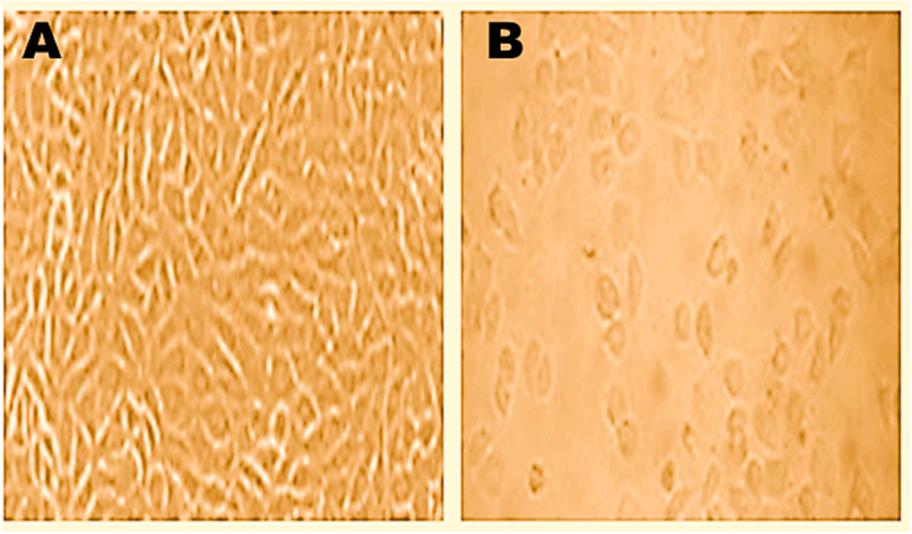

Fig. 6. (A) MCF-7 tumor cells growth without L-methioninase, (B) MCF-7 cells show condensed nuclei and apoptotic bodies in L-methioninase -treated cells for isolate 8.

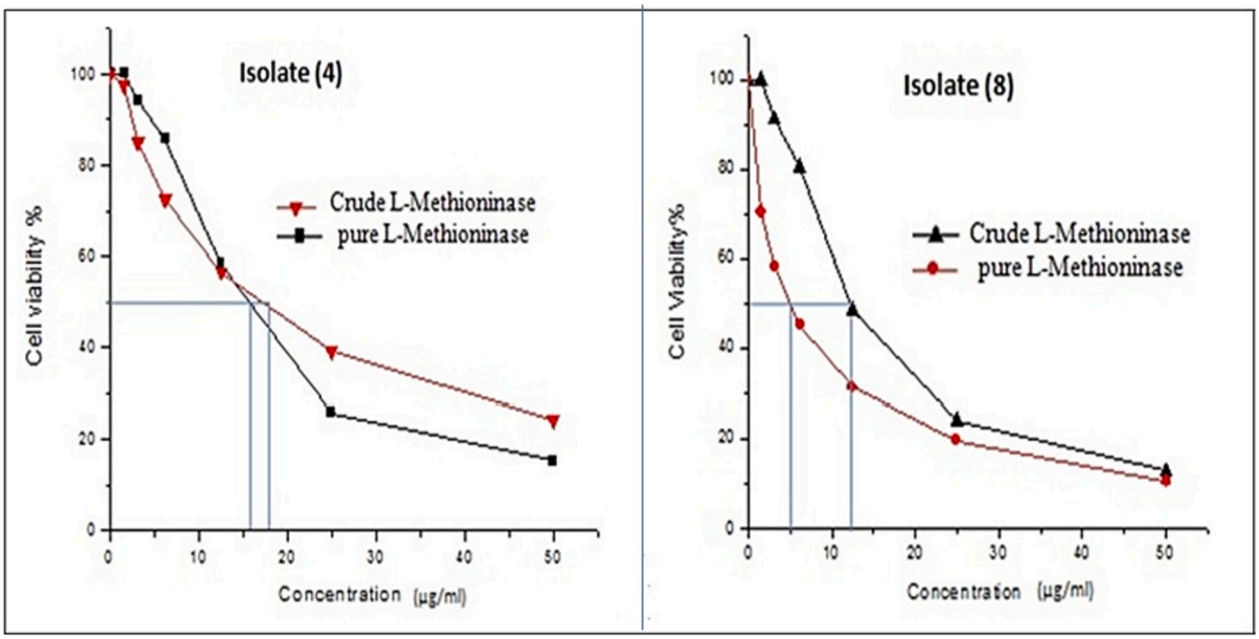

Fig. 7. Cytotoxicity effects of isolates 4 and 8, IC_{50} after L-methioninase purification (15.2 and 5.0 µg/ml).

more methionine than normal cells. This difference could be attributed to increased protein synthesis and accelerated *trans*-methylation processes. This provides that a variety of biochemical processes essential for the rapid growth of cancer cells and that significantly impact DNA result in severe damage to the cell membrane, which triggers apoptosis and ultimately results in the death of the cells. The purified L-methioninase showed a higher affinity towards L-methionine than other substrates, enhancing its anticancer potential.

5. Conclusion

Cancer is the second cause of death. As an alternative to chemical drugs, natural bioactive products are produced by various microbes. It is a resource of compounds that provide hopeful avenues for treating various diseases accurately and properly to target cancer cells. *Trichoderma harzianum* L-methioninase is highly effective against the breast cancer cell line destroyed by crude and purified L-methioninase. The data show that the pure L. methioninase has higher catalytic characteristics, which could be used as a cancer-fighting substance.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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