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Purification and Biochemical Characterization of L-Methioninase Produced from Klebsiella pneumoniae and Evaluation of In Vitro Cytotoxicity Against HepG2 and A549 Cancer Cell Lines with a Potential Pediatric Use

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ABSTRACT: In the present study, L-methioninase was produced from the Klebsiella pneumoniae isolated from clinical samples and was evaluated for its enzymatic and anticancer potential. A total number of 150 clinical samples were collected from different infected areas, from which 27 bacterial isolates were selected. One showed the highest L-methioninase production and was selected for further research.

Screening the modified M9 medium confirmed enzyme production through a characteristic color change, indicating methionine degradation. The quantitative DTNB assay revealed a progressively increased enzyme activity which was correlated with the protein concentration measured by the Bradford estimation method. These results confirmed the efficient enzyme biosynthesis. Optimization studies showed that maximum enzyme production was achieved at pH of 7.0, temperature of 37°C, and incubation period of 24 hours, with glucose and peptone as the most favorable carbon and nitrogen sources, respectively.

Purification of L-methioninase using ammonium sulfate precipitation followed by ion-exchange and gel filtration chromatography could significantly increase specific activity, reaching 55.5 U/mg with a purification fold of 9.2 and yield of 58.3%. The purified enzyme showed optimal activity at physiological conditions (37°C and pH 7.0), proving its suitability for biomedical uses.

The anticancer potential of enzyme was evaluated using MTT assay, showing a clear dose-dependent reduction in viability of A549 and HepG2 cancer cell lines. However, normal HdFn cells showed significantly higher survival rates, indicating selective cytotoxicity. High-Content Screening (HCS) analysis further revealed low cell viability, nuclear damage, increased membrane permeability, and mitochondrial dysfunction.

The results showed that the L-methioninase derived from K. pneumoniae has a strong enzymatic activity, stability under physiological conditions, and significant selective anticancer effects. These findings proved it as a promising therapeutic agent targeting methionine-dependent tumors to support further in vivo and clinical investigations.

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INTRODUCTION

Microorganisms, particularly bacteria, represent a cornerstone resource in biotechnology due to their remarkable metabolic diversity and their capacity to produce a wide range of biologically active compounds [1]. Among these, bacterial enzymes have attracted considerable attention because of their high catalytic efficiency, substrate specificity, and adaptability to diverse environmental conditions. These characteristics have led to a widespread use in industrial processes, medical therapeutics, and biochemical research [2].

Among bacterial species, *Klebsiella pneumoniae* emerged as a metabolically versatile organism capable of producing various extracellular enzymes with significant biomedical potential [3]. Despite its well-documented role as an opportunistic pathogen, its enzymatic machinery offers promising opportunities in biotechnology, particularly in producing enzymes that target specific metabolic pathways [4].

A significant enzyme produced by *K. pneumoniae* is L-methioninase (L-methionine γ-lyase), a pyridoxal phosphate-dependent enzyme that catalyzes the degradation of L-methionine into α-ketobutyrate, ammonia, and methanethiol [5]. This enzyme has attracted considerable interest in cancer research for its ability to selectively deplete methionine, an essential amino acid required for tumor cell proliferation. Many cancer cells show a phenomenon known as methionine dependence, making them highly sensitive to methionine deprivation, whereas normal cells are less affected due to their metabolic flexibility [6].

The catalytic performance and production efficiency of L-methioninase are strongly influenced by culture and reaction conditions, particularly pH, temperature, incubation period, and availability of suitable carbon and nitrogen sources. Enzymes usually show maximum activity within a specific pH and temperature range because these factors directly affect the ionization of amino acid residues, substrate binding, and stability of the enzyme's active site. In contrast, deviation from the optimal pH or temperature may reduce enzymatic activity due to structural changes, impaired substrate interaction, or partial enzyme denaturation. Moreover, nutritional factors such as glucose and peptone enhanced enzyme production by providing readily utilizable carbon and nitrogen sources required for bacterial growth and protein biosynthesis. Therefore, it is essential to optimize these conditions to improve enzyme yield, maintain catalytic efficiency, and support its potential use in anticancer applications [7].

The anticancer potential of L-methioninase is primarily evaluated through *In vitro* cytotoxicity assays. The MTT assay is one of the most widely used colorimetric methods of assessing cell viability based on mitochondrial metabolic activity. In this assay, viable cells reduce MTT into insoluble formazan crystals, which can be quantified spectrophotometrically, providing a reliable quantitative measure of cell survival and proliferation [8].

In addition to conventional assays, advanced techniques such as High-Content Screening (HCS) have been increasingly used for a comprehensive evaluation of cytotoxic effects. HCS combines automated fluorescence imaging with quantitative analysis, enabling simultaneous measurement of multiple cellular parameters, including cell viability, nuclear integrity, mitochondrial membrane potential, and membrane permeability [9]. This multiparametric approach allows for a more detailed understanding of the mechanisms underlying cytotoxicity and enhances the accuracy of anticancer assessments [10].

Therefore, integrating bacterial enzyme production with advanced cytotoxicity evaluation techniques provides a powerful platform for exploring novel anticancer strategies. Investigating L-methioninase derived from bacterial sources, alongside modern analytical approaches such as MTT and HCS, represents a highly promising and emerging direction in the development of selective and effective therapeutic agents.

This study aimed to evaluate the cytotoxic potential of L-methioninase on A549 and HepG2 cancer cell lines in comparison to the normal cell line.

MATERIAL AND METHODS

Sample collection from clinical sources of *Klebsiella pneumoniae*

A total number of 150 clinical bacterial samples were collected from various infection sites, including burns, sputum, wounds, and ear swabs, from patients attending Baghdad Teaching Hospital. The collection process was conducted under strict aseptic conditions to minimize the risk of contamination.

For clarity and reproducibility, the sampling procedure was performed as follows: Sterile swabs and collection containers were prepared prior to sampling. Then, samples were obtained directly from infection sites using aseptic techniques. Next, each sample was immediately labeled with the relevant patient and specimen information. Finally, samples were transported to the laboratory in a cooled container at 4°C and the processing was initiated promptly to preserve microbial viability. Upon arrival at the laboratory, samples were inoculated into Brain Heart Infusion (BHI) broth and incubated at 37°C for 18 hours to promote bacterial enrichment. Subsequently, subculturing was performed on agar Mueller–Hinton agar and MacConkey to obtain isolated pure colonies [11].

Preliminary identification was carried out based on colony morphology, Gram staining, and standard biochemical tests, including oxidase, catalase, citrate utilization, and triple sugar iron (TSI) reactions. A final confirmation of *K. pneumoniae* was performed using the automated VITEK-2 system to ensure accurate identification [12].

Screening for L-Methioninase production from *Klebsiella pneumoniae*

Screening for L-methioninase (MGL) production was performed according to (Sundar and Nellaiah, 2013) using a modified M9 medium composed of (g/L): Na_2HPO_4 (6.0), KH_2PO_4 (3.0), NaCl (0.5), NH_4Cl (1.0), $MgSO_4$ (0.24), and $CaCl_2$ (0.01). The medium was supplemented with L-methionine (0.5%) as the sole sulfur source and phenol red (0.002%) as a pH indicator. Each bacterial isolate was inoculated into 10 ml of sterile medium in test tubes incubated at 37°C for 48 h under shaking conditions (120 rpm). After incubation, enzyme production was indicated by a color change in the medium from red to yellow, resulting from ammonia release during L-methionine degradation.

L-Methioninase activity assay

L-methioninase activity was determined [13] using the DTNB (5,5'-dithiobis-(2-nitrobenzoic acid)) method. The reaction mixture consisted of 0.5 ml of enzyme extract, 0.5 ml of L-methionine solution (20 mM prepared in 0.1 M phosphate buffer, pH 7.0), 0.5 ml of DTNB reagent (10 mM), and 1.0 ml of phosphate buffer (0.1 M, pH 7.0). The mixture was incubated at 37°C for 30 min., after which the reaction was terminated by adding 1.0 ml of 1 N HCl. The absorbance was measured at 412 nm using a UV–Vis spectrophotometer. Enzyme activity was calculated based on methanethiol production using the molar extinction coefficient of DTNB and expressed as µmol/min/ml.

Protein estimation

Protein concentration was determined using the Bradford assay. Briefly, 0.1 ml of enzyme sample was mixed with 5.0 ml of Bradford reagent and incubated at room temperature for 5 min. The absorbance was then measured at 595 nm. A standard calibration curve was constructed using bovine serum albumin (BSA) within the range of 0–100 µg/ml, and the protein concentration was calculated accordingly (fig. 1) [14].

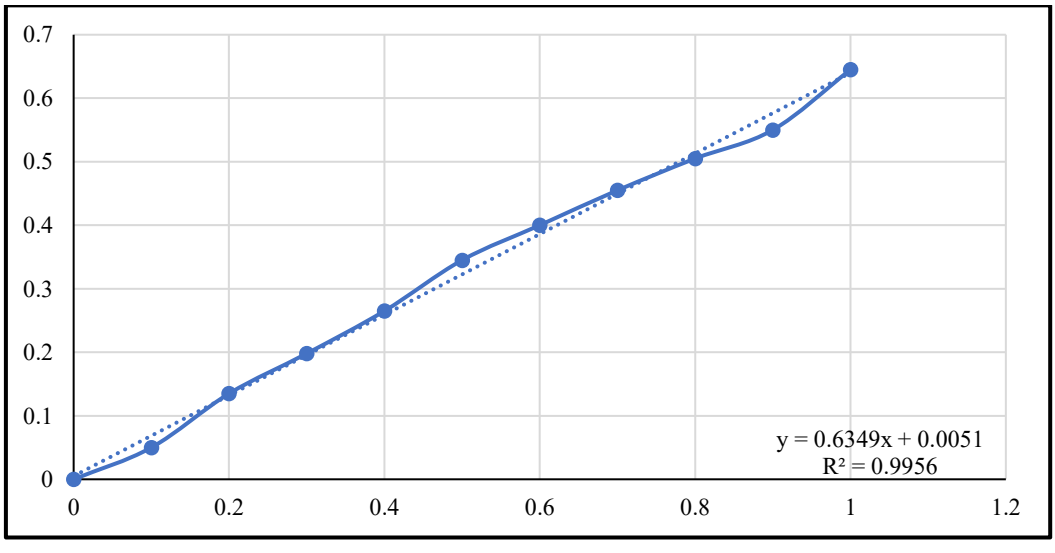


Figure (1): Standard curve of protein concentrations using bovine serum albumin

Optimization of enzyme production (carbon, nitrogen, pH, temperature and incubation periods)

Optimization of L-methioninase production was done in [15] in varying culture conditions in 100 ml Erlenmeyer flasks containing 50 ml of production medium. Different carbon sources were evaluated, including glucose, sucrose, and lactose (1% w/v), as well as nitrogen sources such as peptone, yeast extract, and ammonium sulfate (0.5% w/v). The effect of pH was examined within a range of 4–9, adjusted using HCl or NaOH, while temperature conditions were tested at 27, 30, 37, and 42°C. In addition, incubation periods of 12, 24, 36, and 48 h were investigated. Cultures were incubated under shaking conditions at 120 rpm. After incubation, the cultures were centrifuged at 6000 rpm for 15 min, and the supernatant was collected for enzyme activity determination. The optimal conditions were selected based on the highest enzyme activity observed.

Enzyme purification

The crude enzyme was subjected to ammonium sulfate precipitation at 40-70% saturation and incubated overnight at 4°C. The resulting precipitate was collected by centrifugation at 8000 rpm for 20 mins. and subsequently resuspended in 0.1 M of phosphate buffer (pH 7.0). The dialysis followed against the same buffer for 24 h at 4°C, with the buffer replaced every 6 hours. The dialyzed sample was further purified using DEAE-cellulose ion-exchange chromatography equilibrated with phosphate buffer. Proteins were eluted using a linear NaCl gradient (0–1 M) at a flow rate of 1 ml/min, and the fractions were collected and assayed for enzyme activity. The active fractions were pooled and subjected to gel filtration chromatography using Sephadex G-150 for further purification. Fractions with the highest enzyme activity were collected and stored at 4°C for subsequent analyses [16, 17].

Evolution of the cytotoxic potential of L-methioninase in lungs (A549), liver (HepG2) and normal (HdFn) cell lines

After the cell lines meantime, according to [18], A549, HepG2, and HdFn cells were cultured in 96-well plates at a density of 1×10^4 cells/well incubated for 24 h at 37°C in a humidified atmosphere containing 5% CO_2. Subsequently, the cells were treated with different concentrations of the purified enzyme (25, 50, 100, 200, and 400 µg/ml) and incubated for an additional 24hrs. After that, 20 µL of MTT solution (5 mg/ml) was added to each well and the plates were incubated for 4 h to allow for formazan crystal formation. The culture medium was then carefully removed, and 150 µL of DMSO was added to dissolve the formed crystals. Absorbance was measured at 575 nm using a microplate reader. Cell viability (%) was calculated in comparison with the untreated control cells.

$$\textbf{Total cell } \frac{\textbf{count}}{\textbf{mL}} = \textbf{Cell count} \times \textbf{Dilution factor (sample volume)} \times \mathbf{10^4}$$

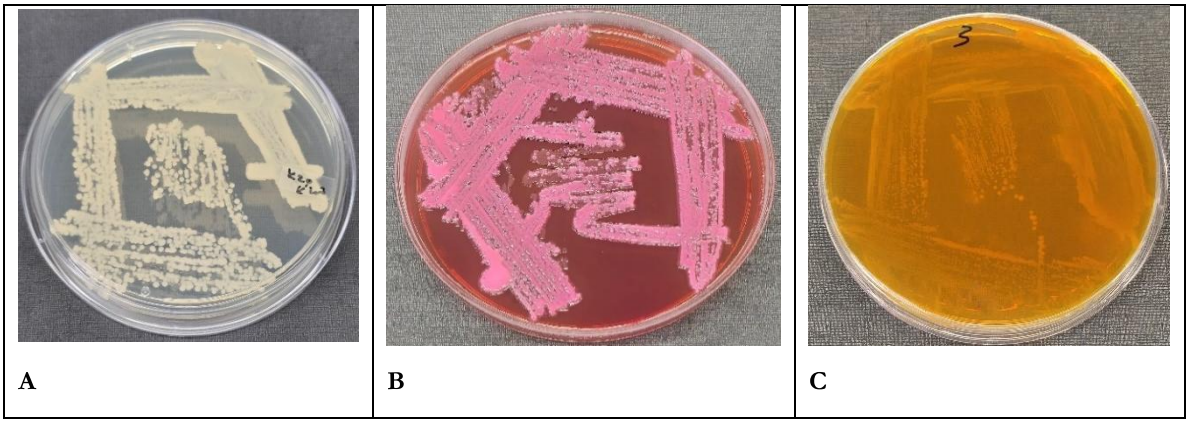
High-content screening test (Multiparametric assay)

High-content screening (HCS) analysis was conducted using fluorescence-based multiparametric assays. The cells were seeded in 96-well plates and treated with the selected concentrations of the purified enzyme. After incubation, the cells were stained with specific fluorescent dyes targeting nuclear components (Hoechst dye), mitochondrial membrane potential, and membrane permeability. Images were captured using an automated high-content imaging system, and quantitative analysis was performed using specialized image analysis software. The parameters of viable cell count, nuclear intensity, mitochondrial function, and membrane integrity were measured. This multiparametric approach enabled a comprehensive and detailed evaluation of cytotoxic mechanisms [19].

RESULTS

Isolation and identification of *Klebsiella pneumoniae* isolates

A total number of 150 clinical samples were processed, from which 27 bacterial isolates were successfully obtained based on the primary culture and purification steps. Preliminary identification using morphological and biochemical characteristics suggested that the majority of isolates belonged to the genus Klebsiella. Final confirmation using the VITEK-2 system identified the selected isolate as *K. pneumoniae*, used for further experimental analysis. Figures 2 and 3 explain the morphology of *K. pneumoniae* on Mueller Hiton agar, MacConkey agar and M9 media.



A B C

Figure (2): *Klebsiella pneumonia* on A) Mueller Hiton agar, B) MacConkey agar C) M9 media

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Patient Name: تهاني Patient ID: 2104
Location: Physician:
Lab ID: 2104 Isolate Number: 1

Organism Quantity:
Selected Organism : klebsiella pneumoniae

Source: **Collected:**

Comments:	

Identification Information	**Analysis Time:**	6.51 hours	**Status:**	Final
Selected Organism	98% Probability	**klebsiella pneumoniae**		
	Bionumber:	25010142545714		
ID Analysis Messages				

Biochemical Details

2	APPA	-	3	ADO	+	4	PyrA	+	5	lARL	-	7	dCEL	-	9	BGAL	+
10	H2S	-	11	BNAG	+	12	AGLTp	+	13	dGLU	+	14	GGT	+	15	OFF	+
17	BGLU	+	18	dMAL	+	19	dMAN	+	20	dMNE	+	21	BXYL	+	22	BAlap	-
23	ProA	-	26	LIP	-	27	PLE	+	29	TyrA	+	31	URE	-	32	dSOR	+
33	SAC	+	34	dTAG	+	35	dTRE	+	36	CIT	+	37	MNT	+	39	5KG	-
40	lLATk	+	41	AGLU	+	42	SUCT	+	43	NAGA	-	44	AGAL	-	45	PHOS	+
46	GlyA	+	47	ODC	-	48	LDC	+	53	lHISa	-	56	CMT	+	57	BGUR	-
58	O129R	+	59	GGAA	-	61	lMLTa	-	62	ELLM	+	64	lLATa	-			

Figure (3): *Klebsiella pneumonia* isolate identification by VITEK 2 system

Screening for L-Methioninase production

Screening of bacterial isolates on modified M9 medium supplemented with L-methionine revealed that several isolates showed positive L-methioninase production, as indicated by a distinct color change in the medium. This color shift reflects ammonia release resulting from L-methionine degradation. Among the screened isolates, sample no. 5 showed the highest enzyme-producing capability, with L-methioninase activity of 1.6 U/ml, protein concentration of 0.4 mg/ml, and specific activity of 4 U/mg protein. Therefore, this isolate was selected for subsequent analyses, suggesting a superior metabolic efficiency in the use of methionine as a sulfur source (Table 1).

Table (1): Activity, protein concentration and specific activity of L-Methioninase enzyme produced from *Klebsiella pneumoniae*

Sample no.	L- Methioninase activity (U/ml)	Protein concentration (mg/ml)	Specific activity (U/mg protein)
1	0.9	0.5	1.8
2	0.87	0.7	1.2
3	1	0.7	1.4
4	1.2	0.4	3
5	1.6	0.4	4

6	**1.5**	**0.5**	**3**
7	**0.8**	**0.5**	**1.6**
8	**0.9**	**0.4**	**2.25**
9	**0.85**	**0.6**	**1.4**
10	**1.3**	**0.4**	**3.2**

Enzyme activity and protein concentration

The selected isolate showed measurable L-methioninase activity as determined by the DTNB assay. Among the tested isolates, sample no. 5 showed the highest enzymatic activity, recording 1.6 U/ml, with a protein concentration of 0.4 mg/ml and a specific activity of 4 U/mg protein. Other isolates showed lower enzyme activities ranging from 0.8 to 1.5 U/ml, protein concentrations ranging from 0.4 to 0.7 mg/ml, and specific activities ranging from 1.2 to 3.2 U/mg protein. These results suggest that sample no. 5 possessed superior L-methioninase-producing capability than the other isolates. Moreover, protein quantification using the Bradford assay supported the enzymatic activity results, confirming that the increased activity was associated with efficient enzyme production rather than assay variability. This correlation reflects effective biosynthesis and secretion of L-methioninase by the selected isolate (Table 1).

Optimization of enzyme production

Optimization studies showed that L-methioninase production was significantly influenced by environmental and nutritional factors. Among the tested carbon sources, glucose supported the highest enzyme yield with a specific activity of approximately 4.7 U/mg protein, followed by sucrose and lactose with about 4.5–4.6 U/mg protein, whereas maltose and starch showed lower activities of approximately 3.5 and 2.7 U/mg protein, respectively. Regarding nitrogen sources, tryptone showed the highest specific activity of approximately 5.1 U/mg protein, followed by peptone and yeast extract with about 4.8–4.9 U/mg protein, while malt extract and NH_4Cl showed lower activities of approximately 3.5 and 3.1 U/mg protein. The optimum pH was 7.0, at which the specific activity reached approximately 5.8 U/mg protein, while activity decreased at acidic and alkaline pH values, reaching about 2.0 U/mg protein at pH 4.0 and 1.5 U/mg protein at pH 9.0. Similarly, the optimum temperature was 37°C, with a specific activity of approximately 6.0 U/mg protein, whereas lower activity was observed at 27°C, 32°C, and 42°C, recording about 4.0, 5.0, and 2.3 U/mg protein, respectively. The incubation period also affected enzyme production, with maximum activity observed after 24 h of incubation, reaching approximately 6.0 U/mg protein, followed by a gradual decrease at 36 h and 48 h, recording about 5.0 and 3.0 U/mg protein, respectively. These findings suggest that optimal L-methioninase production is favored under near-neutral pH, mesophilic temperature, and appropriate nutritional conditions, highlighting the importance of optimizing culture parameters for maximal enzyme yield (Figures 4–8).

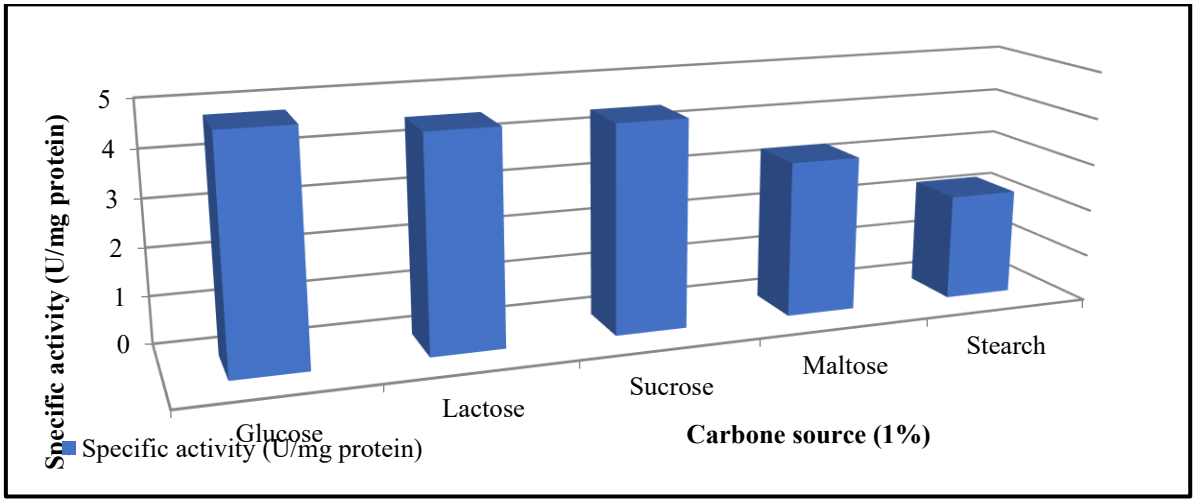


Figure (4): Optimum condition for different carbon sources for L-methioninase production

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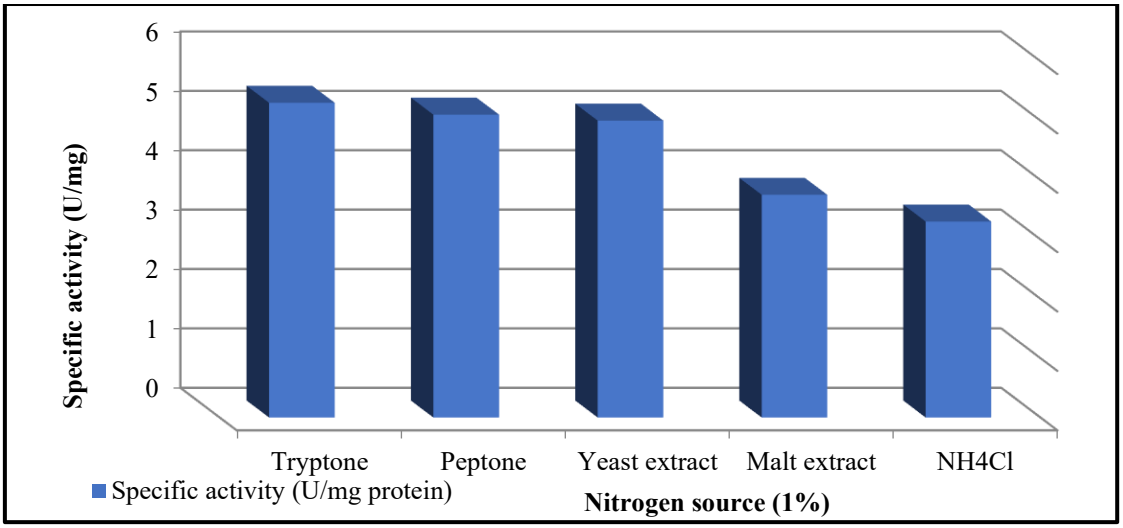


Figure (5): Optimum condition for different nitrogen sources for L-methioninase production

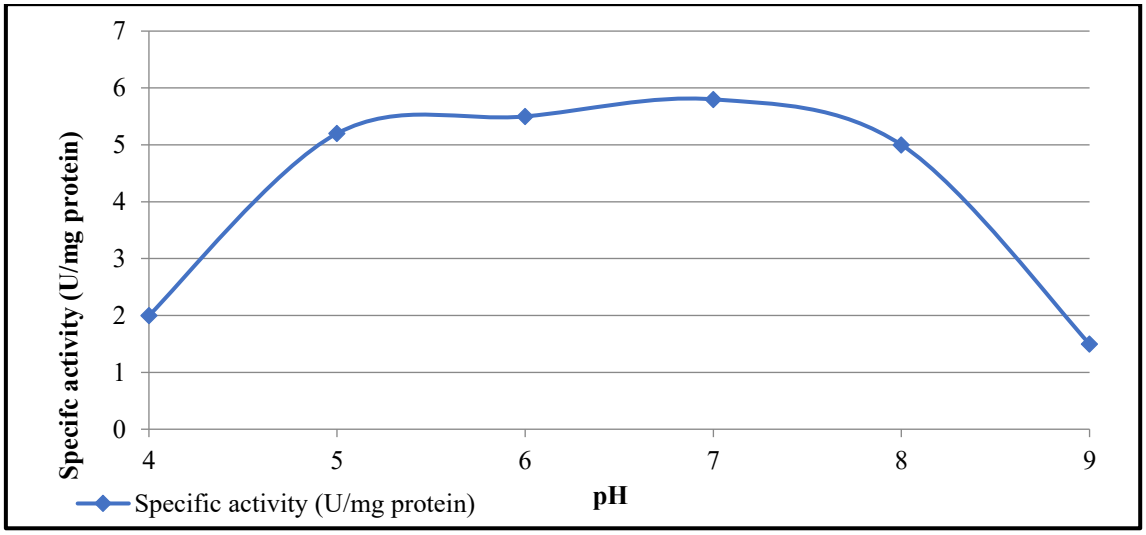


Figure (6): Optimum condition for different pH ranges for L-methioninase production

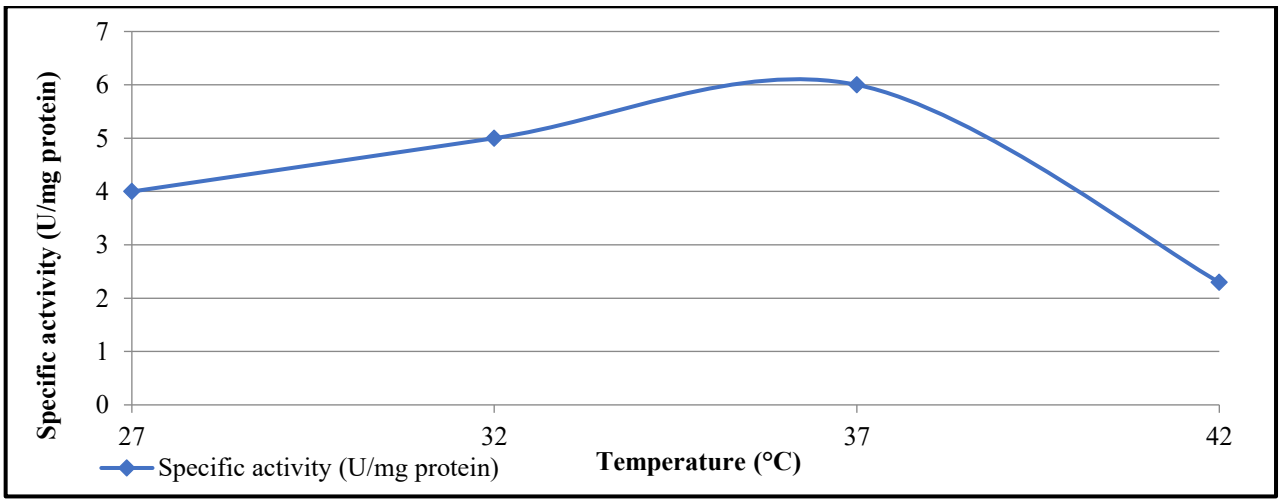


Figure (7): Optimum condition for different temperatures for L-methioninase production

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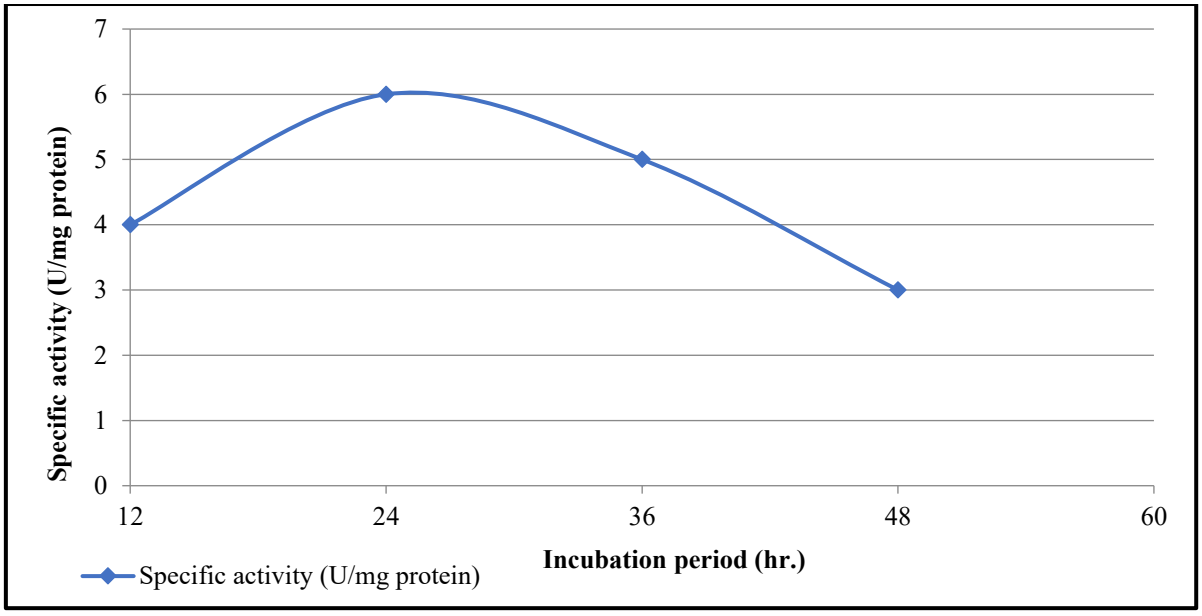


Figure (8): Optimum condition for incubation period for L-methioninase production

Partial purification of L-methioninase resulted in a progressive improvement in enzyme activity and specific activity throughout the purification steps. Ammonium sulfate precipitation effectively concentrated the enzyme, with the highest activity observed at 60% saturation, where enzyme activity increased from 2.4 U/ml in the crude extract to 7.0 U/ml, and specific activity increased from 6 U/mg protein to 14 U/mg protein. Subsequent purification using DEAE-cellulose ion-exchange chromatography further improved enzyme purity, yielding an enzyme activity of 5.5 U/ml and a specific activity of 36.6 U/mg protein, with a purification fold of 6.1 and a yield of 73.3%. Final purification by Sephadex G-150 gel filtration produced the highest specific activity, reaching 55.5 U/mg protein, with enzyme activity of 5.0 U/ml, purification fold of 9.2, and yield of 58.3%. These results prove the successful removal of contaminating proteins and enrichment of the target enzyme. Therefore, the applied purification strategy was effective in enhancing the purity and catalytic efficiency of L-methioninase produced by *K. pneumoniae*, as shown in Tables (2 and 3) and Figures (7 and 8).

Table (2): Effect of different ammonium sulfate saturations on partial purification of L-Methioninase produced from *Klebsiella pneumoniae*

Precipitation with ammonium sulfate (%)	Activity U/ml)	Specific activity (U/mg protein)
Crude Enzyme	2.4	6
40	4.5	10
50	5.0	12.5
60	**7.0**	**14**
70	5.5	13

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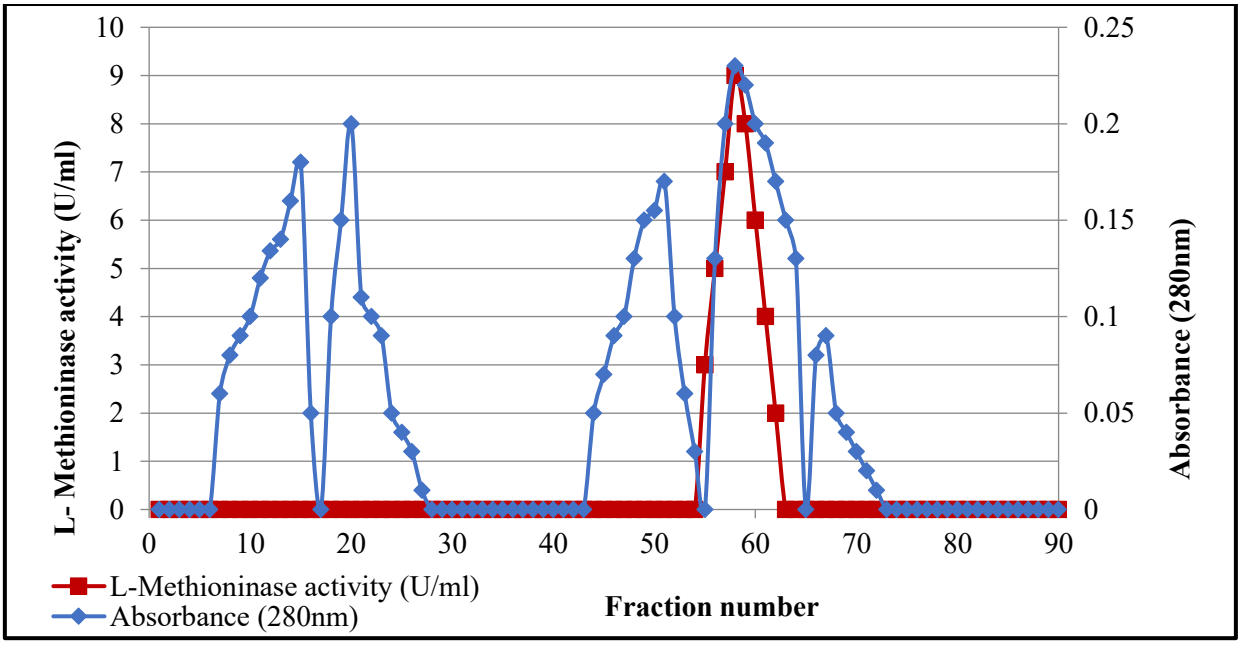


Figure (7): Ion exchange chromatography of L-methioninase using DEAE – cellulose column (3×20 cm) equilibrated with potassium phosphate buffer (pH7) with a flow rate of (30 ml/hr.)

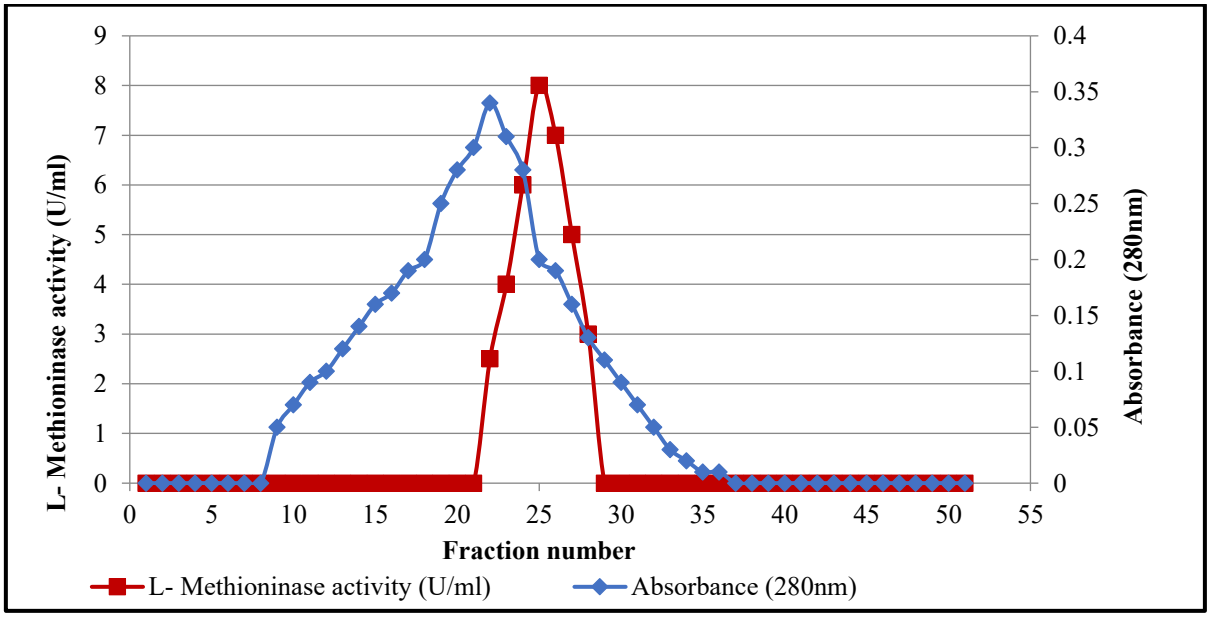


Figure (8): Gel filtration chromatography of L-methioninase using Sephadex G– 150 column (2×40 cm) equilibrated with (0.5 M) potassium phosphate buffer (pH7)

Table (3): Purification steps of L-Methioninase produced from *Klebsiella pneumoniae*

Purification step	Volume (ml)	Enzyme activity (U/ml)	Protein concentration (mg/ml)	Specific activity (U/mg)	Total activity (U)	Purification (folds)	Yield (%)
L- Methioninase Crude enzyme	75	2.4	0.4	6	180	1	100

Ammonium sulphate precipitation (60%)	**20**	**7**	**0.5**	**14**	**140**	**2.3**	**77.7**
DEAE-cellulose	**24**	**5.5**	**0.15**	**36.6**	**132**	**6.1**	**73.3**
Sephadex- G150	**21**	**5**	**0.09**	**55.5**	**105**	**9.2**	**58.3**

Following purification, the enzyme was characterized to determine its optimal temperature and pH conditions. L-methioninase showed maximum activity at 37°C and pH 7.0, indicating stability and efficiency under near-physiological conditions (Figures 9-12).

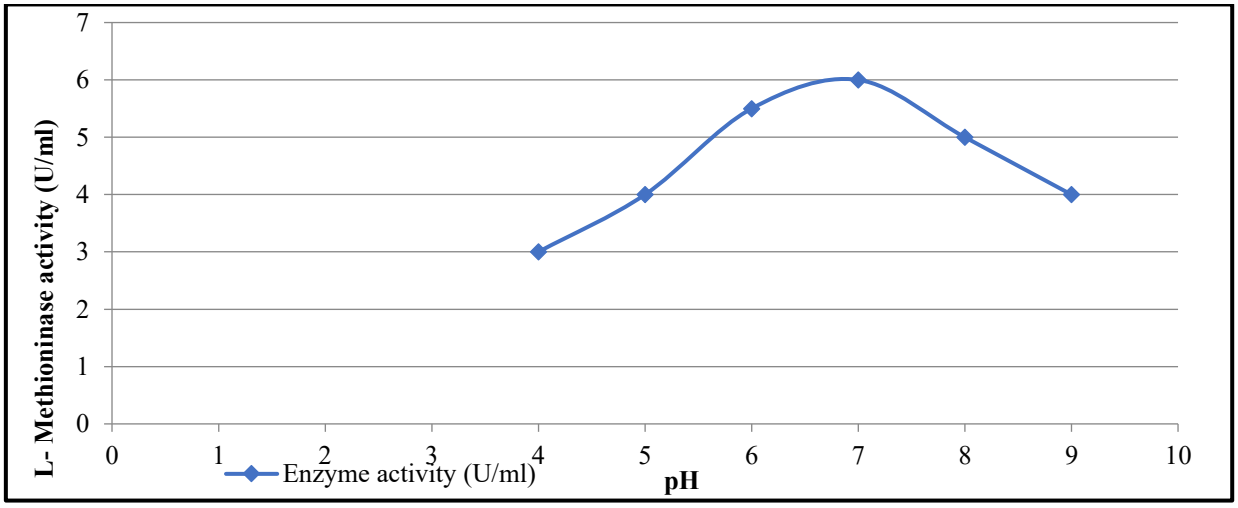


Figure (9): Effect of pH on activity of purifying the L-Methioninase produced from *Klebsiella pneumoniae*

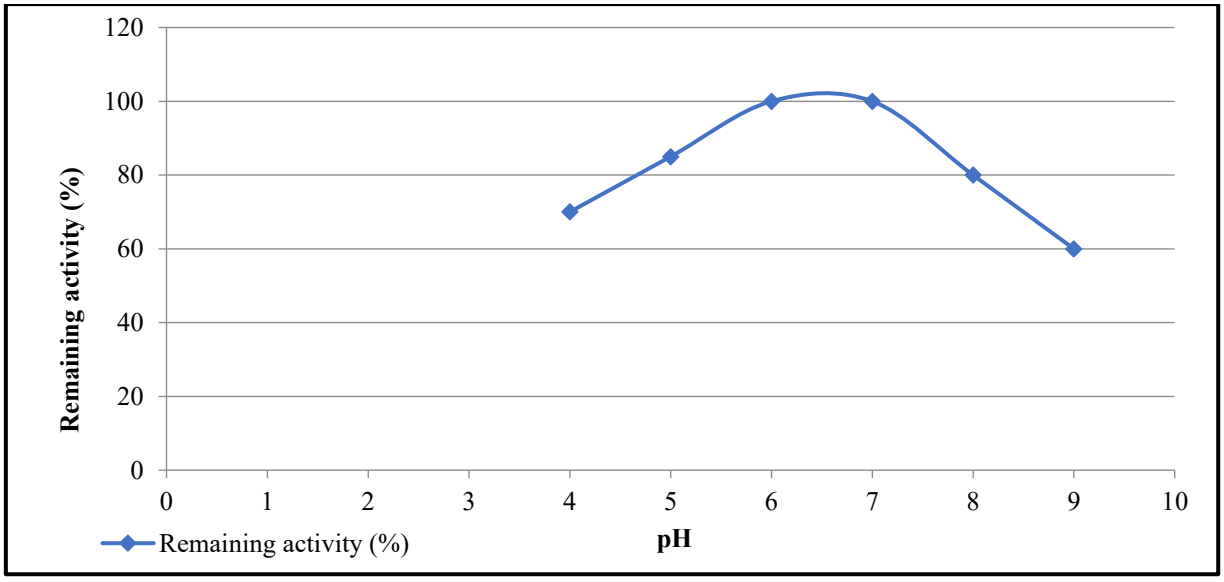


Figure (10): Effect of pH on stability of purifying the L-Methioninase produced from *Klebsiella pneumoniae*

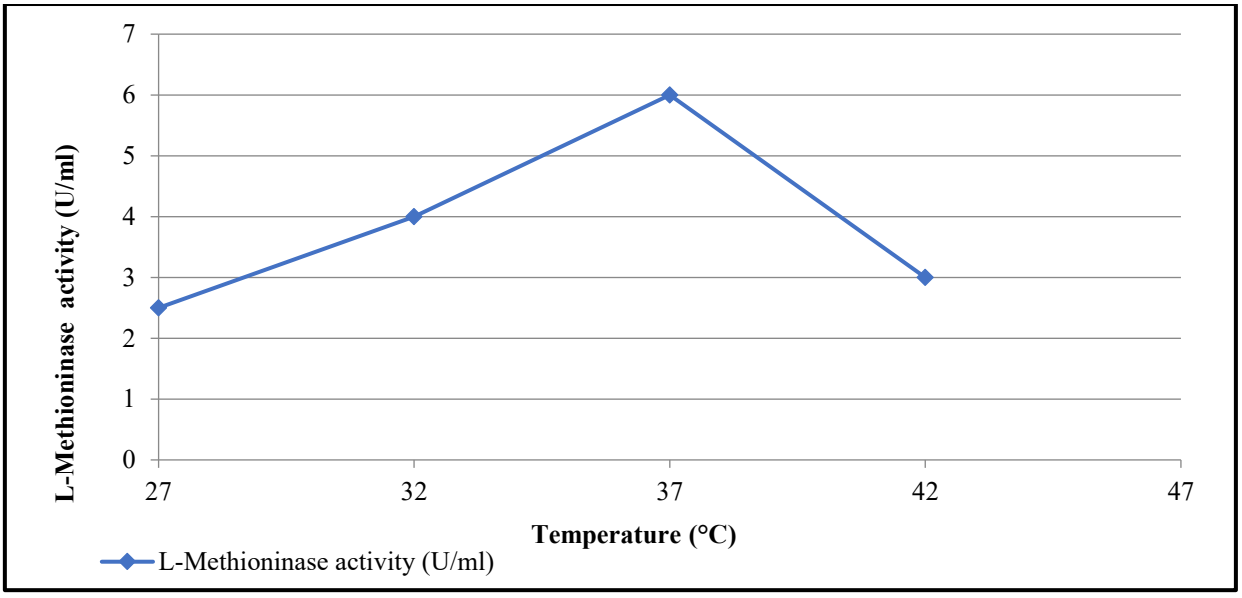


Figure (11): Effect of temperature on activity of purifying the L-Methioninase produced from *Klebsiella pneumoniae*

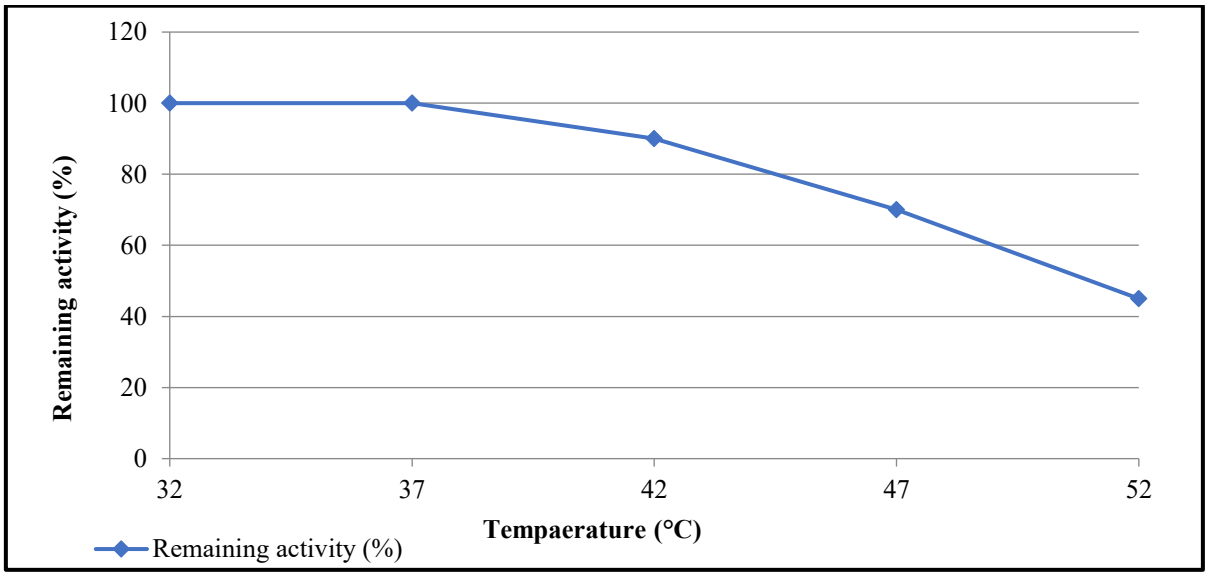


Figure (12): Effect of temperature on stability of purifying the L-Methioninase produced from *Klebsiella pneumoniae*

Cytotoxic potential of L-methioninase on A549 and HepG2 cancer cell line

The cytotoxic activity of purified L-methioninase was investigated against human liver carcinoma cells (HepG2) and human lung adenocarcinoma cells (A549), with human dermal fibroblasts (HdFn) used as a normal cell line control. The MTT assay results showed a concentration-dependent reduction in the viability of both cancer cell lines after 24 hours of treatment with increasing concentrations of L-methioninase (25–400 µg/ml) (Table 4, Figure 13).

A marked decrease in HepG2 and A549 cell viability was observed at higher enzyme concentrations, particularly at 400 µg/ml, where cell viability declined to 36.903 ± 0.341% and 48.909 ± 0.570%, respectively. In contrast, HdFn normal cells maintained relatively higher viability under the same treatment conditions, recording 78.357 ± 0.438% and 83.808 ± 0.753%

viability, respectively. These findings suggested that purified L-methioninase showed selective cytotoxicity toward cancer cells while exerting lower toxic effects on normal cells.

The calculated IC_{50} values further supported this selective anticancer activity. HepG2 cells showed the highest sensitivity to L-methioninase with an IC_{50} value of 94.27 µg/ ml, followed by A549 cells with an IC_{50} value of 118.2 µg/ ml. In comparison, normal HdFn cells showed significantly higher IC_{50} values of 158.3 µg/ ml and 168.9 µg/ml, indicating greater resistance to enzyme treatment. The enhanced susceptibility of cancer cells may be attributed to their dependence on exogenous methionine for rapid proliferation and metabolic activity, making them more vulnerable to the methionine depletion induced by L-methioninase.

Table (4): Cytotoxic effect of L-methioninase on human liver cancer cell line (hepG2) and human lung adenocarcinoma cell line (A549) with human dermal fibroblasts (HdFn) as normal cell line using MTT assay for 24Hrs.

Conc. (µg/ ml)	Cell viability L-methioninase (Mean±SD)			
	HdFn	hepG2	HdFn	A549
400	78.357±0.438	36.903±0.341	83.808±0.753	48.909±0.570
200	82.326±0.841	50.249±0.696	87.847±0.504	57.875±0.528
100	87.925±0.606	62.088±0.747	93.525±0.406	73.011±0.595
50	91.244±0.709	78.253±0.601	95.525±0.406	84.954±.686
25	95.728±0.937	89.778±0.451	97.209±0.930	91.692±0.548

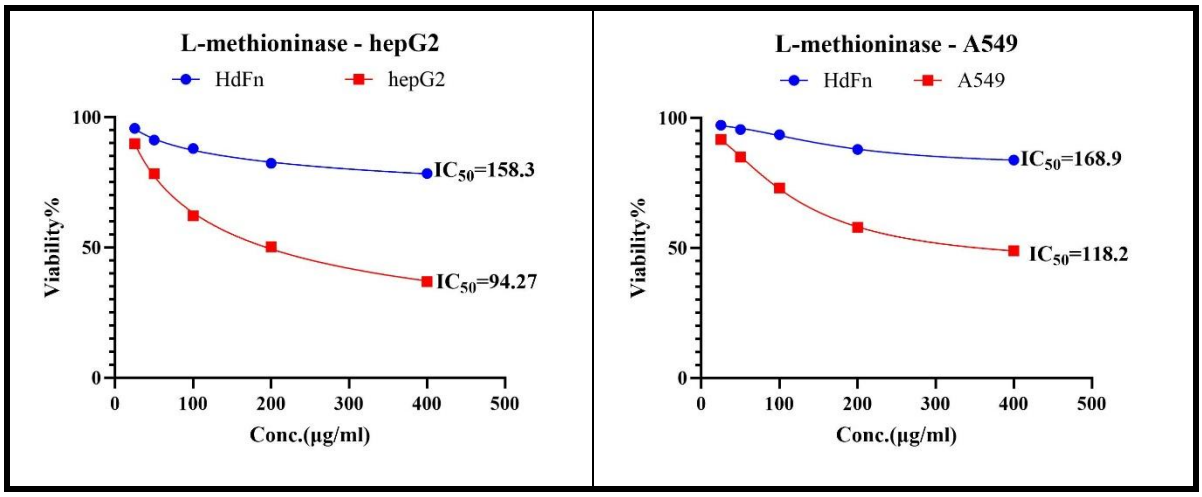


Figure (13): Cytotoxic effect of L-methioninase on human liver cancer cell line (hepG2) and human lung adenocarcinoma cell line (A549) with human dermal fibroblasts (HdFn) using MTT for 24Hrs.

High-content screening (HCS) analysis

High-content screening (HCS) analysis was performed to evaluate the cytotoxic effects of purified L-methioninase, also known as L-methionine γ-lyase, a pyridoxal phosphate-dependent enzyme, on the tested cancer cell lines, particularly HepG2 and A549 cells. The analysis targeted cellular responses through multiparametric assessment of viable cell count, nuclear changes, mitochondrial function, membrane permeability, and cytotoxicity-related alterations. The results suggested that exposure to increasing concentrations of L-methioninase (25–200 µg/mL) triggered progressive alterations in several cellular biomarkers associated with proliferation arrest, mitochondrial dysfunction, membrane destabilization, and apoptosis-associated cell damage (Table 4, Figure 14). Moreover, these alterations became more pronounced at higher L-methioninase concentrations, indicating a clear concentration-dependent cytotoxic response.

One of the earliest and most prominent changes observed was the significant reduction in viable cell count (VCC), which declined progressively following L-methioninase treatment. Untreated cells showed a VCC value of 4679.5 ± 12.021, whereas treatment with 100 and 200 µg/mL L-methioninase reduced viable cell count to 3121 ± 60.812 and 2447.5 ± 98.288, respectively (Table 4, Figure 14). This marked decrease shows a strong inhibitory effect of L-methioninase on cellular proliferation and survival.

Analysis of total nuclear intensity (TNI) revealed a progressive elevation at higher L-methioninase concentrations, increasing from 755.5 ± 9.192 in untreated cells to 829.5 ± 2.121 and 943.5 ± 19.092 at 100 and 200 µg/mL, respectively, as shown in Table (4) and Figure (14). Elevated nuclear intensity is commonly associated with chromatin condensation and nuclear fragmentation, as the characteristic features of apoptosis. Therefore, the observed TNI changes suggest that L-methioninase treatment may induce nuclear alterations related to apoptotic cell death.

Similarly, cell membrane permeability (CMP) analysis showed relatively stable values at lower L-methioninase concentrations, indicating that mild exposure did not immediately compromise plasma membrane integrity. However, higher concentrations produced noticeable membrane-associated alterations, reflecting progression toward advanced cellular injury (Table 4, Figure 14). Consequently, the results of membrane permeability suggest the loss of membrane selectivity and structural integrity under severe cytotoxic stress [20].

A pronounced reduction was also observed in mitochondrial membrane potential (MMP) following treatment with elevated concentrations of L-methioninase. MMP values decreased from 475 ± 19.799 in untreated cells to 340.5 ± 17.678 and 265 ± 7.071 at 100 and 200 µg/mL, respectively, as presented in Table (4) and Figure (14). The loss of mitochondrial membrane potential is considered an important indicator of mitochondrial dysfunction which is closely associated with the activated intrinsic apoptotic pathway. Similar observations have been reported in the literature, showing that methionine depletion may impair mitochondrial function, ATP production, and redox balance in cancer cells [21].

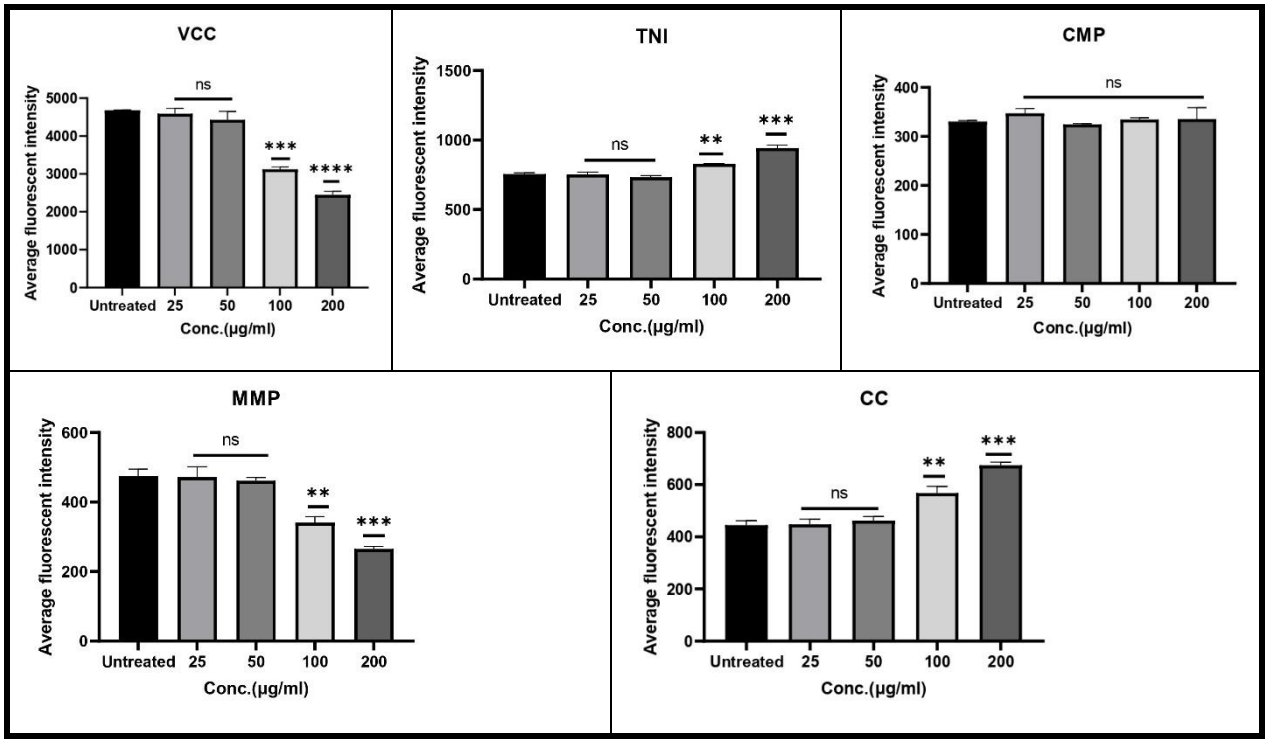
In addition, the cytotoxicity coefficient (CC) increased following L-methioninase exposure, rising from 445 ± 16.971 in untreated cells to 567.5 ± 26.163 and 674.5 ± 12.021 at 100 and 200 µg/mL, respectively (Table 4, Figure 14). This elevation reflects enhanced cellular injury and supports the cytotoxic effect of L-methioninase on treated cancer cells [22].

Collectively, the HCS results suggest that purified L-methioninase exerts its anticancer effect through multiple interconnected cellular responses rather than a single cytotoxic pathway. The simultaneous reduction in viable cell count, alteration in nuclear intensity, disruption of mitochondrial membrane potential, and elevation in cytotoxicity coefficient indicate apoptosis-associated cellular damage (Table 4, Figure 14). These findings are consistent with previous studies showing that methionine depletion can induce metabolic stress, mitochondrial dysfunction, and reduced survival in methionine-dependent cancer cells [23-26]. Therefore, the multiparametric HCS approach provides deeper mechanistic insight into the selective anticancer activity of L-methioninase produced by *K. pneumoniae* and supports its potential as a promising therapeutic candidate for targeting methionine-dependent malignancies.

Table (4): High-content screening analysis of cellular parameters following treatment with purified L-methioninase from *Klebsiella pneumoniae*

Conc (µg/ml)	VCC	TNI	CMP	MMP	CC
	Mean ± SD	Mean ± S	Mean ± S	Mean ± S	Mean ± S
untreated	4679.5±12.021	755.5±9.192	330.5±2.121	475±19.799	445±16.971
25	4591±134.350	753.5±17.678	347.5±9.192	471.5±30.401	448.5±19.092
50	4425±227.688	733.5±12.021	324.5±2.121	461±9.899	462.5±14.849
100	3121±60.812	829.5±2.121	334.5±3.536	340.5±17.678	567.5±26.163
200	2447.5±98.288	943.5±19.092	335.2±3.335	265±7.071	674.5±12.021

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Figures (14): High-content screening analysis of cellular parameters following treatment with purified L-methioninase from *Klebsiella pneumoniae*

ns: non-significant, ** <0.01, ** <0.001

DISCUSSION

Recent advances in microbial biotechnology emphasize the increasing importance of microbial enzymes as multifunctional therapeutic biomolecules, particularly in cancer therapy and metabolic targeting. In the present study, purified L-methioninase produced from *Klebsiella pneumoniae* showed selective cytotoxic activity against HepG2 and A549 cancer cell lines, while normal HdFn cells were less affected. This selective response supports the concept that methionine depletion may represent an effective metabolic strategy for targeting tumor cells that exhibit high methionine dependence [27].

The successful production of L-methioninase from *K. pneumoniae* is consistent with previous research findings indicating that microorganisms can synthesize biologically active extracellular enzymes when appropriate nutritional and environmental conditions are met. The optimization findings suggest that enzyme biosynthesis is highly dependent on culture conditions such as pH, temperature, incubation period, and nutrient composition. Near-physiological conditions appeared to favor enzyme production, which may reflect the compatibility of these parameters with bacterial metabolic activity and protein synthesis. The use of readily metabolizable carbon and nitrogen sources may also enhance enzyme yield by supporting energy generation, amino acid availability, and cellular growth [28].

The purification strategy used in this study pinpoint the importance of sequential purification steps in improving enzyme quality. Ammonium sulfate precipitation served as an effective initial concentration step, while ion-exchange and gel filtration chromatography further enhanced enzyme purity by reducing contaminating proteins. The progressive improvement in specific activity after purification shows that the final enzyme preparation was enriched with active L-methioninase, supporting the reliability of subsequent biological assays. It really matters as the observed cytotoxic effects are more likely attributable to the purified enzyme itself rather than nonspecific bacterial metabolites or impurities [29].

The selective cytotoxicity observed in cancer cells may be explained by differences in methionine metabolism between malignant and normal cells. Many tumor cells depend strongly on exogenous methionine to maintain rapid proliferation, methylation reactions, nucleotide biosynthesis, and redox balance. Thus, the enzymatic depletion of methionine can impose severe metabolic stress on cancer cells, leading to lower growth and survival. In contrast, normal cells generally possess

greater metabolic flexibility, which may explain their comparatively higher tolerance to L-methioninase treatment. This difference supports L-methioninase potential as a metabolic-targeting enzyme rather than a nonspecific cytotoxic agent [30].

The MTT assay findings were further supported by HCS analysis, which provided a broader mechanistic view of enzyme-induced cellular damage. Unlike conventional viability assays, HCS allows simultaneous evaluation of several cellular parameters, including viable cell count, nuclear changes, mitochondrial function, membrane integrity, and cytotoxicity-related alterations. The observed cellular responses suggest that L-methioninase-induced cytotoxicity is not limited to the inhibition of metabolic activity, but involves multiple interconnected events associated with mitochondrial dysfunction, nuclear alteration, membrane destabilization, and apoptosis-related responses [31].

Reduced VCC may be associated with the methionine depletion caused by the enzymatic degradation of extracellular methionine. Such depletion may impair protein synthesis, nucleotide biosynthesis, and essential metabolic pathways required for rapid tumor cell growth. This interpretation is consistent with previous studies reporting that methionine restriction can selectively suppress tumor cell proliferation because many cancer cells show a high dependence on exogenous methionine compared with normal cells [32].

Mitochondrial impairment appears to represent a major mechanism underlying the cytotoxic effect of L-methioninase. Since mitochondria are essential for ATP production, oxidative phosphorylation, and regulation of intrinsic apoptosis, disruption of mitochondrial function can strongly affect cancer cell survival. Methionine depletion may disturb mitochondrial metabolism and increase oxidative stress, thereby contributing to mitochondrial membrane potential loss and activation of apoptosis-associated pathways. These may explain the reduced cancer cell viability observed after enzyme treatment [33].

The nuclear and membrane-associated changes observed by HCS show that enzyme treatment may trigger advanced cellular stress. Increased nuclear alterations may reflect chromatin condensation and nuclear damage, while changes in membrane-related parameters suggest the loss of membrane integrity during later stages of cell injury. Together, these findings show that L-methioninase induces a multiphasic cytotoxic response involving metabolic deprivation, mitochondrial damage, nuclear stress, and membrane disruption. Such combined mechanisms strengthen its potential role as an anticancer biomolecule [34].

When considered in relation to current concepts in metabolic oncology, the present findings support the growing interest in targeting tumor-specific metabolic vulnerabilities. Methionine depletion strategies are increasingly recognized because they interfere with the pathways highly active in many cancer cells. Microbial L-methioninase may, therefore, be a promising candidate for future anticancer approaches, either as an independent metabolic therapy or in combination with chemotherapy, radiotherapy, or immunotherapy to enhance tumor stress and therapeutic selectivity [35].

Future studies should evaluate the safety and efficacy of purified L-methioninase in different populations of patients, including pediatric and adult cancer models. Furthermore, in vivo studies and clinical investigations are warranted to validate its therapeutic potential and establish its safety profile before clinical use.

CONCLUSION

The present study successfully obtained L-methioninase from *Klebsiella pneumoniae*, with isolate no. 5 showing the highest enzyme activity. Optimal enzyme production was achieved at pH 7.0, 37°C, and 24 h incubation, indicating favorable activity under near-physiological conditions.

Purification by ammonium sulfate precipitation, DEAE-cellulose, and Sephadex G-150 improved the enzyme specific activity to 55.5 U/mg. The purified enzyme showed dose-dependent cytotoxicity against HepG2 and A549 cancer cell lines, while normal HdFn cells were less affected.

HCS analysis confirmed the reduced viable cell count, mitochondrial dysfunction, nuclear alteration, and increased cytotoxicity, supporting the selective anticancer effect of the enzyme.

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