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Unravelling the outcome of L-glutaminase produced by *Streptomyces sp*. strain 5 M as an anti-neoplasm activity

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Abstract

Background Actinomycetes are a well-known example of a microbiological origin that may generate a wide variety of chemical structures. As excellent cell factories, these sources are able to manufacture medicines, agrochemicals, and enzymes that are crucial.

Results In this study, about 34 randomly selected *Streptomyces* isolates were discovered in soil, sediment, sea water, and other environments. Using a qualitative fast plate assay, they were tested for L-glutaminase production, and nine of them produced a significant amount of pink L-glutamine. *Streptomyces* sp. strain 5 M was identified by examining the 16S rRNA gene in the promising strain G8. A pH of 7.5, an incubation temperature of 40 °C, and the use of glucose and peptone as the carbon and nitrogen sources, respectively, produced the highest quantities of L-glutaminase. The molecular weight of the isolated L-glutaminase was estimated to be 52 kDa using SDS-PAGE analysis. At pH 7.5 and Temp., 40 °C, the isolated enzyme exhibited its highest levels of stability and activity. The isolated enzyme's K_m and V_{max} values were 2.62 mM and 10.20 U/ml, respectively. Strong toxicity against HepG-2, HeLa, and MCF-7 was observed due to the anticancer properties of the isolated L-glutaminase.

Conclusion Our findings include the discovery of *Streptomyces* sp. strain 5 M, which yields a free L-glutaminase and maybe a possible applicant for extra pharmacological investigation as an antineoplastic drug.

Keywords L-glutaminase, Cytotoxicity, Marine water, *Streptomyces sp.*, Strain 5 M.

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Background

One well-known example of a microbiological source that may produce a variety of chemical structures is actinomycetes, which can produce commercial products including enzymes, agrochemicals, and very valuable pharmaceuticals [7]. Because they produce extracellular enzymes, actinomycetes are regarded as preferable enzyme providers [35].

Actinomycetes produce a variety of enzymes that have been utilized as medications, including L-glutamine amid hydrolase, also known as L-glutaminase [6]. L-glutaminase, an enzyme that hydrolyzes the amide link of L-glutamine to provide glutamate and ammonium ions, has been identified as having anti-neoplasmic properties [31].

L-glutaminase plays a significant role in prokaryotic and eukaryotic nitrogen metabolism [5]. Recently, L-glutaminase has gained a lot of interest due to its widespread application in medications and its ability to combat leukemia [8].

Certain kinds of malignant cells are characterized by an elevated level of glutamine consumption. Experimental treatments that deprive tumor cells of L-glutamine have been developed based on this characteristic [10].

Preventing the tumor cells from absorbing glutamine is one possible method of delaying the growth of tumors; L-glutaminase is an effective tool for this. Tumor cells are selectively deprived in contrast to normal cells because they lack completely functional glutamine biosynthesis machinery [50].

At the same route, *Streptomyces* are well recognized to produce valuable medicines, particularly antibiotics and anticancer mediators, and industrial products like enzymes for revenue-generating discovery platforms [14]. A lot of information has been presented for microbial L-glutaminase producers like *Streptomyces rimosus*, *Streptomyces avermitilis,* and *Streptomyces labedae* [1].

Therefore, the goal of the current study was to identify and screen some actinomycetes for the production of L-glutaminase from soil samples that were gathered from different places around Egypt. The pure enzyme's biological activities were examined and its characteristics were described. Additionally, examine L-glutaminase's antitumor properties against different tumor cell lines had been performed.

Materials and methods

Compounds and sample collection

All analytical-grade chemicals and medium, acquired from Sigma-Aldrich in the US, were used in this investigation. Soil samples were collected from Giza, Dakahlia, and Gharbia between August and December 2021, while water samples were collected from Alexandria and the South Sinai Sea in sterile polyethylene bags before being transported straight to the laboratory for examination. The soil strain was isolated using the serial dilution agar plate technique. Each colony of the soil strain that presented morphologically was sub-cultured, purified, and kept at 4 °C until L-glutaminase analysis after the plates were incubated at 28 °C.

Isolation of *streptomyces* strains

A Millipore membrane filter (0.22 µm) was used to filter the two samples (100 mL each) after they had been serially diluted. The prepared dilutions as 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} were then separately spread out onto an agar medium in 100 µL. In order to promote the development of organisms with slow growth rates, the inoculation plates were then incubated for two weeks at 28 °C. Agar slants with the same composition as the primary plating medium were used to isolate streptomycetes. After isolation, starch nitrate agar was used to purify the isolates.

About 2% agar, at pH 6.5 (w/v), 2% starch, 1% peptone, 0.33% K_2HPO_4, and 0.5% NaCl were the ingredients of the agar. Throughout the purification procedure, streaking was used to inoculate many agar plates with bacteria. For sub-culturing on agar slants, we selected individual colonies [22].

Selection of strains for L-glutaminase assembly

The ability of the bacterial strains to produce L-glutaminase was assessed using the rapid plate assay method [30]. Agar medium with 0.07% phenol red and a pH of 7.0 served as the experimental material. After two to three days of incubation at 37 °C, the pink zone surrounding the colonies was measured to calculate an enzyme index [40].

Determination of L-glutaminase activity

With a few slight modifications, we follow the Thompson and Morrison assay [47], using the Nesslerization method to measure L-glutaminase activity. The standard included 1 mL of the crude enzyme, 0.1 mL of pyridoxal phosphate, and 1 mL of 1% L-glutaminase in (0.2 µM) phosphate buffer at pH 7.0, and was incubated for 1 h at 30 °C.

The reaction was stopped by adding 0.5 mL of (1.5 M) tri-chlor-acetic acid, and the mixture was centrifuged for 10 min at 4025 g. The absorbance of a mixture consisting of 0.1 mL of supernatant, 3.7 mL of deionized water, and 0.2 mL of Nessler's reagent was measured using a UV/VIS-2401 PC visible spectrophotometer (Shimadzu, Kyoto, Japan).

Protein determination

The enzyme concentration was determined by applying the Bradford et al. technique [38]. A stock solution

of 1000 g/mL of bovine serum albumin was prepared as a reference protein. Each sample was injected with the Folin-Ciocalteu reagent, and after 30 min of incubation, the absorbance at 660 nm was measured [20, 23].

Identification tests for isolates of active streptomycetes

The spore chain morphology of cultures grown on inorganic salt-starch agar for 14 days was examined under a light microscope [51]. Spores and their surface ornamentation were examined using a transmission electron microscope [34]. Diffusible pigments were identified using glycerol-asparagine agar, the color of the spore mass, and the pigmentation of the substrate mycelium [16, 41]

Phylogenetic analysis

Molecular genetic identification was done to pinpoint the precise phylogenetic location of the chosen strain, 5 M. The partial 16S ribosomal RNA gene sequence of the *Streptomyces* sp. strain 5 M (800 bp) was searched against the Reference RNA Sequence database (refseq_rna) using the National Center for Biotechnology Information (NCBI) BLAST (Basic Local Alignment Search Tool) [3]. Also, it was searched against a quality-controlled 16S rRNA gene sequences database called EzBioCloud (https://www.ezbiocloud.net) [55], database version 2023.08.23. The highest 50 EzBioCloud hits were downloaded (Supplementary Table 1) and the 16S rRNA sequences were aligned with MUSCLE [15], in MEGA 11 [45], and the longer sequences were trimmed to fit the length of the query (strain 5 M).

The alignment was then used to construct a maximum likelihood (ML) phylogeny in IQ-TREE (http://iqtree.cibiv.univie.ac.at) [48, 53]. The optimal model of sequence evolution (SE) was selected using ModelFinder [26]. Branch support was estimated by performing Ultrafast Bootstrap approximation (UFBoot) [24] of 1000 replicate alignments and single branch tests of 1000 replicates per branch (SH-aLRT) [21].

The phylogenetic tree was viewed and edited on the Interactive Tree of Life version 6 (iTOL; https://itol.embl.de) [29]. The sequencing data for this bacterial strain has the accession number #OL913064. *Streptantibioticus parmotrematis* strain Ptm05 16S ribosomal RNA gene (NR_181850.1) was added to the alignment and the tree as an outgroup because it was the closest relative (95.89% similarity) in the Reference RNA Sequence database (refseq_rna) to the *Streptomyces* sp. strain 5 M from outside the genus, and present as a complete sequence.

Purification of L-glutaminase

The crude enzyme was treated with ammonium sulfate to get a 70% saturation. The mixture was stored overnight at 4 °C and then centrifuged for 20 min at 4025 g. To eliminate the salts (pH 7.4), the precipitate was liquefied in the proper volume of 50 mM Tris–HCl buffer and dialyzed versus the same buffer for an entire night at 4 °C. The dialyzed fraction was loaded onto a Sephacryl S-300 column (100 X 1.6 cm) that had been pre-equilibrated with (0.05 M) Tris–HCl buffer at pH 8.6. About 0.05 M Tris–HCl buffer at pH 7.4 with 0.1 M KCl was used to elute the protein.

Protein and enzyme activity was measured in the fraction 22, and the fraction with the highest degree of enzyme activity has been lyophilized and stored at 4 °C [18].

SDS-PAGE analysis

Singh et al., [43], state that SDS-PAGE was used to ascertain the molecular weight of L-glutaminase. The PageRuler Fermentas unstained protein ladder was used to label it.

Kinetic properties of the purified L-glutaminase

The biochemical characteristics of pure L-glutaminase were found to include substrate selectivity, sensitivity to salt and trace metals, optimal pH, pH sustainability, and heat stability. L-glutaminase's kinetic properties, including Vmax and Km, were assessed using a range of substrate dosages (2–5 mM). The Lineweaver–Burk plot was used to construct the maximum velocity (V_{max}) and Michaelis–Menten constant (K_m) [28, 37].

Anticancer assay

The 3-(4, 5-Dimethylthiazol-2yl)-2, 5-diphenyl tetrazolium bromide (MTT) test (Sigma Aldrich, USA) was used to calculate cellular viability and describe the cytotoxic profile of L-glutaminase. This was a small modification of the Van de Loosdrecht et al. technique [49].

Statistical analysis

The data was analyzed using GraphPad Prism version 8.2.4 (GraphPad Software, Inc., La Jolla, CA, USA). To get the IC_{50} values, a sigmoid-type nonlinear regression was performed using the GraphPad software. The mean, SD, or SEM were used to display the data. Triple testing was used for the majority of the tests.

Results

Isolation and screening of streptomycetes strains

The Streptomycetes isolates were chosen based on their unique morphology, which is frequently spherical and convex in shape with deeply embedded growth into the medium. Dry, powdery spore masses frequently blanket the colonies' surface. Approximately 34 random isolates

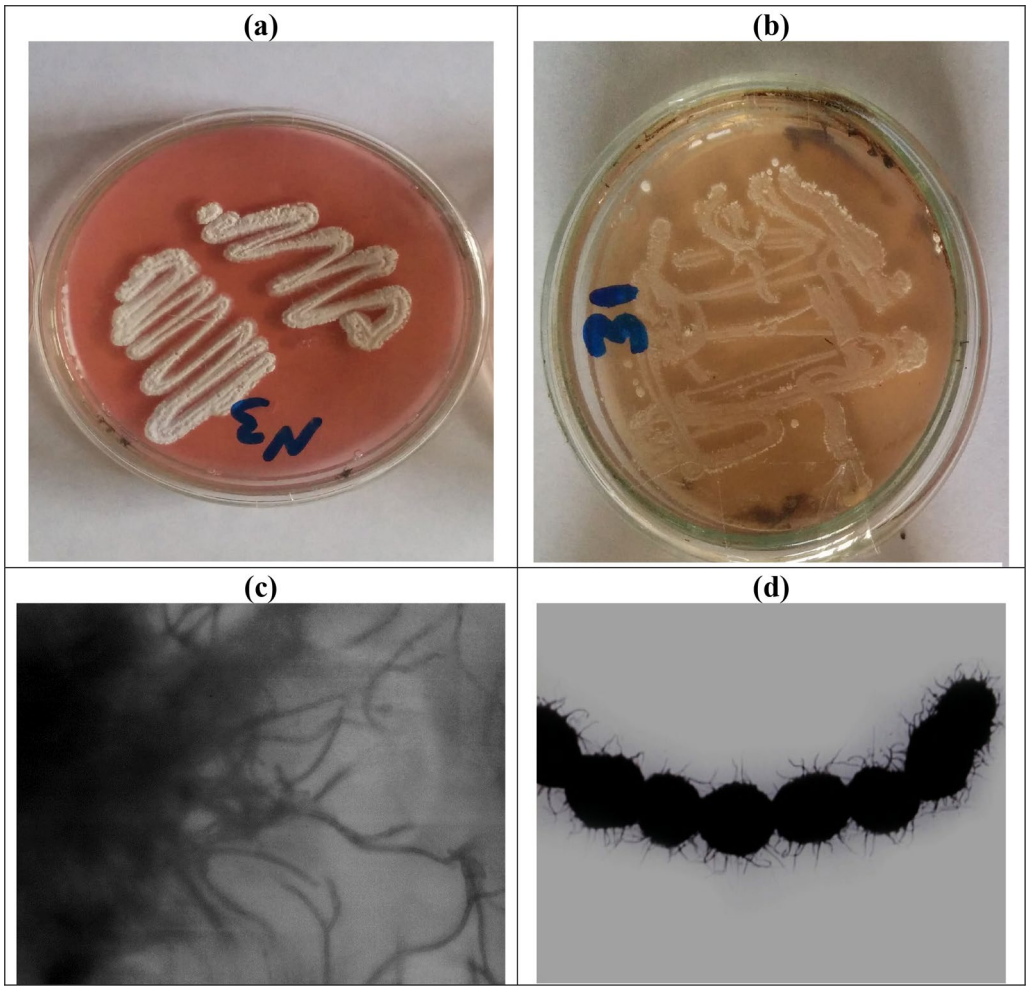

Fig. 1 Screening of isolates by rapid plate assay test on production of L-glutaminase where (**a**) positive result, and (**b**) negative result, (**c**) photomicrograph of isolate G9 showing flexuous sporephores, and (**d**) TEM photomicrograph of isolate G9 showing smooth spore surface

of the genus Streptomycetes were discovered in dirt, silt, and saltwater, among other environments.

The distribution of the Streptomycetes isolates was displayed in Table S2, 3, 4, 5, 6, and 7 supplementary Data. Dakahlia had the most isolates, followed by Gharbia and Giza, while Alexandria and South Sinai had the fewest (Sharm Elshiekh).

When the production of L-glutaminase by 34 *Streptomyces* isolates was examined using a rapid plate assay technique, only nine of them were recognized by the pink color surrounding their colonial growth as evidence and had the highest capacity for creating of extracellular L-glutaminase (Fig. 1a, b.

Screening for L-glutaminase -producing marine streptomycetes

L-glutaminase production was quantitatively examined for six *Streptomyces* isolates (D10, G8, GH3, GH5, Alex4, and SH1), and they were identified by the emergence of a pink tint around their colonial growth. Measurements of specific activity, enzyme synthesis, and protein estimation for each isolation showed that isolate G8 had the highest specific activity (Table 1). The isolates that shown the unique ability to manufacture L-glutaminase were then subjected to further identification, characterization, and optimization techniques.

Table 1 Quantitative screening of *Streptomyces* isolates for L-glutaminase formation

Samples	Total activity (U)	Total protein (mg)	Specific activity (U/mg)
D10	41.35	2.9	14.25
G8	48.20	2.8	17.21
GH3	39.25	3.1	12.66
GH5	49.55	3.9	15.98
Alex 4	51.74	3.3	16.67
SH 1	43.98	3.5	12.56

Table 2 Morphological, physiological, and biochemical characteristics of isolate G8

Morphological and cultural characteristics									
Spore chain Morphology		Spore surface Ornamentation		Colour of spore mass		Pigmentation of substrate mycelium		Diffusible pigment	
Straight/Flexuous > 40		Hairy		Gray		Pale Gray		- ve	
Physiological and biochemical characteristics									
Melanin pigment production		Degradation activities						Nitrate reduction	H_2S production
peptone iron agar	tyrosine agar	Xanthine	Elastin	Arbutin				+ ve	- ve
- ve	- ve	+ ve	- ve	+ ve					
Utilization of sugars									
D-fructose	Sucrose	Rhamnose	D-mannitol	D-xylose	Raffinose	I-inositol	Galactose	L-arabinose	
- ve	+ ve	+ ve	- ve	- ve	+ ve	- ve	- ve	+ ve	

+ ve Positive, - ve Negative

Morphological, physiological, biochemical, and molecular identification of the selected Streptomycetes isolates

The strain (G8) that degrades the most efficiently was identified using morphological, physiological, biochemical, and molecular identification approaches. An overview of the morphological, physiological, and biochemical characteristics is given in Table 2**.** Figure 1c, d show that isolates (G8) had flexuous spore chains and decorated hairy spore surfaces. The bulk of the spores, however, had a gray color. The coloring of the substrate mycelium was light gray/white. Isolation was unable to yield diffuse pigments. It could not, however, produce melanoid pigments. The isolate was unable to produce hydrogen sulfide, but it was able to reduce nitrate. Additionally, Arbutin and xanthine were broken down. The utilization of sugars has been tested using D-glucose as a positive control. The isolate made use of many carbon sources in different ways.

Phylogenetic analysis of the *Streptomyces sp*. strain 5 M

The sequenced part of the 16S ribosomal RNA gene of the strain 5 M extends from the start of the gene to 800 bp afterwards which covers the first four hypervariable regions of the gene (V1, V2, V3, and V4) [19].

The consensus phylogenetic ML tree (Fig. 2), showed that *Streptomyces* sp. 5 M was closely related to *Streptomyces sampsonii* ATCC 25495 (99.50% similarity), *Streptomyces daghestanicus* NRRL B-5418 (99.50%), *Streptomyces albidoflavus* DSM 40455 (99.50%), and *Streptomyces violascens* ISP 5183 (99.48%) and the branch was unresolved enough to be able to discriminate the species level of the strain 5 M.

Given the limitation of partial 16S rRNA gene sequencing that it do not capture sufficient sequence variation to discriminate between closely related species as sequencing the full ~ 1500 bp gene, however the sequenced subregions V1-V3 was found to show the highest resolving power [3, 25].

A BLAST search against the nonredundant nucleotide database showed that strain 5 M was identical in sequence to many strains of *Streptomyces albidoflavus* and *Streptomyces sampsonii* that might be a clue that strain 5 M belongs to one of these species.

Effect of time on the production of L-glutaminase

An incubation time test had an impact on Streptomyces sp. strain 5 M's production of L-glutaminase. According to the results in Fig. 3a, L-glutamine production grows gradually until day 7, when it reaches its maximal level (5.26 U/ml), following which enzyme activity starts to decrease.

Effect of pH on the production of L-glutaminase

pH affects *Streptomyces* sp. strain 5 M's ability to synthesize L-glutaminase. Figure 3b illustrates how the pH of the fermentation medium is said to affect the creation of enzymes. Thus, at pH 7.0, a maximum enzyme output of 5.57 U/mL was noted. A rise or fall in the medium's pH reduced the amount of enzymes produced.

Effect of temperature on L-glutaminase production

Depending on the incubation temperature of the fermentation medium, every microbial strain can develop at a certain pace. The maximum enzyme synthesis (5.85 U/ml) was noted at 30 °C. As shown in Fig. 3c, any change in temperature leads to decreased L-glutaminase production.

Effect of glutamine concentration on L-glutaminase production

The amino acid glutamine triggers the production of L-glutaminase. As a consequence, different amounts of glutamine were added to the enzyme-producing media. According to Fig. 3d, at a concentration of 0.4%, the enzyme production reached its maximum level (6.07 U/ml). However, when glutamine levels increased, enzyme synthesis dropped.

Effect of different carbon sources on L-glutaminase production

Enzyme production rose when different carbon sources were added to the medium at a 1% level. The maximum enzyme yield was provided by glucose (6.21 U/ml), which generated significantly more enzymes than any other carbon source. As a carbon source, arabinose was the least effective (0.95 U/ml) as displayed in Fig. 4a**.**

Effect of different nitrogen sources on L-glutaminase production

The addition of additional organic and inorganic nitrogen sources to the medium resulted in a significant increase in the production of enzymes. Of all the nitrogen sources analyzed, peptone provided the greatest enzyme synthesis (6.33 U/ml). Enzymes were also created in variable levels by all other nitrogen sources. Figure 4b shows that sodium nitrate (1.25 U/ml) was the least effective nitrogen source overall.

Purification of L-glutaminase

After five days, L-glutaminase was purified in order to assess the production and purity of the *Streptomyces* sp. strain 5 M growth. Crude L-glutaminase precipitates fractionally upon salting out (30–80% ammonium sulfate). An overview of the *Streptomyces* sp. strain

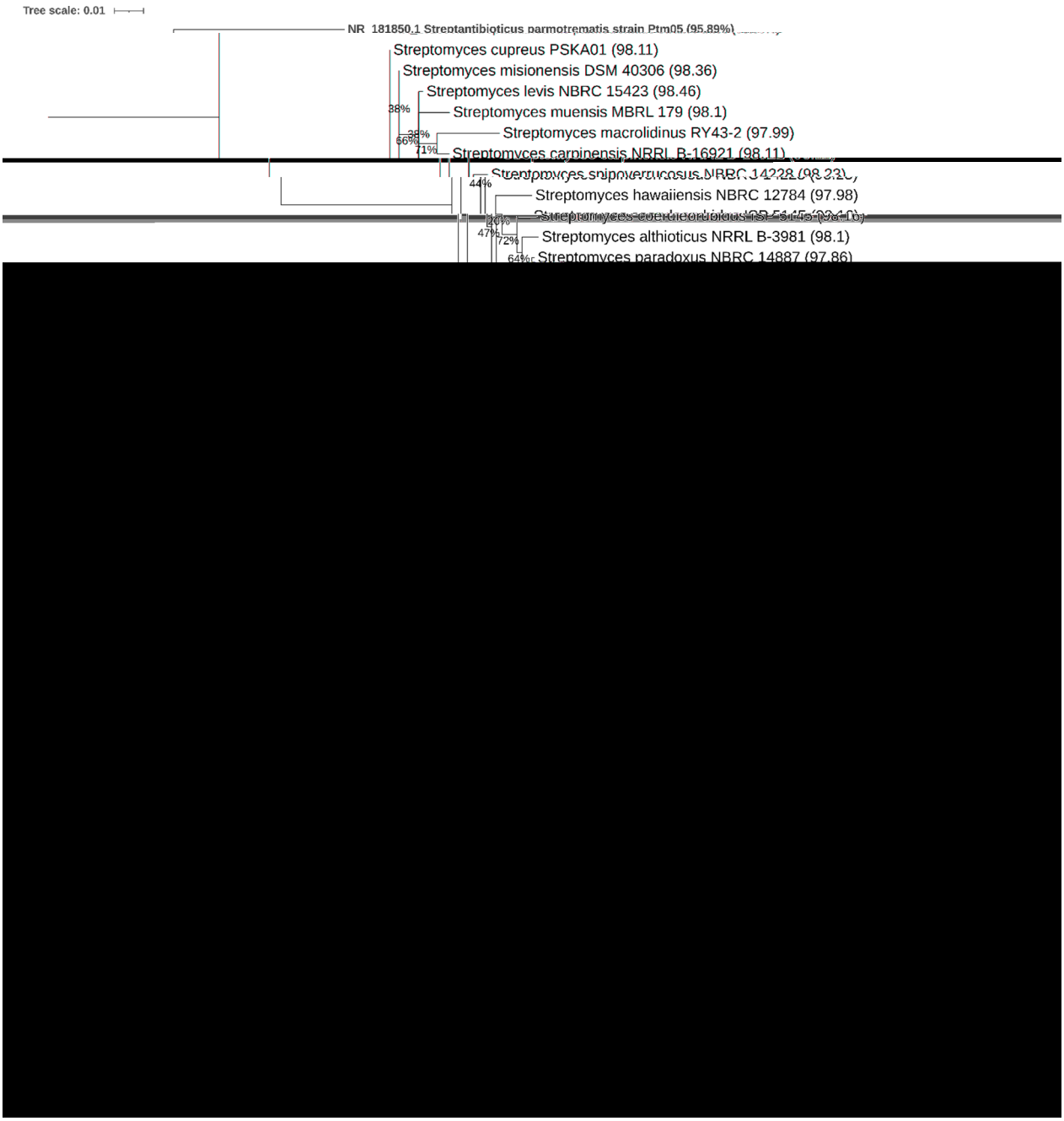

Fig. 2 Consensus phylogenetic tree of the partial sequence of 16S rRNA of *Streptomyces* sp. strain 5 M (in red) with other closely related species of the genus *Streptomyces*. Bootstrap branch support is written on each branching point. The percentage of similarity of each *Streptomyces* species to the strain 5 M are written in parentheses next to each. The tree is drawn to scale

5 M culture's enzyme purification profile is shown in Table 3. About 443.7 mg of protein, 2800.0 unit of the total L-glutaminase activity, and 6.31 units of specific L-glutaminase activity per milligram of protein (U/mg) were present in the crude extract. At each stage of the purification process, the specific activity increased relative to crude. The maximum specific activity of 202.95 U/mg protein with a yield of 55.1% was achieved using Sephacryl S-300 purification.

SDS-PAGE was used to assess the homogeneity of the improved L-glutaminase. Since just one band with an

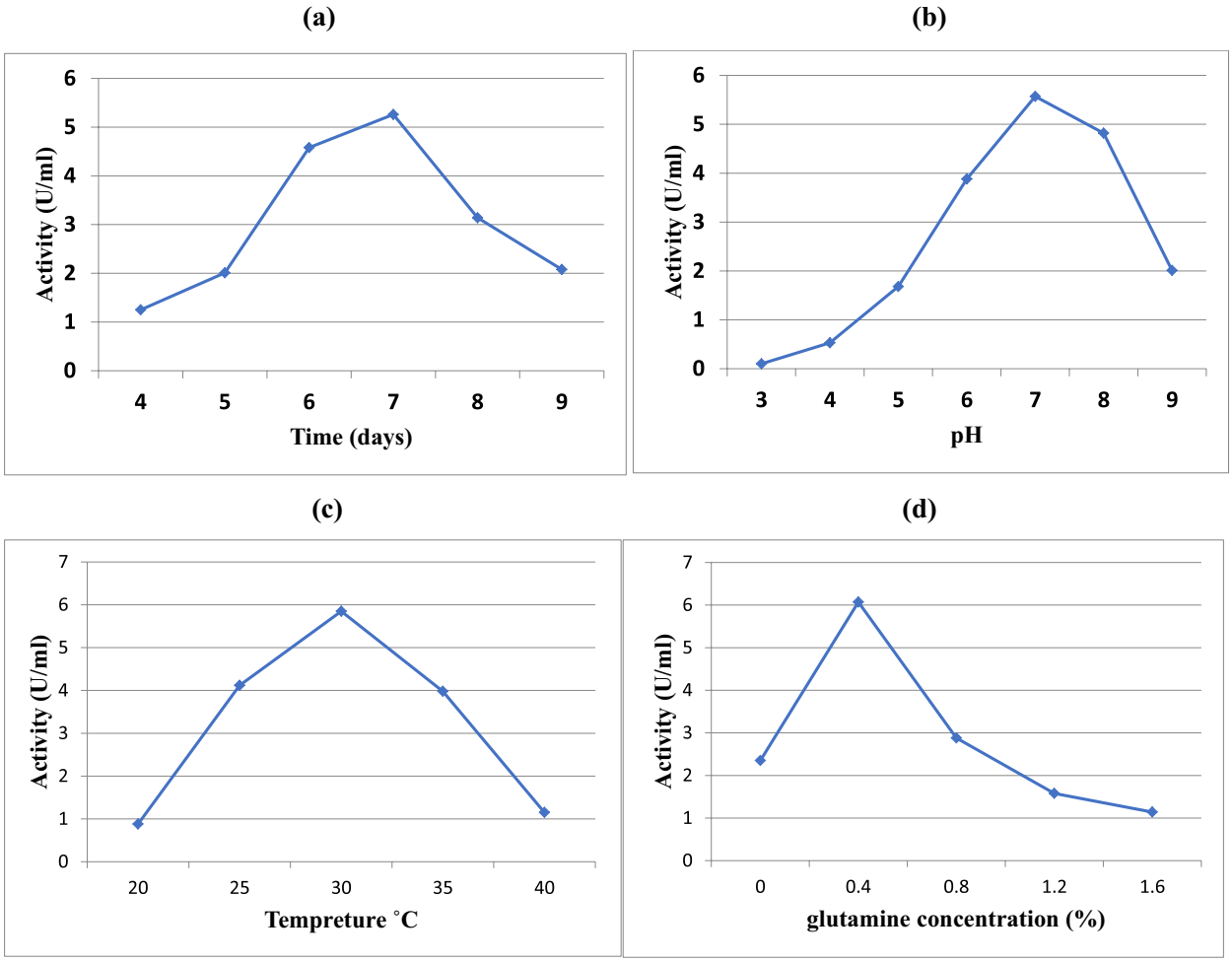

Fig. 3 Effect of incubation time (**a**), pH (**b**), Temp., (**c**), and glutamine concentration (**d**) on L-glutaminase production

apparent mass of 52 kDa was found, the results showed that the L-glutaminase preparation was pure (Fig. 5). The glutaminase enzyme is a monomeric protein, with an estimated molecular weight of 52 KDa, according to the results of gel filtration on Sephacryl S-300, which validated this number. The effectiveness of the enzyme purification process was shown by the outcomes of the L-glutaminase activity test and SDS-PAGE analysis of the L-glutaminase protein.

Kinetic properties of the purified L-glutaminase

The outcome of pH on L-glutaminase

When L-glutamine was used as the substrate, the optimum pH for enzyme activity was 7.5. The initial activity of the enzyme was reduced by 63% at pH 4.0. Additionally, compared to the optimal pH, only 50.00% of the enzyme activity was maintained at pH 10.0.

In contrast to the ideal pH, Fig. 6a demonstrates that the rate of enzyme inactivation was greater in more acidic and alkaline environments. By pre-incubating the enzyme for two hours in the pH range of 3.0 to 11.0 without the substrate, the stability of the enzyme with respect to pH was evaluated. The results indicated that the enzyme was best stable in the pH range of 5.0 to 9.2. Figure 6b illustrates that only 25% and 40%, respectively, of the enzyme's initial activity was preserved after two hours of incubation at pH 3.0 and pH 11.0.

The outcome of temperature on L-glutaminase

It should be mentioned that the optimal temperature for L-glutaminase activity was found to be 40 °C. The enzyme activity peaked at 37 °C and then gradually decreased until it reached 10.0% of its peak at 90 °C as displayed in Fig. 6**c**. The enzyme showed 87.7% catalytic stability at 25.0–55.0 oC for 30 min and 25.0–55.0 °C for 60 min (see Fig. 6d).

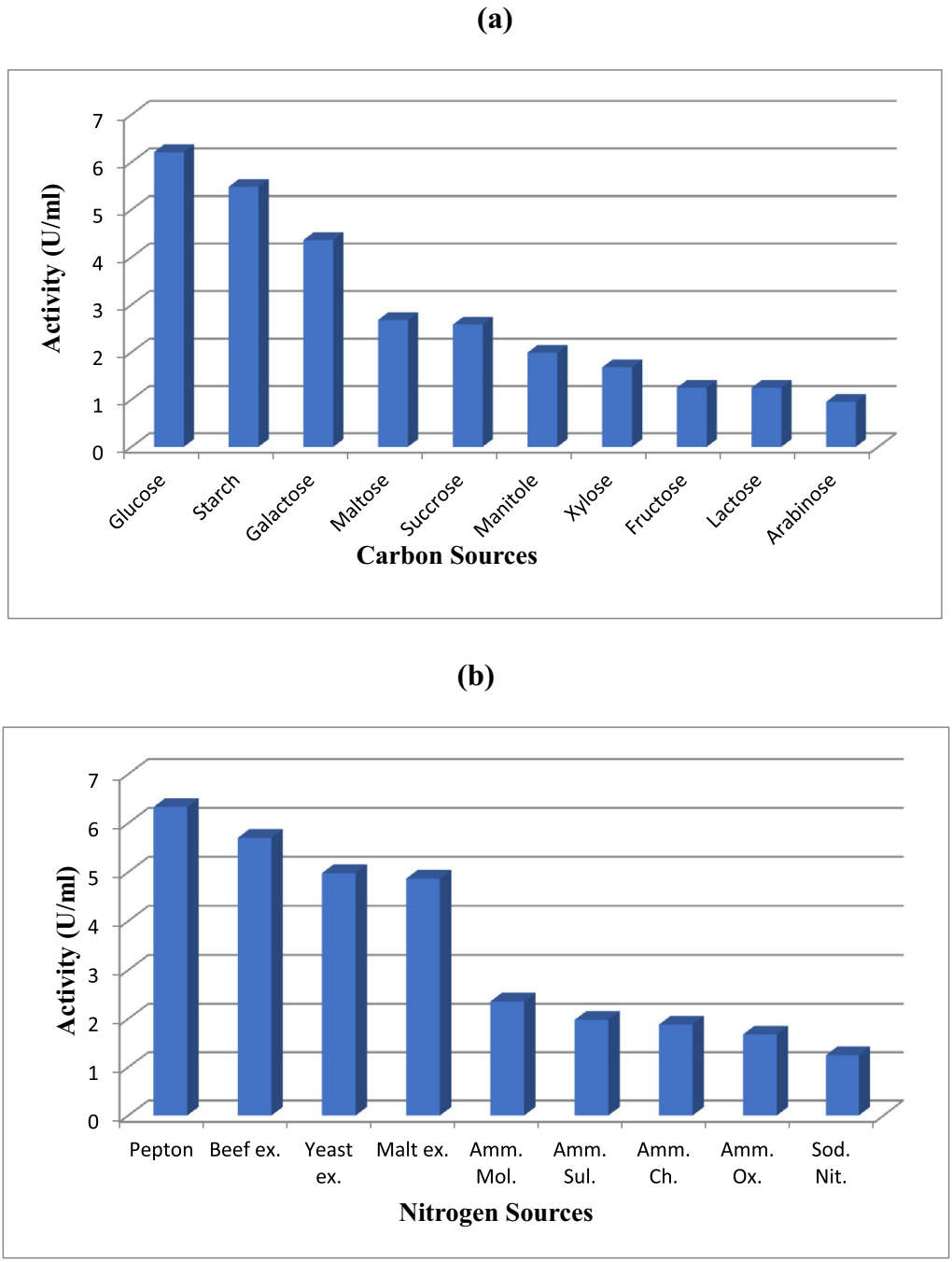

Fig. 4 Effect of different carbon sources (**a**), and nitrogen sources (**b**) on L-glutaminase production

Influence of metals and inhibitors on enzyme activity and stability

The activity of L-glutaminase was also assessed in the presence of different metal ions. As seen in Fig. 7**a**, Na^{+}, K^{+}, Mn^{2+}, Ni^{2+}, and Ba^{2+} functioned as inducers at both 2 and 5 mM, whereas only Mg^{2+}, Co^{2+}, Hg^{2+}, and Cd^{2+} of the ions under investigation exhibited a discernible decline in activity. Figure 7b illustrates how 7.5% NaCl directly impacted the enzyme's activity by causing it to decrease. Tween 80 and Triton X-100 demonstrated the highest residual activity in terms of the effect inhibitors, measuring 112.3 ± 10.1 and $109.4 \pm 11.3\%$, respectively. However, as seen in EDTA at 5 mM, the least residual activity ($46.1 \pm 5.2\%$) was observed (Table 4).

Table 3 A typical purification scheme for L-glutaminase from *Streptomyces sp. strain* 5 M

Purification step	Protein (mg)	Activity (U)	Specific activity (U/mg protein)	Fold purification	Recovery (%)
Crude extract	443.7	2800.0	6.31	1.00	100
Lyophilisation	377.6	2433.2	6.44	1.02	86.9
Amm. Sulphate ppt. (30-80%)	320.2	2214.2	6.92	1.09	79.1
DEAE-Cellulose (0.2 NaCl)	45.8	1904.2	41.58	6.59	68.0
Sephacryl S-300	7.6	1542.4	202.95	32.16	55.1

Kinetics of L-glutaminase

Stable-state kinetic analysis was used to find the values K_m and V_{max} for the pure L-glutaminase. Following the fitting of the Michaelis–Menten equation to the reaction velocity vs substrate absorption, the kinetics constant was determined by the emergence of a characteristic hyperbolic saturation curve. As shown in Fig. 8a, the purified L-glutaminase from *Streptomyces* sp. (strain 5 M) had K_m and V_{max} values of 2.62 mM and 10.2 U/ml, respectively.

This demonstrates that different microbes have varying enzyme affinities for the substrate L-glutamine and can engage in a variety of physiological processes through the activity of the enzyme (Fig. 8b).

Antineoplastic activity of L-glutaminase

A variety of dosages were administered to Hep-G2, HeLa, and MCF-7 cancer cell lines in order to assess the in vitro cytotoxic effects of pure L-glutaminase purified from *Streptomyces* sp. (strain 5 M). Cell inhibition was assessed following incubation, and Fig. 9 illustrates how the enzyme caused cytotoxicity in a dose-dependent manner.

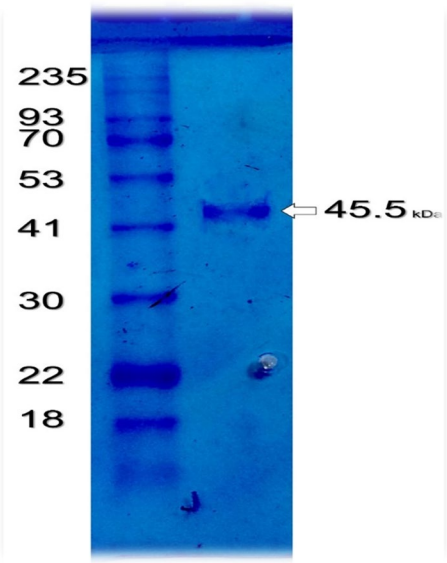

Fig. 5 Protein band with an apparent molecular weight

The IC_{50} values for the pure L-glutaminase against HeLa and MCF-7 cells were 8.96 µg/mL (Fig. 9b), and 7.98 µg/mL (Fig. 9**c**), respectively, indicating an antiproliferative action. The purified L-glutaminase had an IC_{50} value of 13.69 µg/ml and was only slightly cytotoxic to HepG-2 cells (Fig. 9a). Additionally, the morphology of Hep-G2, HeLa, and MCF-7 cells after exposure to L-glutaminase treatment showed membrane meiosis, apoptotic cell shrinkage, and cell fragmentation (Fig. 10).

Discussion

According to the current study, *Streptomyces* sp. (strain 5 M) was identified by a variety of characterization methods. *Streptomyces* sp. (strain 5 M)'s uniqueness was 99% verified by PCR amplification of the 16S rDNA gene. The results of the 16S rDNA gene research are in good agreement with the Streptomyces data that Kannan et al. [27] presented. The tested strain was unique since colonies developed on MGA medium and produced a pink halo around themselves as a result of the amide bond in L-glutamine breaking and ammonia being released [46, 52].

To determine its purity, *Streptomyces* sp. strain 5 M's L-glutaminase was purified. Fractional precipitation of the crude L-glutaminase is caused by salting (30–80% ammonium sulfate). These results were consistent with the ones Reda et al. [36] examined.

Furthermore, following gel filtration, *Bacillus* sp.'s L-glutaminase activity was refined 49 times with a 25% retrieval and a specific activity of 584.2 U/mg protein, according to Kumar et al., [28]. In contrast, Elshafei et al. [18], reported that the intracellular L-glutaminase from *Penicillium brevicompactum* NRC829 was isolated to homogeneousness (162.75 fold) and had a plausible molecular mass of 71 kDa. This indicates that the process used to produce and purify L-glutaminase in the current study was successful.

SDS-PAGE was used to scrutinize the purified L-glutaminase from fermentation conditions and define the molecular homogeneity of the purification process [14].

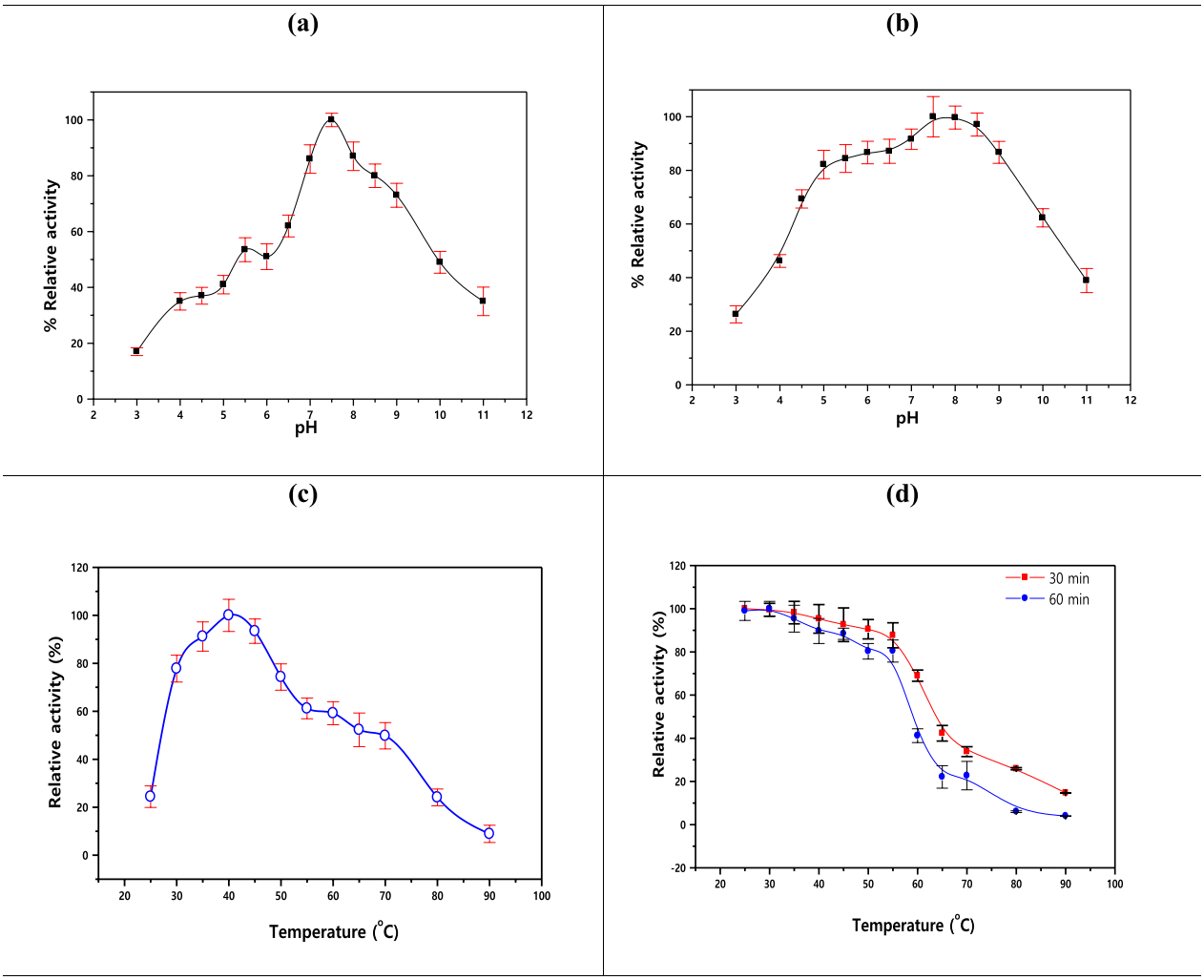

Fig. 6 Effects of pH examined at pH ranged from 3.0–11.0 (**a**), pH stability examined at pH ranged from 2.0–11.0, (**c**) effects of Temperature examined at Temp., ranged from 20–100 °C, and (**d**) Temperature stability examined at Temp., ranged from 20–100 oC

There was just one band at 54 kDa detectable following the final purification step. Similarly, the natural enzyme of *Stenotrophomonas maltophilia* was determined to have a molecular mass of 41 kDa using gel filtration [32].

Additionally, the enzyme was homogeneously purified by purifying L-glutaminase extracellularly produced by *Bacillus cereus* to plausible homogeneousness with a sufficient band, as stated by Singh and Banik [44] The molecular weights of the subunits of native enzymes were found to be around 140 and 35 kDa, respectively, suggesting that they are homo-tetrameric [42]

Regarding the impact of pH stability, the negative effect on L-glutaminase activity at both high and low pH values promotes the ionization of the enzyme, changing the surface charge of L-glutaminase, dissociating its cofactor, and ultimately impairing its ability to bind to the substrate [9]. *Streptomyces* sp. (strain 5 M)'s L-glutaminase activity continuously demonstrated an ideal pH range of 7 to 8, which is a recommended prerequisite for L-glutaminase function. Similarly, L-glutaminase from *Streptomyces gulbargensis* showed more stability at an alkaline pH than an acidic one [4].

Enzymes' extensive use in a variety of sectors is largely due to their ability to withstand severe environments. Orabi et al. [33], reported a similar outcome for the pure L-glutaminase derived from the marine bacterial isolate, which remained stable across the pH range of 3.6 to 9.0. On the other hand, L-glutaminase generated by *Debaryomyces* spp. was only stable in the pH range of 7.5 to 9.0, according to Dura et al. [12].

The enzyme was thermally stable below 60 °C, with a little decrease in activity at 70 °C and a total loss of activity above 80 °C. Theoretically, coenzyme dissociation

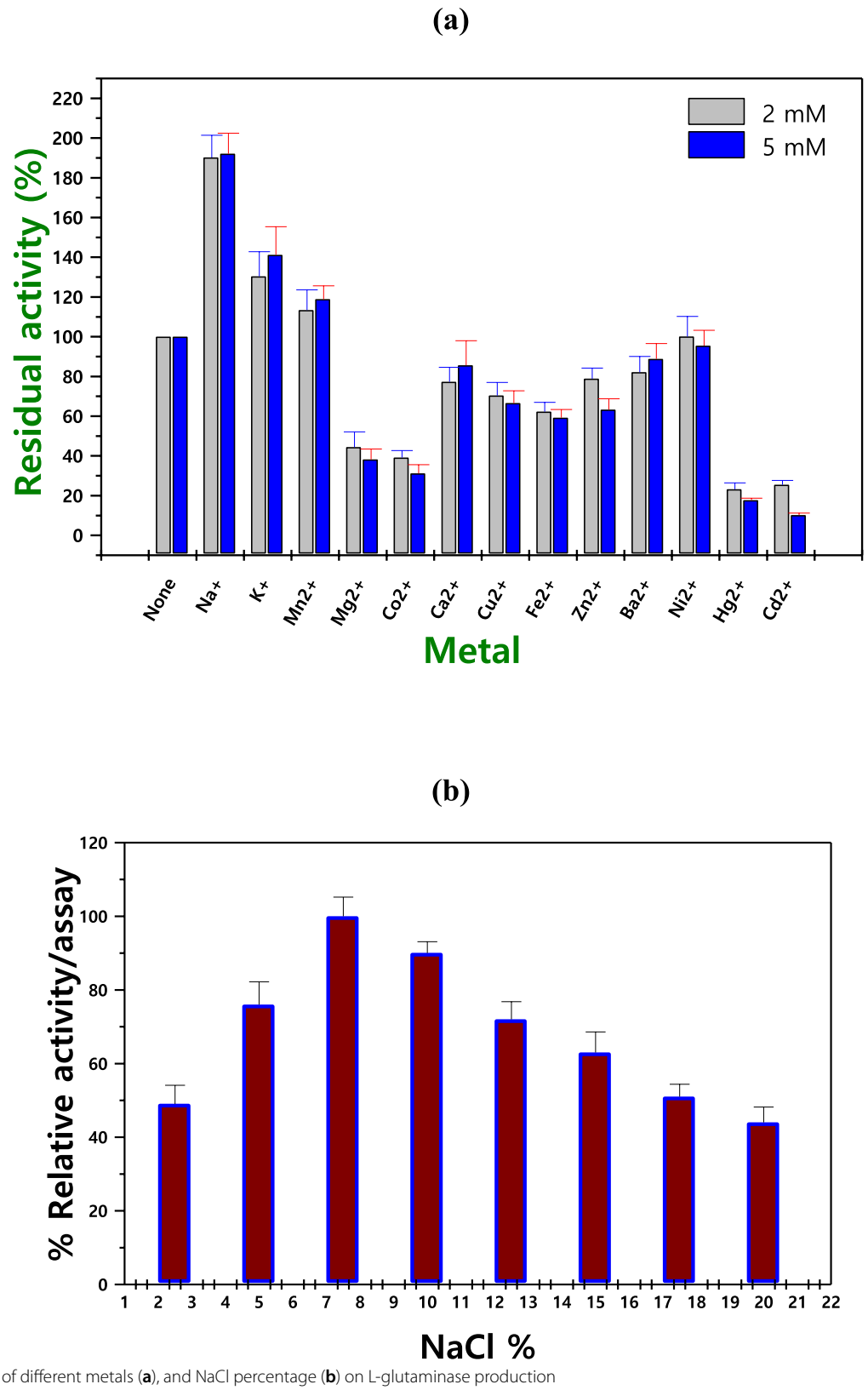

Fig. 7 Effect of different metals (**a**), and NaCl percentage (**b**) on L-glutaminase production

Table 4 Effect of inhibitors on L-glutaminase production

Compound (5 mM concentration)	Residual activity %
Control	100±0.0
β-mercaptoethanol	95.5±7.6
SDS	77.9±6.6
DTT	80.0±8.2
Sodium azide	81.0±7.6
Urea	94.2±7.5
EDTA	46.1±5.2
PMSF	97.5±7.3
Iodoacetate	89.3±5.5
1.10-Phenanthroline	88.3±7.2
β-HMB	83.1±5.6
Tween 80	112.3±10.1
Triton X-100	109.4±11.3
N-ethylmaleimide	97.7±10.1

or denaturation by heating per unit time is indicated by thermal inactivation rates of 50, 60, 70, and 80 °C. These results concurred with those released by the following sources [37, 54].

According to Orabi et al. [33], pure L-glutaminase showed thermostability at 40 °C and retained more than 90% of its activity after an hour. *A. xylosoxidans* RSHG1's L-glutaminase demonstrated thermal stability between 40 and 50 ∘C [39].

The effect of a number of activators and inhibitors on the catalytic potency of the generated L-glutaminase was evaluated using pre-incubation. According to our results, only Mg^{2+}, Co^{2+}, Hg^{2+}, and Cd^{2+} shown a significant decrease in activity, whereas Na^{+}, K^{+}, Mn^{2+}, Ni^{2+}, and Ba^{2+} functioned as inducers at both 2 and 5 mM. These results are consistent with the findings for *S. radiopugnans*-producing L-glutaminase by Singh and Banik [44]. Additionally, Singh and Banik, [44], observed that monovalent cations Na^{+} and K^{+} ions activated the *B. cereus*-producing L-glutaminase, but divalent cations Mg^{2+} and Mn^{2+} hindered its activity.

The enzyme showed a comparatively catalytic activity of K_m 2.62 mM and 10.2 U/ml of V_{max} for the produced L-glutaminase. Numerous studies have previously shown that the *Streptomyces* sp. L-glutaminase's strong affinity for L-glutamine as a substrate [17, 56]. Furthermore, the substrate specificity test showed that L-glutaminase from *Bacillus cereus* MTCC 1305 was specific for L-glutamine, according to Singh and Banik [44].

The isolated glutaminase's kinetic characteristics were compared to those of L-glutaminase derived from *Streptomyces* sp., which had a V_{max} of 7.57 U/ml and a K_m value of 2.8 mM [11]. On the other hand, L-glutaminase generated by *A. xylosoxidans* RSHG1 revealed a K_m value of 0.236 mM [39]. According to Durai et al. [13] [13], *Bacillus* sp. B12 produced L-glutaminase having K_m and V_{max} of 0.4 mmol/L and 0.133 mmol/min, respectively.

The present investigation used the MTT test to assess the effect of pure L-glutaminase on the growth of HepG2, MCF7, and HeLa cancer cells during a 24-h incubation period. HepG-2 cell growth showed an IC_{50} of 13.69 µg/mL, whereas the enzyme undergoing testing was highly efficient towards MCF-7 and HeLa cells (IC_{50}, 7.98 µg/mL, and 8.96 µg/mL, respectively). Likewise, Alrumman et al., [2], showed that purified L-glutaminase from *Bacillus licheniformis* was effective in killing the HepG-2 cell line.

Furthermore, Elshafei et al., [18], discovered that the isolated enzyme from *Penicillium brevicompactum* decreased the growth of the human cell line Hep-G2, which is a representation of hepatocellular carcinoma, and had an IC_{50} value of 63.3 µg/mL. All of these results show that L-glutaminase might potentially be used in cancer chemoprevention and maintains a high level of discriminating against cancer cells.

Conclusion

The potential of the isolated *Streptomyces* sp. (strain 5 M) for L-glutaminase production was investigated using a range of process factors and medium components. The production of L-glutamine increases gradually until day 7, when it reaches its maximum level (5.26 U/ml). There was a maximum enzyme output of 5.57 U/mL at pH 7.0, a maximum enzyme synthesis of 5.85 U/ml at 30 °C, and a maximum enzyme production of 6.07 U/ml at a concentration of 0.4% glutamine. Moreover, glucose had the highest enzyme output (6.21 U/ml), producing a notably greater number of enzymes than any other carbon source. Peptone had the highest enzyme synthesis (6.33 U/ml) of all the nitrogen sources examined. Using pre-incubation, the impact of many activators and inhibitors on the catalytic activity of the produced L-glutaminase was assessed. Our findings indicate that while Na^{+}, K^{+}, Mn^{2+}, Ni^{2+}, and Ba^{2+} worked as inducers at both 2 and 5 mM, particularly Mg^{2+}, Co^{2+}, Hg^{2+}, and Cd^{2+} shown a discernible drop-in activity. For the generated L-glutaminase, the enzyme demonstrated a relatively high catalytic activity of K_m 2.62 mM and 10.2 U/ml of V_{max}. The current study evaluated the impact of pure L-glutaminase on the proliferation of Hep-G2, MCF7, and HeLa cancer cells over the course of a 24 h incubation period using the MTT test. The enzyme under test was very effective against MCF-7 and HeLa cells (IC_{50}, 7.98 µg/mL, and

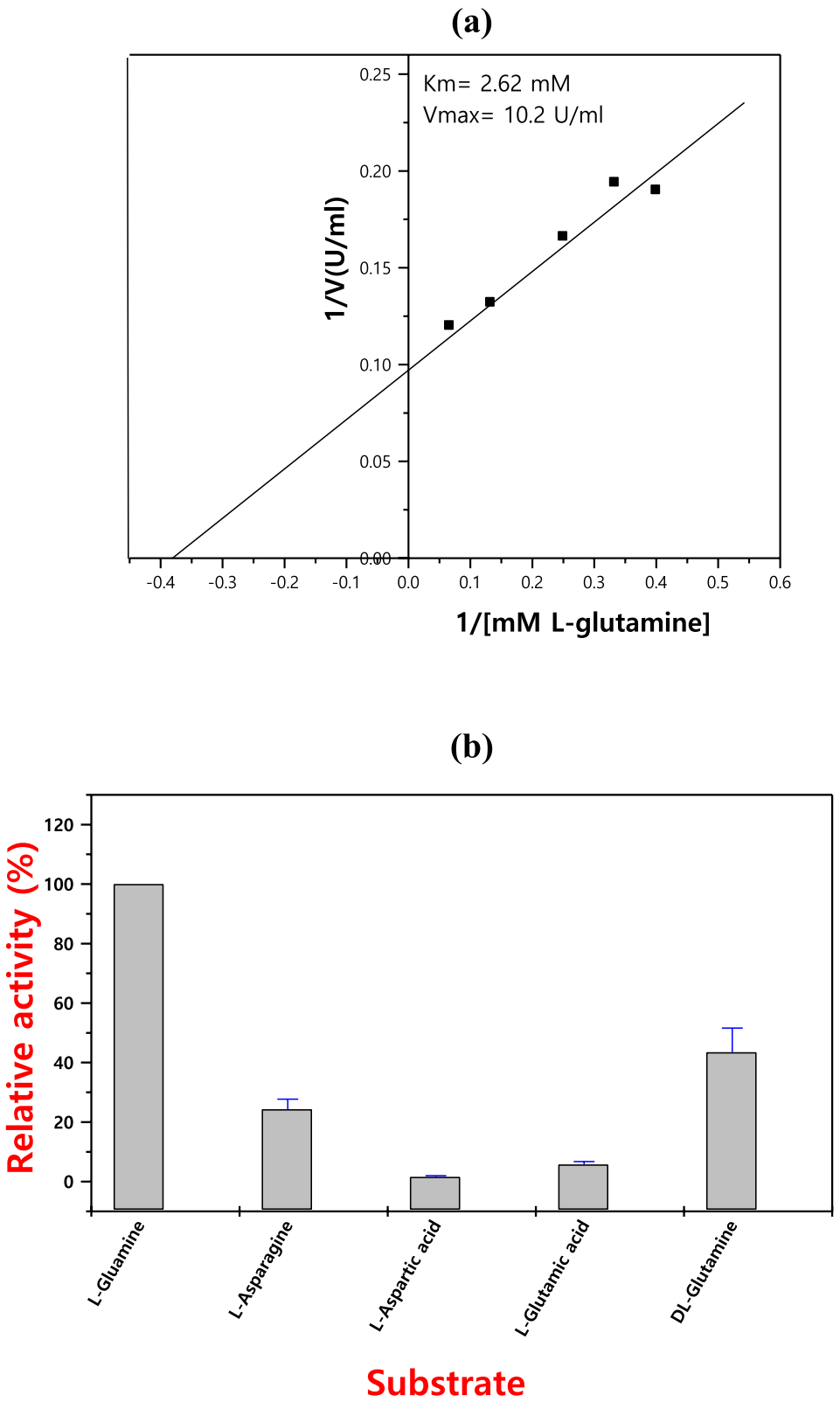

Fig. 8 Steady-state kinetic analysis on the purified L-glutaminase (**a**), and substrate affinities and their physiological roles in the L-glutaminase activity (b)

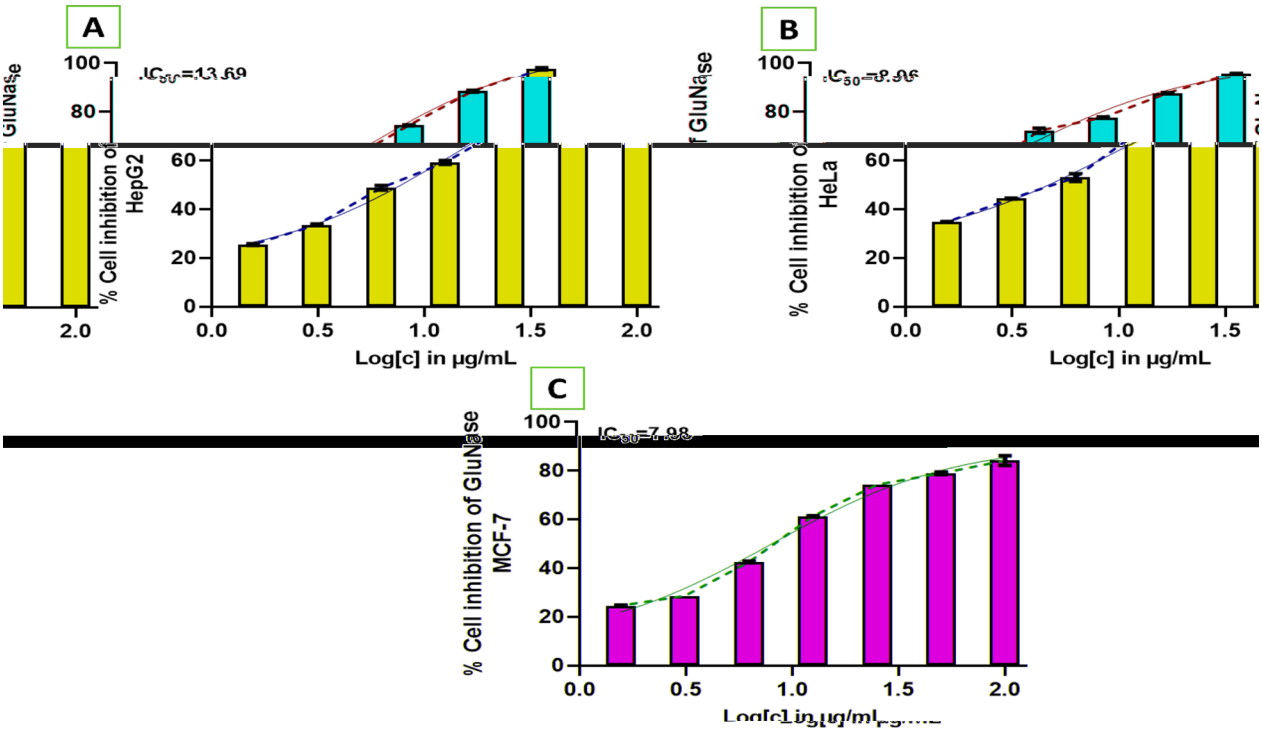

Fig. 9 Effect of GluNase on cell inhibition of HepG-2, HeLa, and MCF-7 cells: MTT test was carried out after the cells had been exposed to different doses of GluNase. Data are provided as mean ± SD, and outcomes are shown as cell viability (% of control)

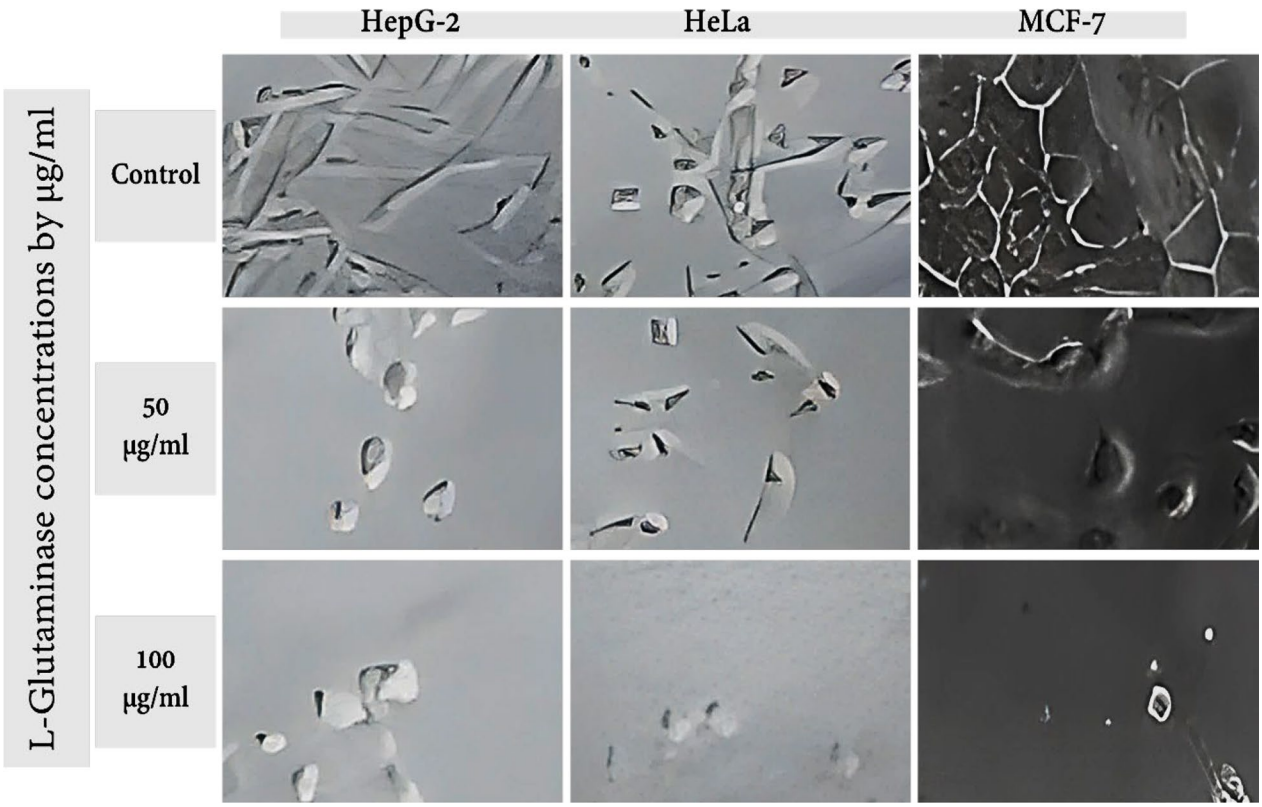

Fig. 10 Illustrative images demonstrating the morphological alterations in HepG-2, HeLa, and MCF-7 cells after being treated with DMSO (50 mg and 100 mg) or left untreated (control); Under an inverted microscope, cells were examined and photographed using a digital camera (50 µm)

8.96 µg/mL, respectively), whereas HepG-2 cell growth had an IC_{50} of 13.69 µg/mL. Additionally, the molecular weight of the purified L-glutaminase must be confirmed using additional techniques, such as mass spectrometry. The produced L-glutaminase can therefore play a key role in cancer treatment and chemoprevention.

Supplementary Information

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Supplementary material 1.

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Author contributions
MGH, GSE, MOA, MNM, MEE, and MIE suggested the research topic, investigated the article, planned the research methodology, wrote the original draft, and participated in data representation and article revising and editing, MAK read the manuscript, participate in the revision and revise the scientific work of the whole manuscript. All the authors read and approved the article.

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Availability of data and materials
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Declarations

Ethics approval and consent to participate
Not applicable.

Research involving human participation and/or animals
Not applicable.

Informed consent
Not applicable.

Competing interests
The authors declare no competing interests.

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