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# Exploration of Bacterial Strains for Production of Glutaminase with Anti-Cancer Potential

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## Abstract

Glutaminase, an amidohydrolase enzyme that catalyzes the hydrolysis of L-glutamine to L-glutamic acid and ammonia, has emerged as a potential anticancer agent due to its ability to deprive cancer cells of glutamine—an essential nutrient for their rapid proliferation. This study explores the isolation, screening, and evaluation of bacterial strains for the production of glutaminase with potential anticancer properties. Soil samples from diverse habitats were collected and processed using serial dilution and selective media techniques to isolate glutaminase-producing bacteria. Preliminary screening was performed using phenol red-glutamine agar, followed by quantitative enzymatic assays to assess glutaminase activity. Crude enzyme extracts were subjected to in vitro cytotoxicity tests against selected human cancer cell lines to assess anticancer potential. Several bacterial strains demonstrated significant glutaminase production with notable cytotoxic effects, indicating their promise as bioresources for enzyme-based cancer therapy. This research contributes to the identification of novel microbial strains capable of producing therapeutic enzymes and provides a foundation for future biochemical and pharmacological investigations of glutaminase as a natural anticancer agent.

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## Keywords

Bacterial Glutaminase, production, anticancer activity

## Introduction

The enzyme industry is one of the most significant sectors worldwide, with a vast and expanding market for enzymes across various applications. Enzymes serve as biological catalysts that enhance the efficiency of biochemical reactions, making them indispensable in various industrial applications. Their ability to speed up chemical processes while maintaining specificity makes them highly desirable for multiple purposes. Given their widespread usage, there is an urgent need to explore novel sources of enzyme production, particularly through microbial means, which can provide cost-effective and sustainable solutions.

Cancer remains one of the most significant health challenges globally, with high mortality rates despite continuous advancements in treatment methodologies. Conventional cancer treatments such as chemotherapy, radiotherapy, and immunotherapy have proven effective to some extent.

One of the promising strategies in cancer treatment involves targeting metabolic pathways that are essential for cancer cell survival and proliferation. Unlike normal cells, cancer cells exhibit altered metabolic dependencies that support their rapid growth and uncontrolled division. Exploiting these metabolic vulnerabilities presents an opportunity for developing novel cancer therapies that

specifically disrupt cancer cell metabolism without significantly affecting normal cells.

Glutaminase is an enzyme responsible for catalyzing the conversion of glutamine to glutamate, an essential reaction in cellular metabolism. Cancer cells, particularly those with high metabolic activity, exhibit an increased dependence on glutamine for survival, a phenomenon known as "glutamine addiction."

Glutamine serves as a vital nutrient for cancer cells, contributing to energy production, biosynthesis, and signaling pathways that promote cell proliferation. By breaking down glutamine, glutaminase effectively depletes this crucial resource, thereby disrupting cancer cell metabolism.

This depletion can inhibit tumor growth and, in some cases, induce programmed cell death (apoptosis) in cancer cells. Due to its potential to interfere with cancer metabolism, glutaminase has emerged as an attractive target for anticancer drug development.

Several studies have explored the anticancer potential of glutaminase inhibitors,

Microorganisms, particularly bacteria, represent a vast and under-explored resource for enzyme production. Bacteria are known for their remarkable metabolic diversity and ability to thrive in diverse environments, making them valuable candidates for the discovery of bio-active compounds, including therapeutic enzymes.

The production of glutaminase by bacterial strains offers several advantages, including cost-effectiveness, scalability, and the possibility of obtaining enzymes with enhanced stability and specificity.

Compared to synthetic or chemically modified enzyme formulations, bacterial-derived glutaminase may possess unique properties that enhance its therapeutic potential. Certain bacterial strains may produce glutaminase with superior enzymatic activity, structural stability, or selective cytotoxicity against cancer cells. These characteristics make bacterial sources of glutaminase a promising avenue for anticancer therapy development.

The current research aims to identify, isolate, and characterize bacterial strains capable of producing glutaminase with potential anticancer properties. This involves the collection and screening of various environmental samples to identify bacterial species that naturally produce the enzyme. Once potential strains are identified, the next step involves optimizing the conditions for maximum enzyme production through fermentation and bioprocessing techniques.

Following enzyme production, the purified glutaminase is subjected to biochemical characterization to determine its structural and functional properties. This includes studying its stability under different pH and temperature conditions, its substrate specificity, and its kinetic parameters. Additionally, molecular techniques are employed to analyze the genetic and regulatory mechanisms governing glutaminase expression in bacterial strains.

To assess the therapeutic potential of bacterial-derived glutaminase, its anticancer activity is evaluated using various cancer cell lines. The enzyme is tested for its ability to inhibit cancer cell growth, induce apoptosis, and interfere with metabolic pathways essential for cancer cell survival. These experiments provide crucial insights into the potential use of bacterial glutaminase as a novel anticancer agent.

Overall, this research represents a step forward in the search for alternative and innovative cancer therapies. By integrating microbiology, biochemistry, and molecular biology, the study aims to bridge the gap between fundamental enzyme research and practical therapeutic applications.

The successful identification and characterization of glutaminase-producing bacteria could contribute to the advancement of enzyme-based treatments, offering new hope for cancer patients worldwide.

Glutaminase is an enzyme that hydrolyzes glutamine into glutamate and ammonia, playing a vital role in cellular metabolism and nitrogen balance. This reaction is particularly important for various physiological processes, including neurotransmitter production, as glutamate serves as a key excitatory neurotransmitter in the brain (*El-Asmar & Greenberg, 1966*). The enzyme exists in different isoforms, primarily located in the mitochondria, where it facilitates the conversion of glutamine to glutamate, a crucial step in the tricarboxylic acid (TCA) cycle for energy production and biosynthesis (Gomaa, 2022).

Glutaminase has also been extensively studied in cancer metabolism, as many cancer cells exhibit glutamine dependence for rapid growth and survival. This

phenomenon, known as glutamine addiction, highlights glutaminase as a potential therapeutic target in oncology (Kothapalli and Lakshmi, 2023). Inhibiting this enzyme can disrupt tumor metabolism, depriving cancer cells of essential nutrients and limiting their proliferation. Given its dual significance in normal and pathological conditions, glutaminase continues to be a focus of biomedical research, with ongoing efforts to develop selective inhibitors for cancer therapy.

This enzyme's role in both neurological function and oncology underscores its importance in advancing therapeutic strategies across multiple disciplines.

Glutaminase is produced by a variety of microorganisms, including bacteria, fungi, and yeast, which serve as key sources for this enzyme.

Among bacterial producers, species belonging to the *Streptomyces, Pseudomonas,* and *Aeromonas* genera are widely recognized for their ability to synthesize glutaminase. These bacteria, commonly found in soil and marine environments, utilize the enzyme as part of their nitrogen metabolism, allowing them to adapt to diverse ecological conditions (Gomaa, 2022; Kothapalli & Lakshmi, 2023).

Fungal species, particularly those from the *Aspergillus* and *Penicillium* genera, also produce substantial amounts of glutaminase. These fungi have gained industrial significance, particularly in the food industry, where glutaminase contributes to flavor enhancement by increasing glutamate levels in fermented products (Kashyap *et al.,* 2002). Additionally, yeasts such as *Saccharomyces cerevisiae* are known to generate glutaminase, though their production is relatively lower compared to bacteria and fungi.

In summary, the exploration of bacterial strains for glutaminase production is a promising approach to developing new, more effective cancer treatments. Since cancer cells have distinct metabolic dependencies, targeting key enzymes such as glutaminase represents an innovative therapeutic strategy. Bacterial sources of glutaminase provide an exciting opportunity to harness microbial diversity for enzyme production, potentially leading to therapies that are safer, more selective, and economically viable. With continued advancements in microbial biotechnology, researchers are working toward uncovering new treatment possibilities that could transform cancer therapy and improve the lives of patients worldwide.

## Materials and Methods

### Isolation of glutaminase producer

### Sample Collection

### Soil Sample

The soil was collected from two different locations and two different type of soil. One agricultural soil sample was collected from the fields of Ratnagiri and second was collected from the Botanical Garden of Bhavan's College in Andheri.
10g of soil was collected and placed in zip-lock bag and stored at $4^0$C.

### Pork Sample

Pork Liver sample was collected from the local meat shop in Santacruz.

After the collection, it was placed in a container and fermented for 12-14 hours.

The sample was then stored at $4^0$C.

### Sample Processing

### Soil Sample

1gm of soil was suspended in 10ml of sterile saline (0.85% NaCl).

The mixture was then vortexed for 1-2 minutes.

The particles were allowed to settle and supernatant was used for further processing.

### Pork Sample

The fermented pork liver sample was directly used to swab the Minimal Glutamine Agar medium. The plates were incubated at $37^0$C for 24-48 hours.

### Screening of the organisms

All observed bacterial colonies were re-streaked onto minimal glutamine agar, which contains L-glutamine as the sole carbon and nitrogen source. The medium also includes phenol red as a pH indicator, with glutamine (1%) serving as the exclusive nutrient source. The pH of the medium was adjusted to 7.0–7.2 before inoculation.

A color change from yellow to pink in the medium indicates extracellular L-glutaminase production by the bacterial colonies.

Each bacterial strain was individually streaked onto minimal glutamine agar plates and incubated at 37°C for 48 hours. After two days, the plates developed a pink hue, confirming that the isolated strains were capable of producing extracellular L-glutaminase. This screening method effectively identifies bacterial strains with potential glutaminase activity for further biochemical and molecular characterization.

### Selection of the highest producing strains

For the selection of the highest producer, Nesslerization reaction was carried out.

The Nesslerization reaction is a chemical test used to detect the presence of ammonia ($NH_3$) or ammonium ions ($NH_4^+$) in a sample. This reaction is commonly used in biochemistry and microbiology to measure enzymatic activity, particularly for enzymes like L-glutaminase, which release ammonia as a byproduct.

Nessler's reagent, a potassium tetraiodomercurate ($K_2HgI_4$) solution, reacts with ammonia or ammonium ions in an alkaline medium.

This reaction forms a yellow to reddish-brown precipitate or a deep orange color in solution.

The intensity of the color is directly proportional to the amount of ammonia present, allowing for quantitative estimation.

### Optimization of the medium for enzyme production

The parameters optimized were:

i. Temperature
ii. pH
iii. Nutritional Requirement

### Preparation of inoculum

5ml of sterile distilled water was aseptically added it 48 hours old Nutrient agar culture slants and cell suspensions was prepared. This suspension was added to 45ml of inoculum medium. The inoculated medium was kept on a rotary shaker at 200rpm for 48 hours. Then 10% inoculation medium is used as inoculums.

### Production

From the inoculum prepared, 5ml was transferred aseptically to 45ml of production medium. The flasks were kept on a orbital shaking incubator at RT and at 120rpm for 120h. The samples withdrawn were centrifuged at 1500rpm for 30 minutes and the clear supernatant was used as crude enzyme preparation for optimization.

Optimum conditions required for maximum L-glutaminase production was determined for incubation temperature, pH of the medium, additional carbon source, by varying and evaluating the rate of L-glutaminase production.

### Temperature

The optimum temperature required for maximal L-glutaminase production was estimated by incubating the inoculated medium at various temperatures (RT, 35 and 550C) for a period of 48 hours. After incubation, the fermented broth was centrifuged, supernatant was collected and the enzyme was assayed.

### pH

Optimal pH required for enhanced level of L-glutaminase production was determined at various levels of pH (5-9) adjusted in the medium using 1N HCl/NaOH. Inoculation and incubation were done. After incubation, the fermented broth was centrifuged, supernatant was collected, and the enzyme was assayed.

### Additional Carbon Source

Need for additional carbon source, along with glutamine, for enhanced enzyme production was tested by incorporating, glucose, sucrose, fructose and lactose in the medium at (0.5-1.5%) (w/v) level. Inoculation and incubation were done as mentioned earlier. After incubation, the culture broth was centrifuged, supernatant was collected, and the enzyme was assayed.

### Crude Enzyme Production & Purification of an enzyme

Production is done as mentioned earlier by applying optimal conditions obtained after optimization. From the inoculum prepared, 10ml was transferred aseptically to 90ml production medium. The production medium has the following components: L-glutamine 20g, D-glucose

10g, distilled water 1000ml and pH 8. the flasks were kept on an orbital shaking incubator at RT and at 120 rpm for 120 hours. Production was carried out with two different selected strains.

### Purification of crude enzyme by ammonium sulphate precipitation

Finely powdered ammonium sulphate (Himedia) was slowly added in to the crude enzyme preparation so as to reach 40% saturation. The whole content was stirred at $4^0$C on a magnetic stirrer. The precipitated protein was removed by centrifugation at 10,000rpm at $4^0$C for 20 minutes. Fresh ammonium sulphate was added to the supernatant to increase the concentration to 50%. The obtained precipitate was re-suspended in a minimal volume of 0.01M phosphate buffer (pH 8). Precipitated protein was removed by centrifugation as described earlier. Once again, the fresh ammonium sulphate was added to the supernatant to increase the concentration to 80%. The obtained enzyme precipitate was re-suspended in a minimal volume of 0.01M phosphate buffer (pH 8) and precipitated protein was recovered by centrifugation.

### Dialysis

The enzyme precipitate obtained after ammonium sulphate precipitation was dialyzed against 0.01M phosphate buffer (pH 8) for 24 hours at $4^0$C with stirring and the buffer was changed occasionally after checking it with 1% $BaCl_2$. Finally, L-glutaminase activity of the dialysate was semi quantitatively assayed by the method mentioned earlier.

### Protein Content

The Folin-Lowry method is a widely used colorimetric assay for protein quantification.

### Application Study of Glutaminase

### Cytotoxicity Testing

Firstly, the enzyme sample was tested for its invitro cytotoxicity on normal cells. Any cyto-toxic effect can be of concern. However, it is primarily an indication of potential for in vivo toxicity and test material cannot be necessarily be considered unsuitable for a given clinical application based solely on cytotoxicity data. Tests for cytotoxicty are designed to determine the biological response of mammalian cells to the test material/ extract of the test material. At the end of the exposure time, the evaluation of the presence and the extent of cytotoxic effect are assessed as biological compatibility of the test material and it's potential to cause cell damage.

Neutral Red Assay was carried out to test the cytotoxcity testing of the enzyme sample.

### Principle

The Neutral Red (NR) Assay is a cytotoxicity test used to assess cell viability and proliferation. It is based on the ability of viable cells to uptake and retain Neutral Red, a weak cationic dye that accumulates in lysosomes under physiological conditions. Living cells actively take up Neutral Red through passive diffusion. Damaged or dead cells lose their ability to uptake and retain the dye due to lysosomal dysfunction. The amount of dye retained is directly proportional to the number of viable cells. After incubation, cells are washed, and the retained dye is extracted using an acid-ethanol or acidified solution. The absorbance of the extracted dye is measured at 540 nm using a spectrophotometer.

The assay was carried out on human lymphocytes and *Candida* cells.

### Protocol

### For control

150 µL lymphocytes was mixed with 150 µL of the neutral red dye. It was incubated at $37^0$C for 10 minutes. The mixture was centrifuged at 1000rpm for 5 minutes. The supernatant was collected and its volume was adjusted to 2ml with the help of sterile saline. The pellet was dissolved in 150 µL of 90% ethanol. The volume was adjusted to 2ml with sterile saline. The OD reading was taken at 540nm.

150 µL *Candida* was mixed with 150 µL of the neutral red dye. It was incubated at $37^0$C for 10 minutes. The mixture was centrifuged at 1000rpm for 5 minutes. The supernatant was collected and its volume was adjusted to 2ml with the help of sterile saline. The pellet was dissolved in 150 µL of 90% ethanol. The volume was adjusted to 2ml with sterile saline. The OD reading was taken at 540nm.

### For Test

150 µL lymphocytes was mixed with 150 µL of the neutral red dye and 1-2 drops of the enzyme sample. It

was incubated at 37$^0$C for 10 minutes. The mixture was centrifuged at 1000rpm for 5 minutes. The supernatant was collected and its volume was adjusted to 2ml with the help of sterile saline. The pellet was dissolved in 150 µL of 90% ethanol. The volume was adjusted to 2ml with sterile saline. The OD reading was taken at 540nm.

150 µL *Candida* was mixed with 150 µL of the neutral red dye and 1-2 drops of the enzyme sample. It was incubated at 37$^0$C for 10 minutes. The mixture was centrifuged at 1000rpm for 5 minutes. The supernatant was collected and its volume was adjusted to 2ml with the help of sterile saline.

The pellet was dissolved in 150 µL of 90% ethanol. The volume was adjusted to 2ml with sterile saline. The OD reading was taken at 540nm.

Cell Viability percentage was calculated by:

$$\text{Cell viability \%} = \frac{OD\ of\ sample}{OD\ of\ control} \text{ X } 100$$

## Anti-Cancer Assay

For checking the activity two of the following cell line was selected:

a. Breast Cancer Cell line
b. Prostate Cancer Cell line
c. Blood Cancer Cell line

Cell Line: MCF-7 breast cancer cell line
Positive Control: 0.01M cisplatin
Concentration: 1:1, 1:10, 1:100, 1:1000 and 1:10000

Cell Line: NIH-3T3 Prostate Cancer Cell line
Positive Control: 0.01M cisplatin
Concentration: 1:1, 1:10, 1:100, 1:1000 and 1:10000

Cell Line: Daudi Cancer Cell line
Positive Control: 0.01M cisplatin
Concentration: 1:1, 1:10, 1:100, 1:1000 and 1:10000

## Incubation conditions

37$^0$C with 5% Carbon dioxide atmosphere.

## Sample preparation

Representative portion of the test sample was used for the assay.

The dilutions to be tested were prepared from both the samples. Serial dilutions of the samples was prepared and assayed in ranges of 1:1, 1:10, 1:100, 1:1000 and 1:10000.

## Assay Principle

MTT Assay: The MTT assay is a colorimetric assay used to assess cell viability, proliferation, and cytotoxicity based on the metabolic activity of living cells. It relies on the ability of mitochondrial dehydrogenase enzymes in viable cells to reduce MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) into formazan crystals, which can be quantified spectrophotometrically. Viable cells take up MTT, a yellow, water-soluble tetrazolium salt. Mitochondrial dehydrogenases in metabolically active cells reduce MTT into insoluble purple formazan crystals. Formazan crystals are dissolved using DMSO (dimethyl sulfoxide) or an acidified isopropanol solution. The absorbance of solubilized formazan is measured at 570 nm using a spectrophotometer. The intensity of the purple color is directly proportional to the number of viable cells.

$$\text{Viability Percentage} = \frac{100\ X\ OD\ at\ 570nm\ for\ extarct}{OD\ at\ 570nm\ for\ blank}$$

## Results and Discussion

## Isolation & Screening of organisms

Previously, the organisms were isolated from soil and marine water samples. Since marine enzyme is a unique protein molecule with novel properties derived from an Organism whose natural habitat comprises saline or brackish water full stop, apart from microorganisms like bacteria including actinomycetes. These property may not be expected in terrestrial sources. As marine organisms thrive in habitats such as hydrothermal vents, oceanic caves and some areas where high pressure and absence of light is obvious. *Halomonas meridiana*: Isolated from the Red Sea, this marine bacterium has demonstrated notable L-glutaminase production.

*Fusarium solani*: A halophilic fungus from marine environments, *F. solani* has been reported to produce L-glutaminase under both submerged and solid-state fermentation conditions.

*Aspergillus sojae*: Utilized in traditional Japanese fermentation processes, such as soy sauce production, *A. sojae* contains multiple glutaminase genes contributing

to its enzymatic activity. *Aspergillus oryzae* and *Trichoderma* species: These fungi are recognized for their glutaminase production capabilities.

Apart from microbial sources, animals and fermented food are also a good source for the glutaminase producer isolation.

Mammalian Liver (e.g., Bovine, Porcine, and Chicken Liver) – A rich source of L-glutaminase due to its role in ammonia detoxification.

Kidneys (e.g., Human, Bovine, Porcine) – Contains L-glutaminase for glutamine breakdown and ammonia production.

Brain Tissue (e.g., Rat, Human, and Bovine) – Plays a role in neurotransmitter synthesis by converting glutamine to glutamate.

So in this study 3 Samples were tested. Two of them were soil samples and one of them was a fermented pork sample.

From these samples, ten different isolates were obtained and screened for the production of L glutaminase enzyme. Among them, two isolates were found to be showing maximum glutaminase after a qualitative test. The cultures were grown onto minimal media supplemented with the dye phenol red for qualitative analysis. The indicator is pH sensitive. Normally it gives yellow colour to the media in acidic and neutral condition and it gives pink colour to the media when the pH changes from acidic to alkaline conditions. Pink colour of the colony indicates the pH alteration which originated from ammonia accumulation in the medium. For quantitative analysis, Nessler's reaction or Nesslerization reaction was carried out with that of the enzyme preparation. On re-streaking of the colonies on the minimal Agar medium, ten isolates were selected and screened for further enzyme production.

## Selection for the highest producing strain

All the isolates were inoculated onto the glutaminase production medium for a period of 96 hours. The glutaminase activity was measured by adding 0.5 ml Nessler's reagent to the culture filtrate. Then the concentration of the enzyme was determined by taking readings colorimetrically at 525nm. Ammonia content was estimated using standard ammonium sulphate solution. Ammonium sulphate was used as the standard. The enzyme L-glutaminase activity was expressed in international units (IU) per milligram of protein. One International Unit (IU) is the amount of enzyme required to liberate 1 micromole ammonia in one minute under experimental conditions.

## Standard curve for ammonium sulphate

Standard: 0.9mg of NH3
Diluent: Distilled Water
Range: 0.1mg-0.9mg of NH3

When the 10 isolates were obtained, nesslerization reaction was carried out. Following are the readings for it.

$$\text{Enzyme activity} = \frac{ammonia\ concentration\ (\mu g/ml)}{time\ (mins)}$$

$$\text{Enzyme activity (IU/ml)} = \frac{Enzyme\ activity(\mu g/ml/min)}{17000} X\ 1000000$$

The isolate 5 and isolate 9 showed maximum glutaminase production as they showed the maximum activity. Hence, they were selected for further production. The following are the colony characteristics observed of the isolates:

## Optimization of medium for enzyme production

The media optimization is an important aspect to be considered in the development of fermentation technology. Large scale production of the industrial enzymes usually involves a wide range of search for optimization of production media. This was achieved by a systematic study on the various nutritional requirements to growing microbes. There are only a few reports available concerning the optimization of media composition. Among physical parameters studied, pH of the medium plays an important role by inducing morphological changes in the microbes and in the enzyme secretion.

## Temperature

The temperature stability of glutaminases showed a wide variation. Glutaminase from *Pseudomonas* showed maximum activity at 37°C and was unstable at high temperatures. Also, *Qian et al.,* proved that *E. coli* L-asparaginase lost its activity more rapidly at higher temperatures among the different temperature. Among

the different temperatures tried, isolate 5 and isolate 9 showed optimal activity at RT. The optimum temperature for the enzyme activity was fine to be 30 to 40°C, at which the enzyme activity was the highest. The significance of incubation temperature in development of a biological process is such that it could determine the effect of protein denaturation, enzyme innovation, cell viability, and death.

## pH

The pH of the medium is one of the most critical environment parameter, affecting the enzyme production and the transport of various components across the cell membrane. The pH under temperature tolerance of glutaminase from various organisms differed greatly. While optimal activities of glutaminase A and B of Pseudomonas aeruginosa were at alkaline pH of 7.5 to 9 and 8.5 respectively (Soda et al., 1972), glutaminase from *Pseudomonas* species as well As for *Escherichia coli* was reported to be active over a broad range of pH (5-9) with them optimum near pH 7. For these experiments, the pH of the solution was adjusted with either 1N HCL or 1N NaOH from pH range of 5-9. Fermentation was carried out for 48 hours. For isolate 5, maximum activity was obtained at pH 8 whereas isolate 9 showed the optimal activity at the pH of 7. However, the pH of the medium strongly affects the growth and the activity of the microorganism. Microbial enzymes are produced in higher yield at a pH near to the maximal for enzyme production.

## Carbon Source

Extracellular enzyme production depends greatly on the composition of the medium.. Presence of additional carbon sources along with L- glutamine in the medium promoted enzyme production. The additional carbon source influenced the biosynthesis of L glutaminase production.

This finding was supported by the earlier researchers the bacteria with *Vibrio costicola* isolated from the marine habitat under solid substrate fermentation

In various studies, when glucose was replaced by various sugars, dextrinand was found to be an effective carbon source for glutaminase production. Isolate 5 and isolate 9 showed maximal enzyme activity when glucose was given as sole carbon source.

## Nitrogen Source

Since any bio-technological process is likely to be based on crude enzymes, selection of best nitrogen sources is important to increase their activities in the culture supernatants. Organic nitrogen sources are preferred for L-glutaminase production. Nitrogen source could be an important limiting factor in the microbial production of the enzyme. Among the various sources tested, and glutamine is considered to be one of the best source for the maximum enzyme production.

## Production & Purification of the enzyme

Crude L- glutaminase was produced at the decided parameters of both the isolates. pH of 8 and 7 was maintained for isolate 5 and isolate 9 respectively. Rest all the parameters remained the same.

Solid state fermentation has been also known to show good enzyme production. In this, sugarcane bagasse is used as substrate for carbon as well as nitrogen source.

## Extraction of enzyme

Ammonium sulfate precipitation is a commonly used method for protein purification based on salting out. The principle behind this technique is that proteins become less soluble at high salt concentrations due to competition between salt ions and protein molecules for water molecules.

This disrupts the hydration shell around the protein, leading to aggregation and precipitation.

## Purification of enzyme

Dialysis is an essential step in the purification of glutaminase following ammonium sulfate precipitation. This process removes excess salts, such as ammonium sulfate, while retaining the enzyme in solution. The precipitated glutaminase is first re-suspended in an appropriate buffer to ensure enzyme stability and activity. The solution is then transferred into a dialysis bag with a suitable molecular weight cutoff, allowing small molecules like salts and impurities to diffuse out while retaining the enzyme. The dialysis bag is placed in a large volume of the 0.01M Phosphate buffer and stirred gently at low temperatures (typically 4°C) to prevent enzyme denaturation. It was dialyzed overnight against the same buffer.

**Table.1** Production medium

| Components | g/L |
|---|---|
| **L-Glutamine** | 20 |
| **D-Glucose** | 10 |
| **Distilled water** | 1L |
| **pH** | 8 |

**Table.2** Enzyme activity of isolates

| Isolate | OD AT 525 nm | Enzyme Activity (IU/ml) |
|---|---|---|
| **Isolate 1** | 1.09 | 2.59 |
| **Isolate 2** | 0.49 | 1.12 |
| **Isolate 3** | 1.15 | 2.73 |
| **Isolate 4** | 1.09 | 2.59 |
| **Isolate 5** | **1.22** | **2.90** |
| **Isolate 6** | 0.63 | 1.46 |
| **Isolate 7** | 0.96 | 2.27 |
| **Isolate 8** | 1.00 | 2.37 |
| **Isolate 9** | **1.30** | **3.10** |
| **Isolate 10** | 1.09 | 2.59 |

**Table.4** Standard Bovine Serum Albumin Table

| S. No. | Concentration mcg/ml | Amount of stock | Amount of diluent | Alkaline CuSO4 | Keep at RT for 10 mins | FC Reagent (ml) | Incubate at RT for 30 mins | OD at 660 nm |
|---|---|---|---|---|---|---|---|---|
| **1** | 40 | 0.2 | 0.8 | 5 | | 0.5 | | 0.16 |
| **2** | 80 | 0.4 | 0.6 | 5 | | 0.5 | | 0.32 |
| **3** | 120 | 0.6 | 0.4 | 5 | | 0.5 | | 0.51 |
| **4** | 200 | 1 | - | 5 | | 0.5 | | 0.62 |
| **5** | Blank | - | 1 | 5 | | 0.5 | | 0.0 |
| **6** | Purified enzyme 1 | 1 | | 5 | | 0.5 | | 0.04 |
| **7** | Purified enzyme 2 | 1 | | 5 | | 0.5 | | 0.03 |

**Table.5** The cell viability percentage from both the enzymes were found to be following

| Enzyme | Cells | %Viability |
|---|---|---|
| **Isolate 5** | Lymphocytes | 93.33% |
| | *Candida* | 90% |
| **Isolate 9** | Lymphocytes | 86.66% |
| | *Candida* | 95% |

**Fig.1** Colonies on Minimal Glutamine Agar

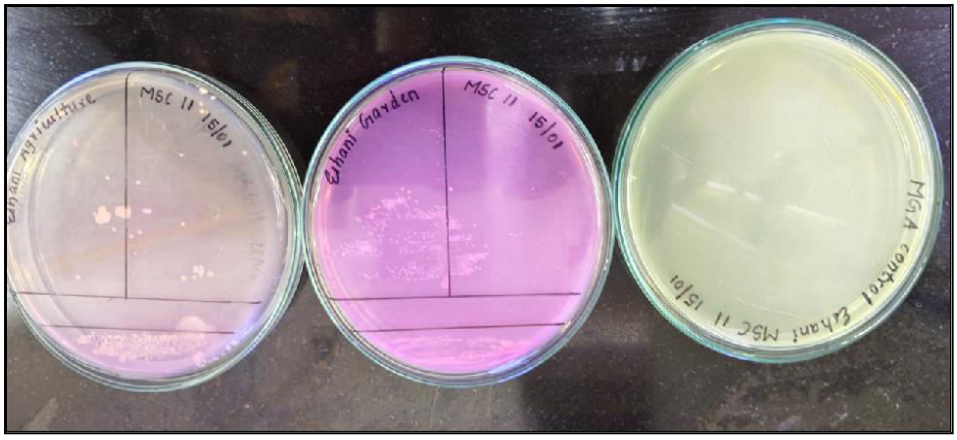
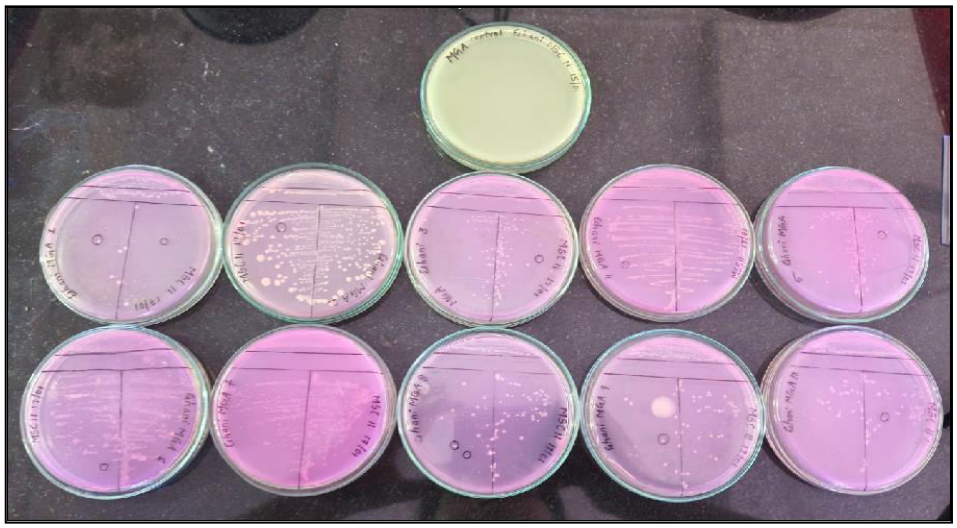
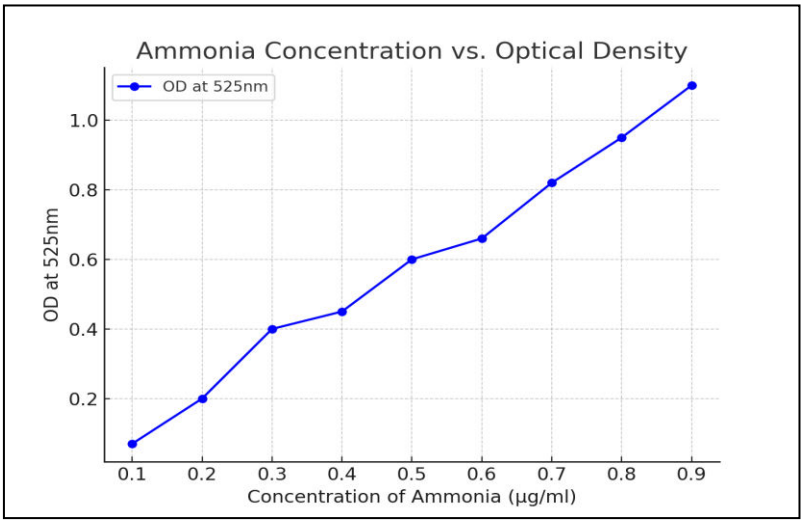


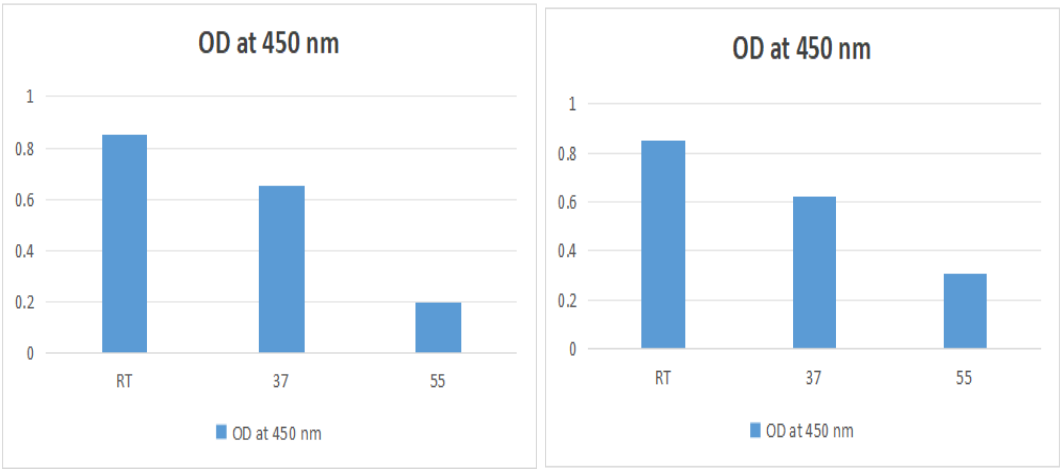

**Fig.2** Screening of organisms



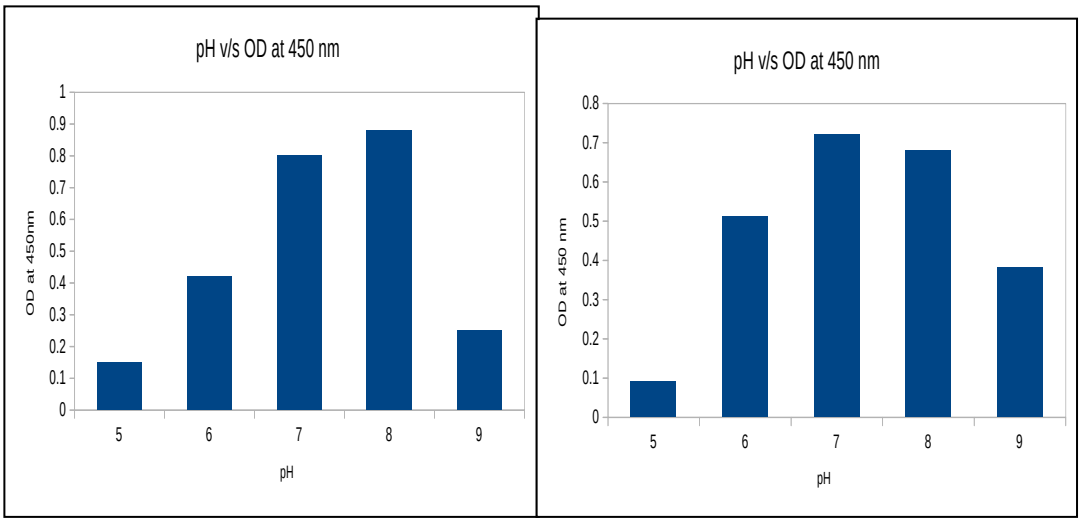
**Graph.1** Standard Ammonium Sulphate Curve



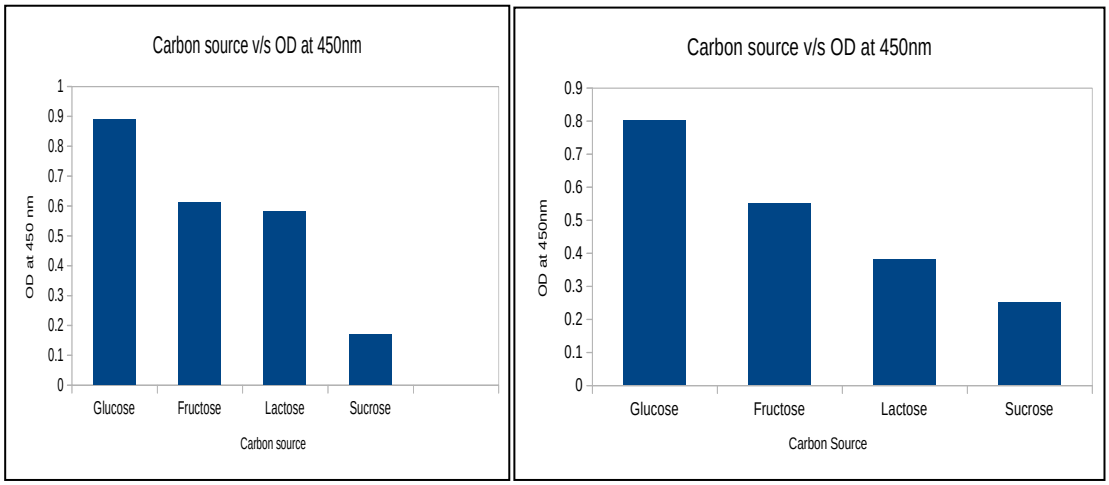
**Graph.2** Effect of temperature on isolate 5 **Graph.3** Effect of temperature on isolate 9



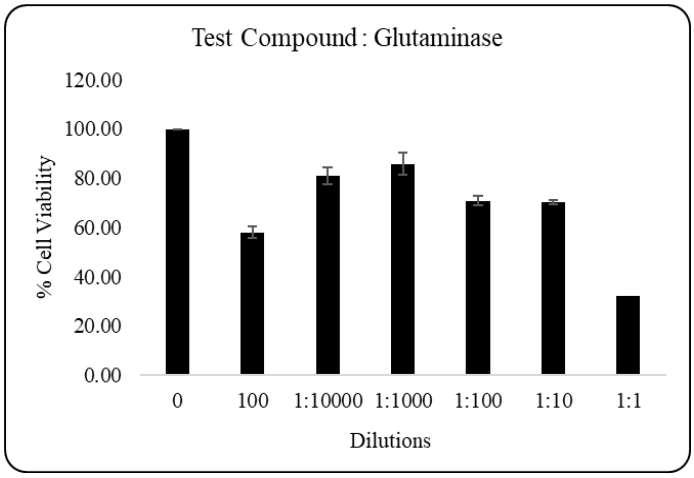

**Graph.4** Effect of pH on isolate 5 **Graph.5** Effect of pH on isolate 9



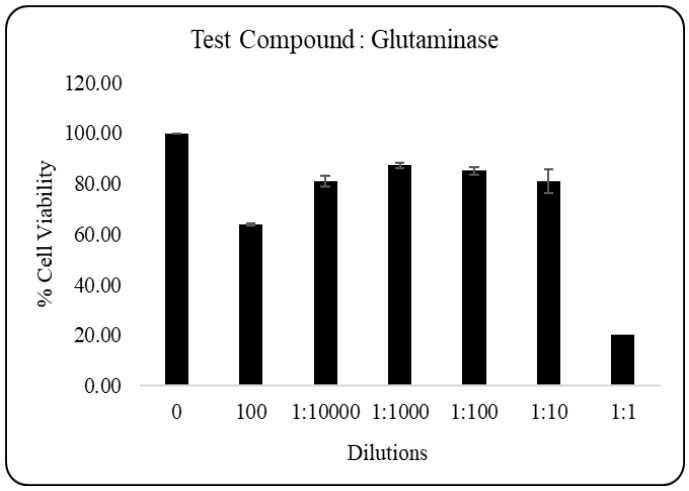

**Graph.6** Effect of carbon source on isolate 5 **Graph.7** Effect of carbon source on isolate 9



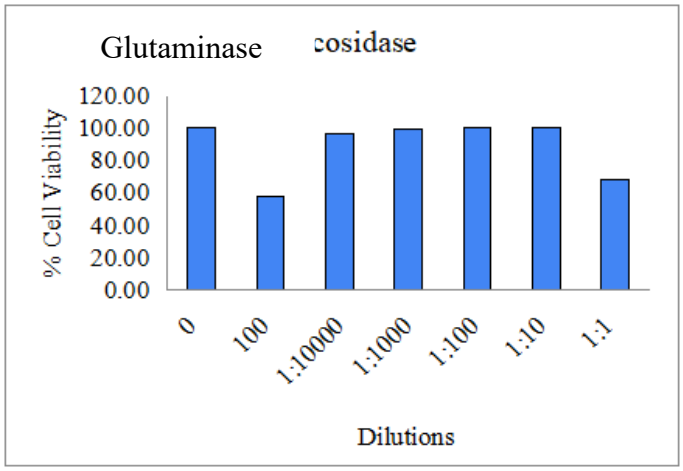
**For MCF-7 cell line**




**For NIH-3T3 cell line**




**For Daudi cell line**




The buffer is periodically changed to maintain the concentration gradient, ensuring efficient removal of salts. After dialysis, the purified glutaminase is collected and can be subjected to further purification techniques

such as ion exchange chromatography or gel filtration to achieve higher purity. Dialysis not only helps in desalting but also stabilizes the enzyme for downstream applications.

The enzyme was assayed by direct nesslerization method according to the method of Sinha *et al.,* and the protein content was determined by modified Lowry's method (Kim *et al.,* 2002).

Standard: Bovine Serum Albumin
Range: 40-200 µg/ml

Specific activity of enzyme = $\frac{enzyme\ activity\ (IU/mL)}{protein\ conyent\ of\ enzyme}$

For purified enzyme 1, the specific activity was calculated to be 1.93 IU/mg.

For purified enzyme 2, the specific activity was calculated to be 1.72 IU/mg.

## Cyto-toxicity testing

The Neutral Red (NR) Assay is a widely used cytotoxicity test that measures cell viability based on the ability of viable cells to incorporate and bind neutral red dye within lysosomes. The principle behind the assay is that healthy, viable cells actively take up and retain the dye in their lysosomes, whereas damaged or dead cells lose this ability. The intensity of neutral red uptake correlates with the number of viable cells, allowing for quantitative assessment of cytotoxicity.

Glutaminase plays a crucial role in cancer cell metabolism by converting glutamine to glutamate, fueling tumor growth. Targeting glutaminase is a potential anti-cancer strategy, and cytotoxicity tests help evaluate its effects on cells.

When testing the cytotoxicity of glutaminase itself, cells are treated with high concentrations of glutaminase to observe its potential toxic effects. If excessive glutamate production disrupts cellular homeostasis, cell viability may decrease. When testing the effect of glutaminase inhibitors, cancer cells are exposed to these inhibitors to assess their ability to reduce glutaminase activity and induce cell death. A reduction in neutral red uptake would indicate effective inhibition of glutaminase, leading to cytotoxic effects. Neutral Red Assay was performed on Human Lymphocytes and *Candida* cells. The cell viability percentage from both the enzymes were found to be following:

The glutaminase enzyme from Isolate 5 showed 93.33% viability in lymphocytes and 90.00% in *Candida* cells, indicating low cytotoxicity. The glutaminase enzyme from Isolate 9 resulted in 86.66% viability in lymphocytes and 95.00% in *Candida* cells, suggesting it may have slightly higher cytotoxic effects on lymphocytes but lower impact on *Candida*.

Since cell viability is above 80%, it suggests that the enzyme does not significantly harm normal cells, making it a promising candidate for therapeutic applications.

Isolate 5 appears less cytotoxic and may be a safer option for therapeutic applications. Isolate 9 shows a slightly higher toxicity towards lymphocytes, which may indicate a stronger biological effect, making it more interesting for potential anti-cancer studies. Further, various studies have shown that glutaminase is nontoxic to normal cells. It was tested against Vero cells (normal cell line) and was estimated by MTT assay. The enzyme showed no inhibition on the viability of Vero cells. Hence, it is considered safe for normal cells and non-toxic in nature.

## Anti-Cancer Assay

Glutaminase from *Bacillus sp.* DV2-37 exhibited strong anticancer activity, inhibiting the proliferation of MCF-7 (breast), HepG-2 (liver), and HCT-116 (colon) cancer cells with $IC_{50}$ values of 3.5, 3.4, and 3.8 µg/ml, respectively (Gomaa, 2022).

Purified preparation of glutaminase showed significant initial retardation in the rate of tumor growth, especially in ascites tumors.

The produced enzyme was tested on the MCF-7, NIH-3T3 and Daudi cell lines. The results observed were:

### For MCF-7 cell line

Based on the experimental observation, it can be concluded that, the MTT data exhibited that the test compound showed dose dependent cytotoxicity throughout the dilution range & at 1:1 dilution exhibited highest cytotoxicity on MCF-7 cells.

### For NIH-3T3 cell line

Based on the experimental observation, it can be concluded that, the MTT data exhibited that the test compound showed cytotoxicity only at the lowest dilution of 1:1 on NIH-3T3 cells.

### For Daudi cell line

Based on the experimental observation, it can be concluded that, the MTT data exhibited that the test compound showed cytotoxicity only at the lowest dilution of 1:1 on NIH-3T3 cells.

Cancer remains one of the most challenging diseases to treat, necessitating the exploration of novel therapeutic strategies. Glutaminase, an enzyme that catalyzes the hydrolysis of glutamine into glutamate and ammonia, has garnered significant attention due to its critical role in cancer metabolism. Many cancer cells exhibit glutamine addiction, relying on glutaminase activity to sustain rapid proliferation and energy production. Targeting glutaminase has emerged as a promising anti-cancer approach, and bacterial-derived glutaminase is considered a valuable resource for therapeutic applications. This research aims to explore bacterial strains capable of producing glutaminase with potential anti-cancer properties. Cancer cells undergo metabolic reprogramming to support their increased energy demands, with glutamine serving as a crucial nutrient. Glutaminase facilitates the conversion of glutamine into glutamate, which subsequently participates in ATP production, nucleotide synthesis, and redox balance. Inhibiting glutaminase activity can effectively starve cancer cells of essential metabolites, leading to growth arrest and apoptosis. Bacterial glutaminases have shown promise as enzymatic drugs for depleting glutamine levels in cancer patients, thereby limiting tumor progression. Bacteria are excellent sources of industrially significant enzymes, including glutaminase.

Various bacterial strains, such as *Pseudomonas*, *Bacillus*, *Escherichia coli*, and *Actinobacteria*, have been reported to produce glutaminase with high activity and stability. Screening bacterial isolates from diverse environments, such as soil, marine ecosystems, and fermented foods, can help identify novel glutaminase-producing strains with enhanced enzymatic properties and potential anti-cancer applications.

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