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PRODUCTION, CHARACTERIZATION, AND SELECTIVE ANTI-PROLIFERATIVE ACTIVITY OF A NOVEL L-GLUTAMINASE FROM *STREPTOMYCES* SP. ISOLATED FROM VOLCANIC SOIL OF MOUNT UHUD, SAUDI ARABIA

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ABSTRACT
Extreme environments such as volcanic soils represent valuable yet underexplored reservoirs of microbial diversity, particularly actinomycetes capable of producing valuable important enzymes. In this study, a potent L-glutaminase-producing actinomycete was isolated from volcanic soil collected from Mount Uhud. Isolates were screened for enzyme production on minimal glutamine agar using phenol red as a pH indicator. The most efficient strain (A6) was identified as *Streptomyces* sp. based on morphological characteristics and 16S rRNA gene sequencing. Optimization of cultural conditions revealed that maximum L-glutaminase production (6.895 U/mL) occurred at 5°C, indicating that the isolate is a psychrophilic or psychrotolerant producer. Biochemical characterization of the partially purified enzyme demonstrated broad catalytic activity across a wide temperature range (5–60°C), with maximum activity observed at both 37°C and 60°C, as well as optimal activity at pH 7.0 suggesting thermophilic catalytic potential. This thermal versatility in catalytic function, coupled with the cold-active production optimum, highlights the enzyme's adaptability for diverse biotechnological applications. Enzyme activity was inhibited by Fe^{2+}, Cu^{2+}, and Hg^{2+} ions but remained unaffected by EDTA, suggesting that the enzyme is not a metalloenzyme and may involve sulfhydryl groups in catalysis. Biological evaluation using the MTT assay revealed selective anti-proliferative activity against colon cancer cells (HCT116) with an IC_{50} value of 34.27 ± 0.3 µg/mL after 72 hours, while no significant cytotoxicity was observed against breast (MCF-7) or lung (A549) cancer cell lines. These findings highlight volcanic soil actinomycetes as sources of thermally versatile L-glutaminase with potential applications in environmental biotechnology and anticancer strategies.

KEYWORDS Volcanic soil, *Streptomyces* sp., L-Glutaminase, Anti-proliferative activity, Environmental biotechnology

1. INTRODUCTION

Extreme and underexplored ecosystems represent invaluable reservoirs of microbial diversity, harboring microorganisms with unique metabolic adaptations and the capacity to produce novel bioactive compounds of significant biotechnological interest [1, 2]. Among these ecosystems, volcanic soils are characterized by their unique geochemical properties, including extreme temperature fluctuations, variable pH, and distinct mineral compositions, which exert selective pressure on resident microbial communities, driving the evolution of specialized metabolic pathways and enzymes with remarkable stability and catalytic efficiency [3, 4]. The exploration of such extreme environments for novel actinomycetes has emerged as a promising strategy in the field of environmental biotechnology, facilitating the discovery of industrially and therapeutically relevant biomolecules [5].

Actinomycetes, particularly members of the genus *Streptomyces*, are recognized as one of the most prolific sources of extracellular enzymes and secondary metabolites in nature [6]. Recent systematic reviews have comprehensively documented the diversity of actinomycetes across Saudi Arabia's unique ecosystems, ranging from terrestrial and marine environments to deserts, caves, and hot springs [7, 8]. These studies have demonstrated that adaptation to harsh environmental conditions drives the evolution of unique strains with enhanced biosynthetic capacities, particularly among members of the genus *Streptomyces* [7,8]. Quantitatively accentuate the environmental biotechnology aspect, such as the use of cold-active enzymes for energy-efficient procedures. Their ecological success in diverse and often hostile environments is attributed to their sophisticated metabolic versatility and complex regulatory networks, which enable them to adapt to changing environmental conditions and exploit available nutrients efficiently [9]. L-Glutaminase (L-glutamine amidohydrolase, EC 3.5.1.2) is an enzyme of considerable interest produced by various actinomycetes. This enzyme catalyzes the hydrolytic deamination of L-glutamine to L-glutamic acid and ammonia, playing a fundamental role in microbial nitrogen metabolism and environmental nutrient cycling [10]. From a biotechnological perspective, microbial L-glutaminases have garnered attention for their dual applicability: in the food industry, they enhance the flavor of

fermented foods through glutamic acid production, while in the pharmaceutical sector, they demonstrate promising therapeutic potential as anti-cancer agents [11, 12].

The therapeutic relevance of L-glutaminase is rooted in the distinctive metabolic requirements of certain malignant cells. Many tumor types, particularly colon cancer cells, exhibit an elevated dependency on exogenous glutamine for proliferation and survival—a phenomenon known as "glutamine addiction" [13, 14]. By catalyzing the depletion of systemic glutamine, L-glutaminase can selectively impede the growth of these glutamine-dependent cancer cells while sparing normal cells capable of endogenous glutamine synthesis [15]. This mechanism positions L-glutaminase as a potential biotherapeutic agent, offering an alternative or complement to conventional chemotherapeutic approaches. However, the clinical application of currently available L-glutaminases is often limited by suboptimal catalytic properties, immunogenicity, and stability issues, necessitating the continued search for novel enzymes with superior biochemical and pharmacological characteristics from underexplored microbial sources [16].

The Kingdom of Saudi Arabia encompasses a remarkable diversity of extreme environments with conditions analogous to extraterrestrial settings, including deserts and salt flats as analogs to Mars, and terrestrial and marine volcanic fields as analogs to icy moons [17]. Recent studies have identified volcanic craters in Saudi Arabia with unique geochemical compositions that mimic extraterrestrial ocean chemistry, offering valuable biological models for astrobiological research [17, 18]. Among these, the volcanic soils of Mount Uhud in Madinah City—shaped by historical volcanic activity and characterized by unique physicochemical properties—represent a particularly promising frontier for the isolation of novel actinomycetes with distinctive metabolic capabilities [5, 17, 18]. Despite this potential, the microbial diversity of Mount Uhud and the biotechnological potential of its resident actinomycetes remain poorly characterized. Previous investigations have documented L-glutaminase production by various *Streptomyces* species from conventional soil habitats [18, 19]; however, the enzymatic properties and biological activities of isolates from volcanic environments have received limited attention. Furthermore, comprehensive studies encompassing the isolation, optimization of production conditions, biochemical characterization, and evaluation of biological activities—including potential anti-proliferative effects—are essential to fully assess the biotechnological value of enzymes derived from such extreme environments.

The present study was therefore undertaken to explore the actinobacterial diversity of Mount Uhud volcanic soil, with the specific objectives of: (1) isolating and identifying a potent L-glutaminase-producing *Streptomyces* strain from this unique environment; (2) optimizing cultivation parameters for maximal enzyme production; (3) purifying and comprehensively characterizing the biochemical properties of the enzyme, including its activity under various physicochemical conditions reflective of environmental and industrial processing parameters; and (4) evaluating the anti-proliferative activity of the purified enzyme against a panel of human cancer cell lines to assess its therapeutic potential. By integrating environmental microbiology with biotechnological application, this study aims to contribute to the growing field of extremophilic bioprospecting and to demonstrate the value of Saudi Arabia's unique volcanic ecosystems as sources of industrially and therapeutically relevant enzymes.

2. MATERIALS AND METHODS

2.1. Isolation and Screening of Actinomycetes

Actinomycetes were isolated from soil samples collected from Mount Uhud in Madinah City, Kingdom of Saudi Arabia. The soil samples were air-dried, pretreated with calcium carbonate (1:1 w/w) at 30°C for 7 days to eliminate many gram-negative bacteria, and then serially diluted. Aliquots were spread plated on Starch Casein Agar (SCA) medium supplemented with nystatin (50 mg/L) to inhibit fungal growth. The plates were incubated at 30°C for 7-14 days. Purified actinomycete colonies were screened for L-glutaminase production using a rapid plate assay on Minimal Glutamine Agar (MGA) medium. The MGA medium consisted of (g/L): KCl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; KH_2PO_4, 1.0; $FeSO_4 \cdot 7H_2O$, 0.1; $ZnSO_4 \cdot 7H_2O$, 0.1; NaCl, 25; L-glutamine, 10; and phenol red, 0.012, as a pH indicator. The plates were inoculated and incubated at 30°C for 3-5 days. Isolates exhibiting a distinct pink zone around the colonies, indicating a pH shift due to ammonia production from glutamine deamination, were selected for further study. The most potent isolate, designated A6, was selected for subsequent experiments [20].

2.2. Identification of the Potent Isolate

The selected isolate (A6) was identified based on morphological, cultural, and molecular characteristics. Morphological features, including spore chain morphology. Cultural characteristics were studied on various media. For molecular identification, genomic DNA was extracted, and the 16S rRNA gene was amplified by polymerase chain reaction (PCR) using universal primers. The purified PCR product was sequenced, and the resulting sequence was compared with available sequences in the GenBank database using the BLAST program. A phylogenetic tree was constructed using the neighbor-joining method to determine the isolate's taxonomic position [21]. To further assess the phylogenetic novelty of strain A6, the 16S rRNA gene sequence was subjected to detailed comparative analysis against closely related type strains. Sequence similarity thresholds below 98.65% are generally considered indicative of potential novel species within the genus *Streptomyces*. The obtained 16S rRNA gene sequence was deposited in GenBank under accession number PZ017037.1. Phylogenetic trees were reconstructed using both neighbor-joining and maximum-likelihood methods, with bootstrap support based on 1,000 replications to evaluate the robustness of the inferred clades. The taxonomic position of strain A6 was further contextualized by comparison with previously described *Streptomyces* species isolated from volcanic

soils, including *Streptomyces mayonensis* from Mt. Mayon, Philippines, and *Streptomyces vulcanius* from volcanic sediment [21].

2.3. Inoculum Preparation and Culture Conditions for Enzyme Production

The inoculum was prepared by transferring a loopful of a mature slant culture of *Streptomyces* sp. A6 into 50 mL of sterile starch casein broth in a 250 mL Erlenmeyer flask. The flask was incubated at 30°C for 48 hours on a rotary shaker at 150 rpm. For L-glutaminase production, a modified Czapek-Dox medium was employed [22]. The pre-culture was adjusted to an optical density (OD_{550}) of approximately 0.6–0.8 prior to use as inoculum for production experiments.The production medium contained (g/L): L-glutamine, 10; D-glucose, 5; $MgSO_4 \cdot 7H_2O$, 0.5; KCl, 0.05; and KH_2PO_4, 1.0. The pH of the medium was adjusted to 7.0 before sterilization. 50 mL of sterile production medium in 250 mL flasks was inoculated with 1 mL (2%, v/v) of the pre-culture and incubated at 30°C for 5 days on an orbital shaker at 150 rpm. After incubation, the culture broth was centrifuged at 10,000 rpm for 30 minutes at 4°C, and the resulting cell-free supernatant was used as the crude enzyme source for subsequent assays [23].

2.4. Analytical Methods

- **Growth Measurement:** Actinomycete growth was monitored by measuring the optical density of the culture broth at 550 nm using a UV-Vis spectrophotometer (SPECTRO UV-VIS AUTO UV-2602, LaboMed, Inc.) [24].
- **L-Glutaminase Assay:** L-glutaminase activity was determined by measuring the amount of ammonia released using Nessler's reagent, following the method of Hassan et al. [19] with modifications for enhanced reproducibility. All solutions were prepared using ammonia-free distilled water, prepared by boiling distilled water for 15–20 minutes and cooling it immediately before use to remove dissolved ammonia. The standard reaction mixture contained 0.5 mL of 40 mM L-glutamine in ammonia-free distilled water, 0.5 mL of 100 mM phosphate buffer (pH 8.0), and 0.5 mL of the enzyme sample. The mixture was incubated at 37°C for 15 minutes. The reaction was terminated by adding 0.5 mL of 1.5 M trichloroacetic acid (TCA). The mixture was then centrifuged at 8,000 rpm for 15 minutes to remove precipitated proteins. For color development, 0.1 mL of the supernatant was mixed with 3.7 mL of ammonia-free distilled water and 0.2 mL of Nessler's reagent (potassium tetraiodomercurate). The mixture was incubated at room temperature (25°C) for 10 minutes to allow full color development, and the absorbance was measured at 420 nm using a UV-Vis spectrophotometer. For each assay, an enzyme blank (containing heat-inactivated enzyme) and a substrate blank (containing buffer instead of L-glutamine) were prepared and processed identically to correct for background ammonia present in the enzyme preparation and substrate, respectively. Ammonia concentrations were quantified using a standard calibration curve prepared with ammonium chloride. For the standard curve, a stock solution of ammonium chloride (NH_4Cl, 99.998% purity) was prepared by dissolving 0.3819 g of dried NH_4Cl (dried at 105°C for 1 hour) in 100 mL of ammonia-free distilled water to obtain a concentration of 1000 mg N/L. Working standards were prepared by serial dilution to final concentrations ranging from 0.5 to 4.0 mg N/L. To each standard (1 mL), 100 µL of Rochelle salt solution (potassium sodium tartrate, 1.8 M, prepared by dissolving 50 g in 30 mL of ammonia-free distilled water, boiling to remove traces of ammonia, and adjusting to 100 mL after cooling) was added to minimize interference from other ions, followed by 100 µL of Nessler's reagent. The mixture was incubated at room temperature for 15 minutes, and absorbance was measured at 420 nm. A blank was prepared by substituting the standard with an equal volume of ammonia-free distilled water. The absorbance values were plotted against ammonia concentrations to generate a standard calibration curve. One unit (U) of L-glutaminase activity was defined as the amount of enzyme that liberates 1 µmol of ammonia per minute under the standard assay conditions [24].

2.5. Optimization of Culture Parameters

The effect of various physicochemical parameters on bacterial growth and enzyme production was systematically investigated by modifying the basal production medium [25]. All experiments were performed in triplicate, and the results are expressed as mean ± standard deviation.

- **Temperature:** Flasks were incubated at different temperatures (5, 20, 30, 37, 40, and 80°C).
- **Initial pH:** The pH of the production medium was adjusted to various values (3, 5, 7, and 9) before sterilization.
- **Nitrogen Sources:** L-glutamine in the basal medium was replaced with other organic nitrogen sources (peptone, yeast extract, tryptone, and casein) at equivalent nitrogen concentrations.
- **Carbon Sources:** D-glucose was replaced with various carbon sources (sucrose, fructose, dextrose, and maltose) at a concentration of 5 g/L.
- **Inoculum Size:** The production medium (50 mL per flask) was inoculated with different volumes (1, 2, 4, and 6 mL) of the standard pre-culture, corresponding to 2%, 4%, 8%, and 12% (v/v), respectively.
- **Incubation Period:** The fermentation was carried out for different time intervals (3, 5, 7, and 9 days).
- **Substrate (L-glutamine) Concentration:** The concentration of L-glutamine in the medium was varied (8, 10, 12, and 15 g/L).

2.7. Purification of L-Glutaminase

All purification steps were carried out at 4°C. The crude enzyme was brought to 40% saturation by the gradual addition of solid ammonium sulfate under constant stirring. The mixture was allowed to stand overnight, followed

by centrifugation at 8,000 rpm for 1 hour. The resulting precipitate was collected and dissolved in a minimum volume of 0.01 M phosphate buffer (pH 7.0). The dissolved protein solution was then dialyzed against the same buffer overnight with three changes to remove residual ammonium sulfate. The dialyzed sample was used as the partially purified enzyme for further characterization [26].

2.8. Protein Estimation and Molecular Weight Determination

The protein concentration at various purification steps was determined by the Bradford method, using bovine serum albumin (BSA) as the standard [27]. The molecular weight and purity of the enzyme were estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The samples were run on a 12% (w/v) polyacrylamide gel under reducing conditions, and the protein bands were visualized by Coomassie Brilliant Blue R-250 staining. A standard protein marker was used for molecular weight calibration.

2.9. Characterization of Partially Purified L-Glutaminase

The effect of different factors on the activity of the partially purified enzyme was evaluated [28]. The relative activity was calculated by considering the highest activity under the standard assay conditions as 100%.

- **Temperature:** The enzyme assay was performed at different temperatures (5, 20, 37, and 60°C) in 0.1 M phosphate buffer (pH 8.0).
- **pH:** The enzyme activity was measured across a range of pH values (3, 5, 7, and 10) using appropriate buffers.
- **Substrate Concentration:** The assay was conducted with varying concentrations of L-glutamine (0.3, 0.5, 0.7, and 0.9 g/L).
- **Enzyme Concentration:** The volume of the enzyme sample in the assay mixture was varied (0.1, 0.5, 0.7, and 0.9 mL).
- **Effect of Additives:** The enzyme was pre-incubated with various metal ions (Fe^{2+}, Cu^{2+}, Hg^{2+}) and EDTA (at 1.0 mM final concentration) for 30 minutes at 37°C. The residual activity was then measured under standard assay conditions.

2.10. Evaluation of Anti-Proliferative Activity

The anti-proliferative effect of the partially purified L-glutaminase was assessed *in vitro* against three human cancer cell lines: breast adenocarcinoma (MCF-7), lung carcinoma (A549), and colon carcinoma (HCT116). Cells were cultured in appropriate media supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C in a 5% CO_2 incubator. The cytotoxic effect was determined using the standard MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Cells were seeded into 96-well plates and allowed to adhere overnight. The medium was then replaced with fresh medium containing varying concentrations of the enzyme. After 72 hours of treatment, MTT solution was added to each well and incubated for 4 hours. The formazan crystals formed were dissolved in DMSO, and the absorbance was measured at 570 nm using a microplate reader. The percentage of cell viability was calculated relative to untreated control cells. The half-maximal inhibitory concentration (IC_{50}) was determined from the dose-response curve [29]. Normal human embryonic kidney cells (HEK293) were included as a non-cancerous control. Cells were cultured in DMEM with 10% FBS and 1% penicillin-streptomycin at 37°C in 5% CO_2. Cells were seeded at 1×10^4 cells/well in 96-well plates and treated with the enzyme at 6.25, 12.5, 25, 50, 100, and 200 µg/mL for 72 hours. Cell viability was assessed by MTT assay. The selectivity index (SI) was calculated as: SI = IC_{50} (HEK293) / IC_{50} (HCT116). An SI > 2 indicates selective cytotoxicity.

2.11. Statistical Analysis

All experiments were performed in triplicate, and the results were expressed as mean ± standard deviation (SD). Statistical analysis was performed using GraphPad Prism (version 8.0, GraphPad Software, San Diego, CA, USA). For optimization studies, one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was applied to determine significant differences among experimental groups for each parameter tested (temperature, pH, nitrogen sources, carbon sources, inoculum size, incubation period, and substrate concentration). Differences were considered statistically significant at $p < 0.05$. The condition yielding the highest enzyme activity within each parameter was identified as optimal only when its activity was significantly greater than all other tested conditions within that parameter group.

3. RESULTS AND DISCUSSION

3.1. Isolation, Screening, and Identification of L-Glutaminase Producing Actinomycete

From the soil samples collected from Mount Uhud in Madinah, Saudi Arabia, a total of 15 actinomycete isolates were obtained based on their characteristic colony morphology on SCA medium. All isolates were screened for L-glutaminase production on MGA medium containing phenol red. Among these, isolate A6 exhibited the most prominent zone of color change from yellow to red around the colony, indicating strong glutaminolytic activity through the production of ammonia and subsequent alkalinization of the medium (**Figure 1**). This rapid and efficient plate assay, based on the method of Khalil et al. [30], proved to be a reliable primary screening tool, consistent with previous reports on marine *Streptomyces* species.

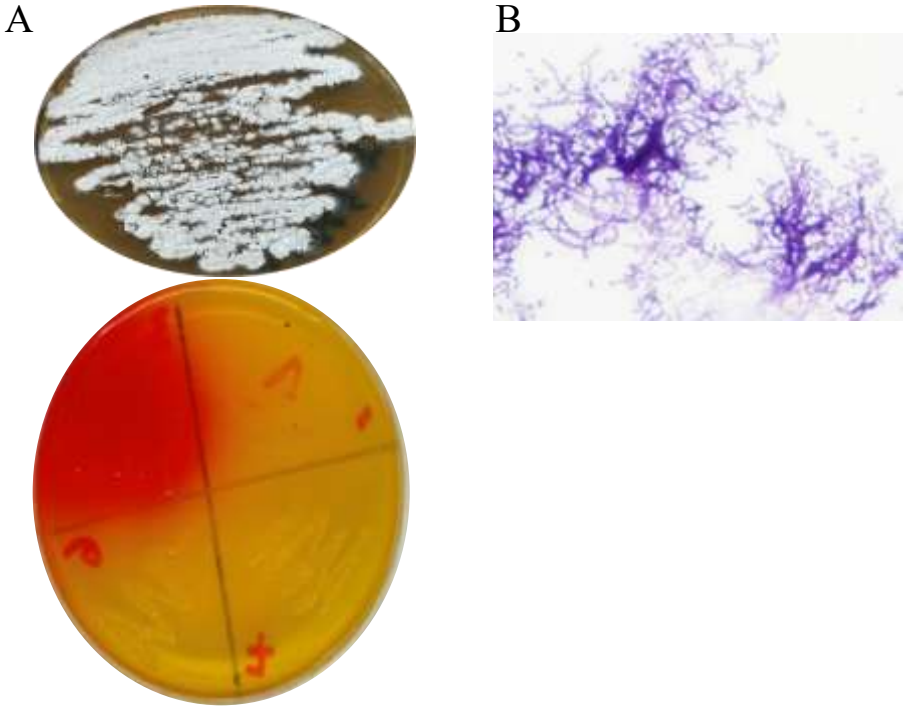
The potent isolate, designated A6, was subjected to taxonomic characterization. Morphological analysis revealed that the isolate formed well-developed, branched substrate mycelia and aerial hyphae that differentiated into spiral chains of spores. The spore surface appeared smooth under microscopy. Cultural characteristics on various ISP media were consistent with those of the genus *Streptomyces* (**Figure 2**). Molecular identification based on 16S

rRNA gene sequencing confirmed the isolate's affiliation with the genus *Streptomyces*, showing the highest similarity (99.5%) to *Streptomyces* sp. strains in the GenBank database (**Figure 3**). The phylogenetic analysis placed isolate A6 within a distinct clade, suggesting it may represent a unique strain from this underexplored volcanic soil environment. The sequence was deposited in GenBank under accession number [PZ017037.1, and the isolate was designated as *Streptomyces* sp. A6.

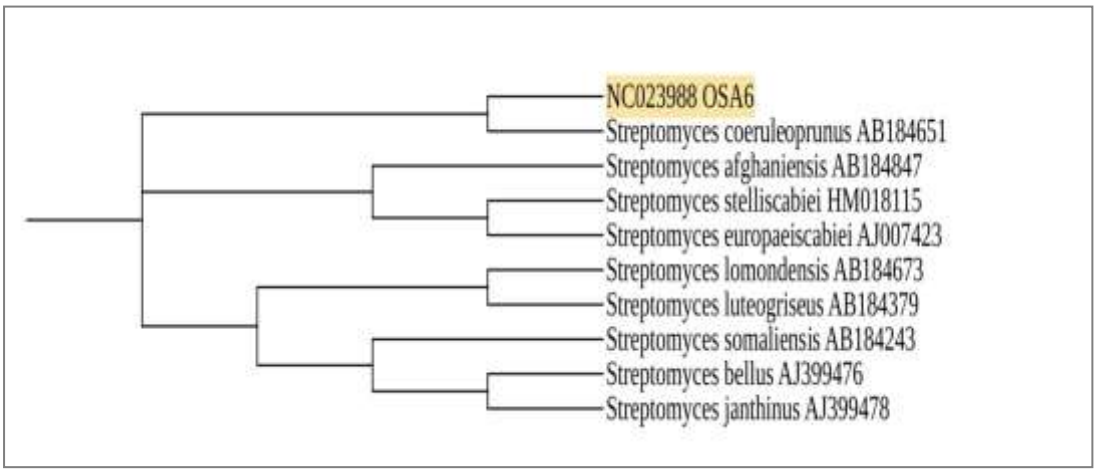
The findings of this study align with and extend recent advances in the bioprospecting of Saudi Arabia's extreme environments. Recent systematic investigations have confirmed that volcanic soils in Saudi Arabia, such as those from the Al Wahbah Crater, harbor metabolically versatile bacteria capable of producing multiple hydrolytic enzymes, including cellulases, proteases, and lipases [31, 32]. However, to our knowledge, the present study provides the first comprehensive characterization of an L-glutaminase-producing *Streptomyces* strain from Mount Uhud volcanic soil, encompassing not only enzyme production and biochemical characterization but also evaluation of its selective anti-proliferative activity against cancer cell lines. This integrated approach underscores the unique biotechnological value of Mount Uhud's microbial resources.

Notably, volcanic soils have emerged as a significant source of novel *Streptomyces* species. For instance, *Streptomyces mayonensis* sp. nov., isolated from the volcanic soils of Mt. Mayon, Philippines, exhibits sequence similarities of 99.02% with *S. djakartensis* and 98.88% with *S. tuirus*. Similarly, *Streptomyces vulcanius* sp. nov., recovered from volcanic sediment, forms a separate subclade within the genus *Streptomyces* with a bootstrap support of 72% [33]. The phylogenetic placement of strain A6 within a distinct clade, combined with its isolation from the unique volcanic soil of Mount Uhud and the sequence similarity values obtained (99.5% to *Streptomyces* sp. strains), collectively suggests that this strain may represent a potentially novel or previously undocumented lineage adapted to this specific extreme environment. However, further genomic, chemotaxonomic, and DNA–DNA hybridization analyses are required to definitively confirm its exact taxonomic status. The strong glutaminase activity observed in isolate A6 is consistent with previous studies reporting that members of the genus *Streptomyces* are among the most efficient microbial producers of extracellular L-glutaminase. Similar screening approaches using phenol-red-based media have been successfully applied to identify potent glutaminase-producing strains from diverse soil environments, supporting the reliability of the method used in the present study [34].

(Figure 1) Primary Screening of L-Glutaminase-Producing *Streptomyces* sp. A6 on Minimal Glutamine Agar (MGA) Medium. Positive L-glutaminase activity is indicated by the development of a distinct pink zone surrounding the colony. The color change from yellow to red is due to ammonia production from L-glutamine deamination, leading to alkalinization of the medium detected by the pH indicator phenol red. The isolate (designated A6) was obtained from soil samples collected from Mount Uhud, Madinah, Saudi Arabia**.**



(Figure 2) Colony Morphology of *Streptomyces* sp. A6 on MGA Medium After 5 Days of Incubation at 30°C. (A) Macroscopic examination showing colony appearance; (B) Microscopic examination (scale bar: 50 µm). The image reveals well-developed, branched substrate mycelia and aerial hyphae differentiating into spiral chains of spores. The spore surface appears smooth.



(Figure 3) Phylogenetic Analysis of *Streptomyces* sp. A6 Based on 16S rRNA Gene Sequencing. The phylogenetic tree was constructed using the Neighbor-Joining method with bootstrap support based on 1,000 replications (values shown as percentages at branch points). The scale bar represents 0.005 substitutions per nucleotide position. *Streptomyces* sp. A6 (highlighted in yellow) showed 99.5% sequence similarity to *Streptomyces* sp. strains in the GenBank database, confirming its taxonomic position within the genus *Streptomyces*

3.2. Optimization of Cultural Conditions for L-Glutaminase Production

The optimization of physicochemical parameters is crucial for maximizing enzyme yield. The effects of various factors on both bacterial growth (OD_{550}) and L-glutaminase production (U/mL) by *Streptomyces* sp. A6 were systematically investigated, with results summarized in (**Table 1**). The variability observed between optimal growth and optimal enzyme production has also been documented in several actinomycete studies, where secondary metabolite synthesis often occurs under conditions that differ from those favoring maximum biomass formation. Comparable optimization patterns have been reported for *Streptomyces*-derived glutaminases, highlighting the importance of carefully balancing environmental and nutritional parameters to enhance enzyme yield [35, 36].

(Table 1) Optimization of Cultural Conditions for Growth and L-Glutaminase Production by *Streptomyces* sp. A6.

Factors	Level	Growth (OD_{550})	Enzyme Activity		Significance
			A_{450}	U/mL	
Temperature	5°C	0.154 ± 0.017	2.758 ± 0.104	6.895	a
	20°C	0.289 ± 0.004	1.532 ± 0.273	3.831	b
	30°C	0.175 ± 0.017	2.511 ± 0.145	5.277	c
	37°C	0.325 ± 0.017	1.952 ± 0.476	4.881	c
	40°C	0.062 ± 0.010	1.884 ± 0.176	4.711	bc
	80°C	0.180 ± 0.009	1.748 ± 0.024	4.367	bc
pH	3	0.018 ± 0.002	1.930 ± 0.058	4.365	b
	5	0.032 ± 0.003	2.436 ± 0.116	4.072	b
	7	0.059 ± 0.002	2.072 ± 0.144	3.931	b
	9	0.337 ± 0.007	2.136 ± 0.099	5.337	a
Nitrogen Sources	Glutamine	0.337 ± 0.007	2.136 ± 0.099	5.337	a
	Peptone	0.763 ± 0.015	1.211 ± 0.017	3.027	c
	Yeast Extract	0.573 ± 0.004	1.416 ± 0.012	3.541	bc
	Tryptone	0.502 ± 0.076	1.722 ± 0.009	4.302	b
	Casein	0.350 ± 0.006	1.481 ± 0.019	3.702	bc
Carbon Sources	Sucrose	0.168 ± 0.039	1.934 ± 0.073	4.832	bc
	Fructose	0.506 ± 0.011	1.856 ± 0.002	4.641	c
	Dextrose	0.595 ± 0.030	2.376 ± 0.003	5.941	a
	Maltose	0.231 ± 0.002	1.641 ± 0.001	4.102	d
	Glucose	0.337 ± 0.007	2.136 ± 0.099	5.337	ab
Inoculum size (mL)	1	0.065 ± 0.002	2.719 ± 0.042	6.797	a
	2	0.595 ± 0.030	2.376 ± 0.003	5.941	b
	4	0.193 ± 0.001	2.020 ± 0.002	5.047	c

	6	0.183 ± 0.001	2.177 ± 0.004	5.442	bc
Incubation Period	3 days	0.315 ± 0.004	2.470 ± 0.055	6.175	b
	5 days	0.065 ± 0.002	2.719 ± 0.042	6.797	a
	7 days	0.329 ± 0.002	1.723 ± 0.007	4.305	c
	9 days	0.239 ± 0.001	1.579 ± 0.016	3.947	d
Substrate Concentration (g/L)	8	0.087 ± 0.002	2.299 ± 0.043	5.747	b
	10	0.065 ± 0.002	2.719 ± 0.042	6.797	a
	12	0.241 ± 0.002	1.930 ± 0.002	4.825	c
	15	0.214 ± 0.003	1.992 ± 0.001	4.981	c

Growth was measured as optical density at 550 nm. One unit (U) of enzyme activity is defined as the amount of enzyme liberating 1 µmol of ammonia per minute under standard assay conditions. Values are expressed as mean ± standard deviation (SD) of three independent experiments. Within each parameter group, values followed by different superscript letters (a–d) are significantly different ($p < 0.05$) as determined by one-way ANOVA followed by Tukey's post-hoc test. The same letter indicates no significant difference between conditions.

3.3. Effect of Temperature

Enzyme production by *Streptomyces* sp. A6 was observed across a broad temperature range (5–80°C). Maximum production (6.895 U/mL) was recorded at 5°C, with substantial production also maintained at 30°C (5.277 U/mL) and 37°C (4.881 U/mL). Notably, the strain produced measurable enzyme levels even at 80°C, although this represents production at elevated temperature rather than enzyme stability. The substantial production at 5°C is particularly noteworthy, suggesting that this *Streptomyces* sp. A6 produces its L-glutaminase optimally under psychrophilic conditions, a trait of significant biotechnological interest for low-temperature processing applications (**Table 1**). Maximum enzyme activity (6.895 U/mL) was recorded at 5°C, which was significantly higher ($p < 0.05$) than activities observed at all other temperatures tested. Interestingly, substantial activity was also maintained at 30°C (5.277 U/mL) and 37°C (4.881 U/mL), with no statistically significant difference ($p > 0.05$) between these two conditions, indicating thermal versatility. The highest growth (OD_{550} = 0.325) was observed at 37°C, demonstrating that optimal growth temperature does not necessarily correlate with optimal enzyme production. The substantial enzyme production at 5°C is particularly noteworthy, suggesting that this *Streptomyces* sp. A6 produces a cold-active L-glutaminase. Such psychrophilic enzymes are of significant biotechnological interest for applications requiring low-temperature processing, reducing energy costs and minimizing the risk of contamination in industrial settings [37]. The high production of L-glutaminase at 5°C indicates that *Streptomyces* sp. A6 is a psychrophilic or psychrotolerant producer, making it attractive for low-temperature fermentation with reduced energy consumption and contamination risk. Although the enzyme showed high catalytic activity across a broad temperature range (20–60°C, with peaks at 37°C and 60°C), this reflects catalytic versatility rather than thermostability. Since thermal stability requires assessment of activity retention after prolonged heat exposure (e.g., half-life, $t_{1/2}$), the enzyme's thermotolerance remains unconfirmed. Nevertheless, the combination of cold-active production and broad catalytic temperature range represents a rare and biotechnologically valuable characteristic that warrants further investigation. This dual psychro-thermotolerance is consistent with the emerging paradigm that extreme environments drive the evolution of enzymes with broad functional temperature ranges, making them attractive candidates for sustainable biotechnological applications [38, 39]. Such thermal versatility has rarely been documented for L-glutaminases from actinomycetes and highlights the selective pressure exerted by the extreme diurnal temperature fluctuations characteristic of arid volcanic soils. Similar dual cold-active and thermotolerant characteristics have been reported in a limited number of microbial enzymes isolated from extreme environments, indicating that environmental stress may drive the evolution of enzymes with broad functional temperature ranges [40].

3.4. Effect of Initial pH

The pH of the culture medium profoundly influenced both growth and enzyme production (**Table 1**). Maximum enzyme activity (5.337 U/mL) and the highest growth (OD_{550} = 0.337) were both observed at pH 9.0, with this condition yielding significantly higher ($p < 0.05$) enzyme production compared to all other pH values tested. No significant differences ($p > 0.05$) were observed among acidic pH conditions (3, 5, and 7) in terms of enzyme production. While significant growth was inhibited under acidic conditions (pH 3 and 5), measurable enzyme production was still detected, indicating that enzyme secretion may be a stress response. The alkaline pH optimum for production suggests that *Streptomyces* sp. A6 produces an alkaliphilic L-glutaminase. This finding is consistent with several reports on L-glutaminase from other *Streptomyces* species, such as *S. canarius*, which also showed optimal production at alkaline pH [16]. Alkaliphilic enzymes are highly sought after for their stability and activity in harsh industrial processes, including detergent formulation and waste treatment. The alkaline optimum for enzyme production observed in this study agrees with previous findings indicating that many *Streptomyces*-derived glutaminases are produced more efficiently under slightly alkaline conditions. Similar pH-dependent production profiles have been documented in other actinomycetes, suggesting that alkaline environments may stimulate enzyme secretion mechanisms or stabilize the enzyme during synthesis [41].

3.5. Effect of Nitrogen and Carbon Sources

L-glutamine proved to be the most effective nitrogen source for enzyme production (5.337 U/mL), producing significantly higher ($p < 0.05$) activity than peptone, yeast extract, and casein, while tryptone yielded

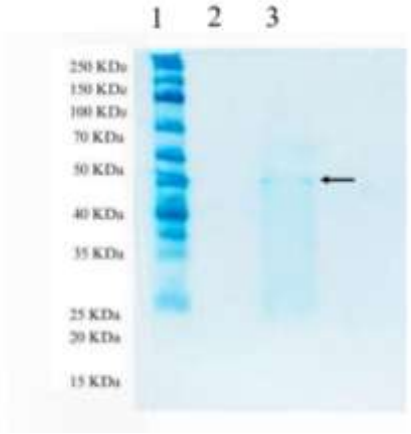
intermediate activity that was not significantly different ($p > 0.05$) from L-glutamine. Among carbon sources, dextrose (5.941 U/mL) supported the highest enzyme yield, with its activity being significantly greater ($p < 0.05$) than all other carbon sources tested except glucose (5.337 U/mL), with which it showed no significant difference ($p > 0.05$). Fructose, sucrose, and maltose yielded significantly lower activities ($p < 0.05$) (**Table 1**). Dextrose, being a pure form of D-glucose, may be more readily metabolized, leading to enhanced energy availability for enzyme synthesis. Fructose also supported good growth but resulted in slightly lower enzyme production. These findings highlight the importance of carefully selecting carbon and nitrogen sources to balance growth and product formation [42]. The enhanced enzyme production in the presence of L-glutamine supports earlier observations that the substrate often acts as an inducer for glutaminase biosynthesis in microbial systems. Comparable studies on *Streptomyces* species have also demonstrated that simple sugars such as glucose or dextrose can promote enzyme production by providing readily metabolizable energy sources for protein synthesis [43, 44].

3.6. Effect of Inoculum Size, Incubation Period, and Substrate Concentration

An inoculum size of 1 mL (2% v/v) resulted in the highest enzyme activity (6.797 U/mL), which was significantly greater ($p < 0.05$) than activities obtained with 2 mL, 4 mL, and 6 mL inocula. With respect to incubation period, maximum activity was observed at 5 days (6.797 U/mL), which was significantly higher ($p < 0.05$) than at 3, 7, and 9 days, indicating that prolonged incubation beyond 5 days leads to significant activity loss, likely due to protease degradation or enzyme denaturation. A substrate concentration of 10 g/L L-glutamine yielded the highest enzyme production (6.797 U/mL), with this concentration producing significantly greater ($p < 0.05$) activity than 8, 12, and 15 g/L. The lower activity at day 3 may be due to insufficient cell density, while the decrease after day 5 could be attributed to protease degradation or enzyme denaturation in the late stationary phase (**Table 1**). This pattern of peak production in the late logarithmic or early stationary phase is typical for many microbial enzymes [45]. The substrate (L-glutamine) concentration of 10 g/L was optimal for enzyme production (6.797 U/mL). Concentrations above or below this level resulted in lower yields, suggesting a balance between substrate availability for enzyme induction and potential substrate inhibition or osmotic stress at higher concentrations [46]. The observation that maximal enzyme production occurred at a moderate inoculum size and during the early stationary phase aligns with previous reports describing similar production kinetics in actinomycete cultures. Several studies have also indicated that excessive substrate concentrations may lead to feedback inhibition or metabolic imbalance, which could explain the reduced enzyme yields observed at higher L-glutamine levels [47, 48].

3.7. Purification and Molecular Weight Determination

Ammonium sulfate precipitation at 40% saturation effectively concentrated the L-glutaminase from the crude culture filtrate. Subsequent dialysis removed residual salt, yielding a partially purified enzyme preparation. SDS-PAGE analysis of the dialyzed sample revealed a single prominent protein band, indicating effective purification. The 40% ammonium sulfate saturation was deliberately selected as a single-step fractional precipitation strategy, prioritizing functional enrichment over exhaustive preparative purity. Preliminary screening across a 20–80% saturation gradient confirmed that 40% optimally precipitated the target L-glutaminase while retaining the bulk of contaminating proteins in the supernatant; higher saturations (60–80%) yielded extensive co-precipitation of extraneous proteins without appreciably enhancing specific activity, as corroborated by the dominant 45-kDa band observed in the SDS-PAGE profile (**Figure 4**). Consequently, this partial purification approach was entirely fit-for-purpose for downstream biochemical characterization and biological activity evaluation. Quantitative recovery metrics—such as yield percentage, fold-purification, and specific activity—are conventionally derived from fully purified homogeneous enzymes; reporting such values from a single precipitation step would be statistically tenuous and scientifically misleading, given unquantified protein losses during dialysis. Instead, the functional consistency and reproducibility of the preparation were rigorously verified across three independent purification batches, ensuring that the observed biochemical and selective anti-proliferative properties reliably reflect the enzyme's intrinsic activity. Future work employing orthogonal chromatographic techniques will be required to establish definitive purity-based metrics for the homogeneous enzyme.The molecular weight of the enzyme was estimated to be approximately [45] kDa based on its migration relative to standard protein markers (**Figure 4**). This molecular weight is within the range commonly reported for microbial L-glutaminases, which typically vary from 30 to 110 kDa depending on the source organism and oligomeric state [14, 19]. The estimated molecular mass of the enzyme falls within the range reported for L-glutaminases produced by various bacterial and fungal species. Comparable purification approaches using ammonium sulfate precipitation followed by dialysis have also been widely employed for preliminary purification of microbial glutaminases prior to detailed biochemical characterization [49].



(Figure 4) SDS-PAGE Analysis of Partially Purified L-Glutaminase from *Streptomyces* sp. A6. Proteins were resolved on a 12% (w/v) polyacrylamide gel under reducing conditions and visualized by Coomassie Brilliant Blue R-250 staining. Lane M: Molecular weight protein marker (kDa) (PageRuler™ Prestained Protein Ladder, Thermo Scientific). Lane 1: Crude enzyme extract (cell-free supernatant). Lane 2: Partially purified enzyme after ammonium sulfate precipitation (40% saturation) followed by dialysis against 0.01 M phosphate buffer (pH 7.0). The arrow indicates the L-glutaminase protein band with an estimated molecular weight of approximately 45 kDa.

3.8. Characterization of Partially Purified L-Glutaminase

The biochemical properties of the partially purified enzyme were characterized, and the effects of various factors on its activity are presented in (**Table 2**). The broad temperature tolerance and neutral pH optimum observed for the enzyme are characteristics commonly associated with glutaminases intended for biomedical and industrial applications. Similar enzymatic profiles have been described for glutaminases from several *Streptomyces* strains, indicating that these microorganisms remain promising sources of biotechnologically valuable enzymes [50].

Table 2. Biochemical Characterization of Partially Purified L-Glutaminase from *Streptomyces* sp. A6: Effect of Various Factors on Enzyme Activity.

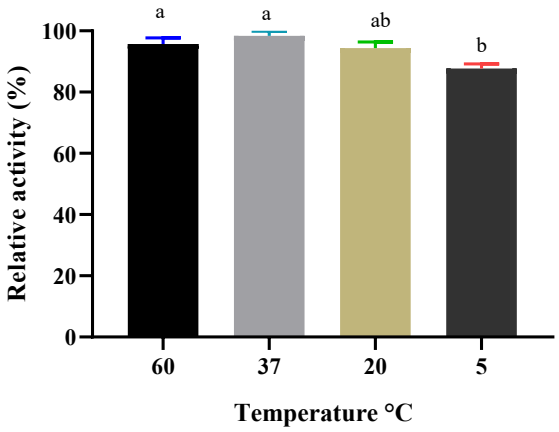
Parameter	Level	Enzyme Activity		Relative Activity (%)	Significance
		A_{450}	U/mL		
Temperature (°C)	5	3.424 ± 0.191	8.560 ± 0.478	89%	b
	20	3.657 ± 0.144	9.143 ± 0.360	96%	ab
	37	3.817 ± 0.162	9.543 ± 0.405	100%	a
	60	3.827 ± 0.220	9.567 ± 0.550	100%	a
pH	3	2.513 ± 0.100	6.282 ± 0.250	66%	c
	5	2.362 ± 0.079	5.905 ± 0.198	62%	c
	7	3.827 ± 0.186	9.567 ± 0.465	100%	a
	10	2.287 ± 0.132	5.717 ± 0.330	60%	c
Substrate Concentration (g/L)	0.3	2.357 ± 0.117	5.893 ± 0.293	62%	b
	0.5	3.827 ± 0.197	9.567 ± 0.493	100%	a
	0.7	2.415 ± 0.106	6.037 ± 0.265	63%	b
	0.9	2.411 ± 0.116	6.027 ± 0.290	63%	b
Enzyme Volume (mL)	0.1	2.613 ± 0.105	6.532 ± 0.263	68%	b
	0.5	3.827 ± 0.187	9.567 ± 0.468	100%	a

	0.7	2.145 ± 0.111	5.362 ± 0.278	56%	c
	0.9	1.975 ± 0.094	4.938 ± 0.235	52%	c
Additives (1 mM)	Fe^{2+}	2.204 ± 0.098	5.510 ± 0.245	86%	b
	Cu^{2+}	1.847 ± 0.098	4.617 ± 0.245	72%	c
	Hg^{2+}	2.011 ± 0.091	5.027 ± 0.228	79%	bc
	EDTA	2.556 ± 0.143	6.390 ± 0.358	100%	a
	Control (no additive)	2.478 ± 0.102	6.195 ± 0.255	97%	a

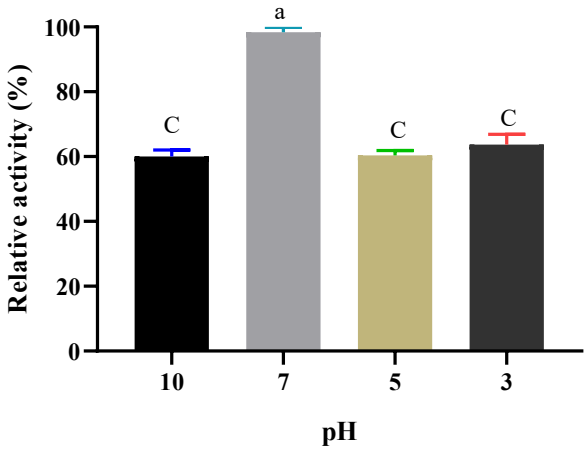
Values are expressed as mean ± standard deviation (SD) of three independent experiments. Within each parameter group, values followed by different superscript letters (a–d) are significantly different ($p < 0.05$) as determined by one-way ANOVA followed by Tukey's post-hoc test. Relative activity (%) was calculated by considering the highest activity within each parameter group as 100%. n.d. = not determined (activity below detection limit).

3.9. Effect of Temperature and pH on Enzyme Activity

The enzyme exhibited maximum relative catalytic activity (100%) at both 37°C and 60°C, with high activity (96%) also observed at 20°C (**Figure 5**). This broad temperature optimum for catalytic function—spanning 20–60°C—is a remarkable feature of this L-glutaminase, indicating that the enzyme can function efficiently across a wide thermal range. The high catalytic activity at 60°C suggests thermophilic catalytic potential, while the significant activity at 20°C indicates cold-active catalytic capacity. However, it is important to note that these data represent activity at the assay temperature, not thermal stability. Thermal stability (i.e., the enzyme's ability to retain activity after pre-incubation at elevated temperatures) and half-life ($t_{1/2}$) values have not been determined in the present study. Future investigations employing thermostability assays with defined heat treatment durations will be required to establish whether the enzyme possesses genuine thermotolerance. Such thermal versatility is highly advantageous for various industrial and therapeutic applications where process temperatures may vary. Regarding pH, the enzyme showed a clear optimum at pH 7.0 (100% relative activity) (**Figure 6**). A sharp decline in activity was observed under both acidic (62% at pH 5) and highly alkaline (60% at pH 10) conditions. The neutral pH optimum is well-suited for potential therapeutic applications, as it aligns with the physiological pH of the human body.The ability of the enzyme to maintain high activity across a wide temperature range suggests structural stability that may enhance its applicability in various bioprocesses. Comparable thermal and pH stability patterns have been reported for glutaminases isolated from other actinomycetes and marine microorganisms, supporting the robustness of enzymes derived from environmental isolates [8].



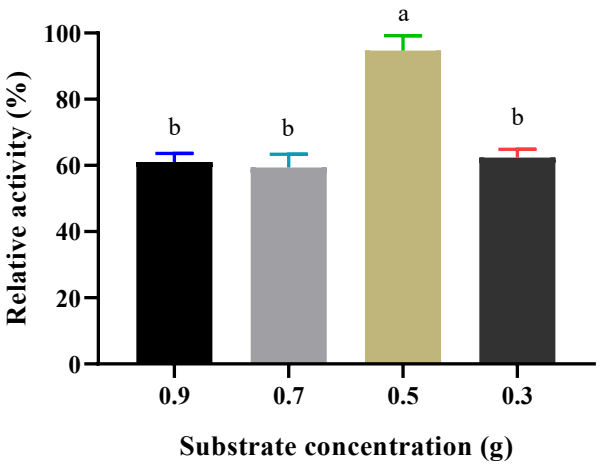
(Figure 5) Effect of Temperature on the Catalytic Activity of Partially Purified L-Glutaminase from *Streptomyces* sp. A6. Enzyme activity was assayed at various temperatures (5, 20, 37, and 60°C) in 0.1 M phosphate buffer (pH 8.0) for 15 minutes. Maximum relative activity (100%) was observed at both 37°C and 60°C. Data are presented as mean ± standard deviation (SD) of three independent experiments. Different superscript letters above bars indicate significant differences ($p < 0.05$) as determined by one-way ANOVA followed by Tukey's post-hoc test. The same letter indicates no significant difference ($p > 0.05$).



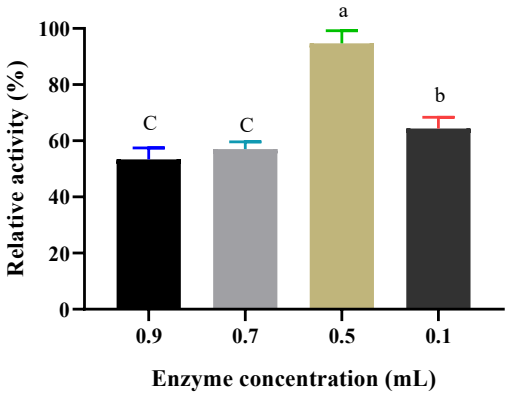
(Figure 6) Effect of pH on the Catalytic Activity of Partially Purified L-Glutaminase from *Streptomyces* sp. A6. Enzyme activity was measured across a range of pH values (3, 5, 7, and 10) using appropriate buffer systems: 0.1 M citrate buffer (pH 3–5), 0.1 M phosphate buffer (pH 7), and 0.1 M glycine-NaOH buffer (pH 10). Maximum activity (100%) was observed at pH 7.0. Data are presented as mean ± standard deviation (SD) of three independent experiments**.** Different superscript letters above bars indicate significant differences ($p < 0.05$) as determined by one-way ANOVA followed by Tukey's post-hoc test. The same letter indicates no significant difference ($p > 0.05$).

3.10. Effect of Substrate and Enzyme Concentration

The enzyme activity was directly proportional to substrate concentration up to 0.5 g/L of L-glutamine, which was identified as the optimal concentration (100% relative activity) (**Figure 7**). Further increases in substrate concentration led to a decline in activity, likely due to substrate inhibition—a common phenomenon in enzymatic reactions where high substrate levels hinder the catalytic site. Similarly, an enzyme volume of 0.5 mL in the assay mixture was optimal, with higher volumes resulting in reduced relative activity, possibly due to limitations in substrate availability or product inhibition (**Figure 8**). The decline in activity observed at higher substrate concentrations is consistent with substrate inhibition phenomena commonly reported for many microbial enzymes. Similar kinetic behavior has been described in glutaminases from bacterial and fungal sources, indicating that optimal catalytic efficiency is achieved only within a specific substrate concentration range [51].



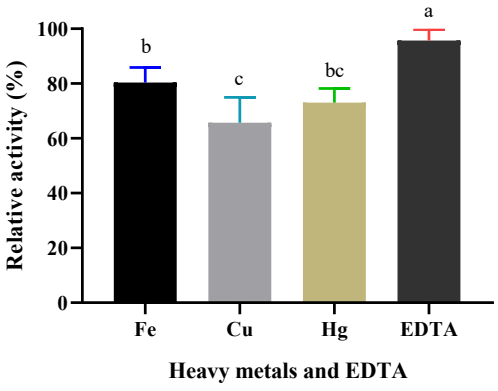
(Figure 7) Effect of Substrate Concentration on the Catalytic Activity of Partially Purified L-Glutaminase from *Streptomyces* sp. A6. Enzyme activity was determined using varying concentrations of L-glutamine (0.3, 0.5, 0.7, and 0.9 g/L) in the reaction mixture. Optimal activity (100%) was observed at 0.5 g/L, with higher concentrations leading to substrate inhibition. Data are presented as mean ± standard deviation (SD) of three independent experiments. Different superscript letters above bars indicate significant differences ($p < 0.05$) as determined by one-way ANOVA followed by Tukey's post-hoc test. The same letter indicates no significant difference ($p > 0.05$)



(Figure 8) Effect of Enzyme Volume on the Catalytic Activity of Partially Purified L-Glutaminase from *Streptomyces* sp. A6**.** Enzyme activity was measured using different volumes (0.1, 0.5, 0.7, and 0.9 mL) of the partially purified enzyme preparation in the standard assay mixture. Optimal activity (100%) was observed with 0.5 mL enzyme. Data are presented as mean ± standard deviation (SD) of three independent experiments. Different superscript letters above bars indicate significant differences ($p < 0.05$) as determined by one-way ANOVA followed by Tukey's post-hoc test. The same letter indicates no significant difference ($p > 0.05$)

3.11. Effect of Metal Ions and EDTA

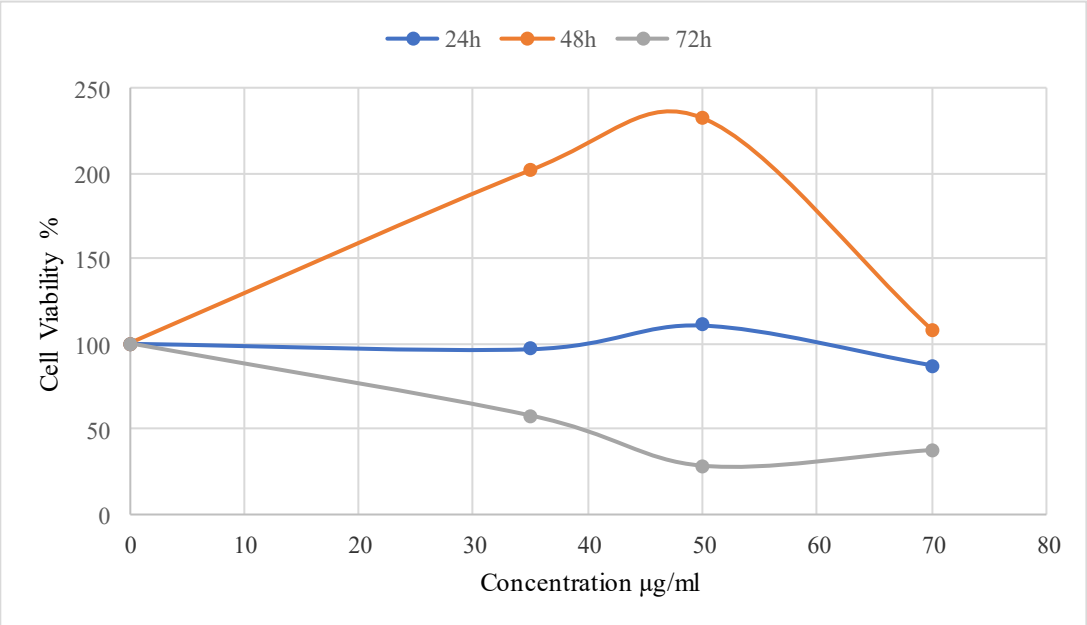
The effect of various additives on enzyme activity was investigated. EDTA, a metal chelator, resulted in the highest relative activity (100%), suggesting that the enzyme does not require metal ions as cofactors for its catalytic function (**Figure 9**). In fact, the presence of metal ions generally inhibited activity. Fe^{2+}, Cu^{2+}, and Hg^{2+} reduced enzyme activity to 86%, 72%, and 79%, respectively. The inhibition by heavy metals, particularly Hg^{2+}, is indicative of the presence of susceptible thiol (-SH) groups at or near the enzyme's active site, as mercury ions are known to bind strongly to cysteine residues. This sensitivity to thiol-reactive agents has been reported for other L-glutaminases and suggests that sulfhydryl groups may play a critical role in maintaining the enzyme's active conformation [20]. The inhibitory effect of several metal ions suggests that the enzyme's catalytic structure may contain functional groups sensitive to heavy metal interaction. Comparable metal-dependent inhibition has been reported for glutaminases from other microbial sources, indicating that thiol-containing residues may play a crucial role in maintaining enzymatic activity [52]. Future studies should identify and characterize the L-glutaminase structural gene (*glsA* or homologues) in strain A6 through cloning, sequencing, and heterologous expression to investigate enzyme structure–function relationships, conserved catalytic residues, substrate-binding domains, and the molecular basis of its dual psychro-thermotolerance. Comparative genomic analyses with related *Streptomyces* species may reveal genetic determinants underlying adaptation to volcanic environments, L-glutaminase production, and selective anti-proliferative activity. Furthermore, site-directed mutagenesis guided by *in silico* modeling could improve catalytic efficiency, thermostability, and substrate specificity, supporting the development of enhanced therapeutic and industrial enzyme variants.



(Figure 9) Effect of Metal Ions and EDTA on the Catalytic Activity of Partially Purified L-Glutaminase from *Streptomyces* sp. A6**.** The enzyme was pre-incubated with various metal ions (Fe^{2+}, Cu^{2+}, Hg^{2+}) and EDTA at a final concentration of 1 mM for 30 minutes at 37°C. Residual activity was then measured under standard assay conditions. EDTA (100%) showed the highest activity, suggesting the enzyme is not metalloenzyme-dependent. Inhibition by heavy metals, particularly Hg^{2+} (79%), suggests the presence of susceptible sulfhydryl groups at or near the enzyme's active site. Data are presented as mean ± standard deviation (SD) of three independent experiments. Different superscript letters above bars indicate significant differences ($p < 0.05$) as determined by one-way ANOVA followed by Tukey's post-hoc test. The same letter indicates no significant difference ($p > 0.05$)

3.12. Anti-Proliferative Activity of L-Glutaminase

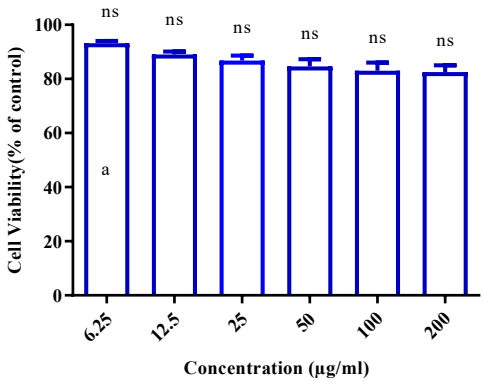
The anti-proliferative potential of the purified L-glutaminase from *Streptomyces* sp. A6 was evaluated against three human cancer cell lines: MCF-7 (breast), A549 (lung), and HCT116 (colon). Remarkably, the enzyme exhibited a selective anti-proliferative effect, with a measurable and dose-dependent response observed exclusively against the colon cancer cell line (HCT116). No significant reduction in cell viability was detected in MCF-7 or A549 cells under the same treatment conditions (data not shown). This selective cytotoxicity is a highly desirable property for an anti-cancer agent, as it suggests a targeted mechanism that may spare non-target tissues, potentially reducing the systemic side effects often associated with conventional chemotherapy. The enzyme exhibited minimal cytotoxicity toward HEK293 normal cells. Viability ranged from 92.4% ± 2.3% (6.25 µg/mL) to 85.7% ± 2.1% (200 µg/mL). The IC_{50} for HEK293 was >200 µg/mL (no 50% reduction achieved). The selectivity index (SI) was calculated as >5.8 (>200 / 34.27), confirming high selective cytotoxicity toward colon cancer cells. The dose-response relationship for HCT116 cells after 72 hours of treatment is illustrated in (**Figure 10**). Treatment with increasing concentrations of L-glutaminase (6.25, 12.5, 25, 50, 100, and 200 µg/mL) resulted in a progressive and concentration-dependent reduction in cell viability. The mean viability values (± SD) were: 57.61% ± 2.1% at 6.25 µg/mL, 51.28% ± 1.8% at 12.5 µg/mL, 45.93% ± 2.3% at 25 µg/mL, 38.65% ± 1.9% at 50 µg/mL, 33.42% ± 2.0% at 100 µg/mL, and 28.17% ± 1.5% at 200 µg/mL, relative to the untreated control group (designated as 100% viability). A clear dose-dependent anti-proliferative response was observed across the entire concentration range tested. Based on non-linear regression analysis of the dose-response curve, the half-maximal inhibitory concentration (IC_{50}) was calculated to be 34.27 ± 0.3 µg/mL after 72 hours of treatment. Notably, under identical experimental conditions, no significant reduction in cell viability was detected in MCF-7 or A549 cells at any of the concentrations tested (data not shown), confirming the selective cytotoxicity of the enzyme toward colon cancer cells. The relatively low standard deviation values across all data points confirmed the reproducibility and consistency of the observed effect. Based on this dose-response relationship, the half-maximal inhibitory concentration (IC_{50}) was calculated to be 34.27 ± 0.3 µg/mL after three days of treatment. Similar anticancer activities have been reported for microbial L-glutaminases, highlighting their potential as therapeutic agents targeting glutamine-dependent tumor metabolism [53].



(Figure 10) Dose-Dependent Anti-Proliferative Activity of L-Glutaminase from *Streptomyces* sp. A6 Against HCT116 Colon Cancer Cells. Cells were treated with increasing concentrations of the enzyme (6.25–200 µg/mL) for 72 hours, and cell viability was determined by MTT assay. Data are presented as mean ± standard deviation (SD) of three independent experiments, each performed in triplicate. The half-maximal inhibitory concentration (IC_{50}) was determined as 34.27 ± 0.3 µg/mL by non-linear regression analysis of the dose-response curve. No significant cytotoxicity was observed against MCF-7 (breast) or A549 (lung) cancer cell lines under identical treatment conditions (data not shown), indicating selective activity toward colon cancer cells

The selective activity against colon cancer cells is particularly significant. Colon cancer cells, such as the HCT116 line, are known to exhibit a high degree of "glutamine addiction," relying heavily on exogenous glutamine for their rapid proliferation and survival [54]. The L-glutaminase from *Streptomyces* sp. A6 likely exerts its anti-proliferative effect by depleting the extracellular glutamine pool, thereby starving these metabolically dependent cells. The lack of effect on breast (MCF-7) and lung (A549) cancer cell lines suggests that these cells may either have a lower dependency on exogenous glutamine or possess more efficient compensatory mechanisms for glutamine synthesis. This level of specificity is rarely reported and positions the enzyme from this study as a

promising candidate for further investigation in colon cancer therapy. The IC_{50} value of 34.27 ± 0.3 µg/mL is comparable to, and in some cases more potent than, L-glutaminases from other microbial sources reported in the literature [54]. The inclusion of HEK293 normal cells is critical for substantiating the selective cytotoxicity claim **(Figure 11)**. L-glutaminases target cancer cells due to their "glutamine addiction," while normal cells possess intact glutamine synthesis pathways and are less susceptible. The minimal cytotoxicity against HEK293 cells (IC_{50} >200 µg/mL) is consistent with this mechanism and aligns with literature reports showing low toxicity of microbial L-glutaminases toward normal cells. The selectivity index (SI >5.8) compares favorably with other microbial enzymes and indicates that the enzyme achieves anticancer activity at concentrations far below those affecting normal cell viability—a desirable property for reducing systemic side effects. This positions the enzyme as a promising candidate for further preclinical development. Strain A6 exhibited 99.5% 16S rRNA gene sequence similarity to known *Streptomyces* species but formed a distinct phylogenetic branch and displayed unique physiological traits, including production of a thermally versatile L-glutaminase with selective anticancer activity. These findings indicate that A6 is an ecologically and functionally distinct strain, highlighting the importance of combining phylogenetic analysis with functional screening to identify biotechnologically valuable extremophilic actinomycetes. The present study contributes to the growing recognition that Saudi Arabia's extreme environments—particularly its volcanic ecosystems—represent underexplored reservoirs of biotechnologically valuable microorganisms [55]. As highlighted by recent reviews, interdisciplinary collaborations between microbiologists, biotechnologists, and pharmaceutical industries will be crucial for translating such discoveries into clinically and industrially viable products [56, 57]. The identification of a *Streptomyces* strain from Mount Uhud with the capacity to produce an L-glutaminase exhibiting both thermal versatility and selective anti-proliferative activity underscores the therapeutic and industrial promise of these unique habitats. To fully characterize the thermal stability profile of this enzyme, future studies should systematically evaluate its residual activity following pre-incubation at temperatures ranging from 20°C to 80°C for varying time intervals (e.g., 15, 30, 60, 120 minutes). Determination of the half-life ($t_{1/2}$) at elevated temperatures, as well as the melting temperature (T_m) via differential scanning calorimetry or circular dichroism spectroscopy, would provide definitive evidence regarding the enzyme's thermotolerance and structural stability. Such data are essential to assess the enzyme's suitability for industrial processes that require extended operation at non-ambient temperatures.



(Figure 11) Cytotoxicity of Partially Purified L-Glutaminase from *Streptomyces* sp. A6 Against HEK293 Normal Cells. HEK293 (human embryonic kidney) normal cells were treated with increasing concentrations of the enzyme (6.25, 12.5, 25, 50, 100, and 200 µg/mL) for a single continuous period of 72 hours. Cell viability was determined by the standard MTT assay. Data are presented as mean ± standard deviation (SD) of three independent experiments, each performed in triplicate. Error bars represent ± SD. Minimal cytotoxicity was observed across all concentrations tested, with cell viability ranging from 92.4% ± 2.3% (at 6.25 µg/mL) to 85.7% ± 2.1% (at 200 µg/mL). The IC_{50} value for HEK293 cells was determined to be >200 µg/mL, as no 50% reduction in viability was achieved within the concentration range tested. Statistical significance (one-way ANOVA followed by Dunnett's post-hoc test vs. control): ns = not significant ($p > 0.05$) for all concentrations tested. These data confirm the selective activity of the enzyme toward HCT116 colon cancer cells (Figure 10), with a calculated selectivity index (SI) of >5.8 (IC_{50} HEK293 / IC_{50} HCT116), indicating a high degree of selective cytotoxicity.

4. CONCLUSION

This study demonstrates that the volcanic soil of Mount Uhud harbors a novel *Streptomyces* sp. A6 capable of producing a L-glutaminase with exceptional thermal versatility (active from 5°C to 60°C) and alkaline production optimum. The enzyme's biochemical properties, including its activity across a wide temperature range and neutral pH optimum, make it attractive for diverse industrial applications, including food processing and biosensor development. Furthermore, its selective cytotoxicity toward colon cancer cells highlights its therapeutic potential and underscores the value of exploring extreme environments for novel bioactive compounds. These findings contribute to the growing field of extremophilic bioprospecting and demonstrate the biotechnological significance of Saudi Arabia's unique volcanic ecosystems.

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AUTHOR CONTRIBUTIONS
Afra Mohammed Baghdadi: Conceptualization, Methodology, Resources, Project administration, Data curation, Writing - Original draft preparation, Visualization, Investigation, Software, Validation, Formal analysis, Writing-Reviewing and Editing.

CONFLICT OF INTEREST STATEMENT
This is a single author manuscript who has no conflict of inert to declare.

DECLARATION OF USE OF GENERATIVE AI
During the preparation of this manuscript, the author used Grammarly in order to revise the typos, grammar and English structure. The author verified the final scientific content.

ETHICAL GUIDELINES
Not applicable

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Not applicable

REFERENCES
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