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Original Article

Isolation, Screening, and Optimization of L-Glutaminase Production by Bacterial Isolates from Najaf, Iraq

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ABSTRACT

L-glutaminase (EC 3.5.1.2) is an industrially important enzyme with considerable applications in food biotechnology, pharmaceutical research, and industrial bioprocessing. The present study focused on the isolation of bacterial strains from soil in Najaf Province, Iraq, and the screening of these isolates for L-glutaminase production. In addition, the effects of various physicochemical and nutritional factors on enzyme production were investigated to identify the optimal conditions for enhanced L-glutaminase productivity. Initially, ten bacterial isolates were screened, exhibiting enzyme activities ranging from 6.8 to 26.7 U/mL. Among them, NPI-7 demonstrated the highest activity (26.7 U/mL), followed by NPI-6 (21.1 U/mL) and NPI-8 (17.9 U/mL). These three promising isolates were selected for further optimization studies. The effects of incubation period, pH, temperature, carbon sources, nitrogen sources, and metal salts were evaluated. Maximum enzyme production was observed at 72 h of incubation, pH 7.0, and 40°C, with NPI-7 exhibiting activities of 28.7, 29.7, and 28.7 U/mL, respectively. Among the carbon sources investigated, sucrose supported the highest enzyme activity (28.7 U/mL), followed by fructose (24.7 U/mL). Yeast extract was identified as the most favorable nitrogen source, producing 32.7 U/mL in NPI-8, while $MgCl_2$ supported the highest activity (32.7 U/mL) among the tested metal salts. $MnCl_2$ also promoted considerable enzyme production (31.5 U/mL). Overall, the findings identified NPI-7 and NPI-8 as promising L-glutaminase-producing isolates and demonstrated the influence of nutritional and environmental factors on enzyme productivity. Further molecular identification, biochemical characterization, and statistical optimization are warranted to establish their biotechnological potential.

Keywords: L-glutaminase, bacterial isolates, enzyme production, fermentation optimization, nutritional factors, metal salts, industrial biotechnology

INTRODUCTION

Microbial enzymes offer substantial and increasingly important advantages over chemical catalysts in that, they are derived from renewable resources. Microbial communities are an important biological component of soil function, and all organisms in an ecosystem rely on the activity of microorganisms for their role in improving soil quality and regulating nutrient availability Imhoff et al. (2011). Producing extracellular enzymes which are needed to break down organic material for sustainable soil productivity. L-glutaminase plays a major role in the nitrogen metabolism of both prokaryotes and eukaryotes Vijayan et al. (2017). It is an important enzyme therapy for a variety disease of the lymphatic system especially acute lymphocytic leukemia Cachumba et al. (2016).

The enzyme has also been applied in the treatment of Hodgkin 's disease, lymphosarcoma, and acute myelocytic leukemia Hohaus et al. (2003). L-glutamine is not an essential amino acid for the animal cell, and the neoplastic cells are not able to induce the synthesis of glutamine synthetize.

However, they are dependent on the extracellular level of L-glutamine to protein synthesis Pavlova et al. (2018). As L-glutamine is withdrawn from plasma by L-glutaminase, this amino acid leads to inhibit the growth in neoplastic cells Dias et al. (2016). There is a great demand to produce chemotherapy, a variety of nutritional and physiochemical factors influences the production of enzymes. For this reason, this enzyme has been developed to use as therapy to tumor cells Parker (2009). Nowadays optimization of different production parameter would enhance the quality and quantity of enzyme production, and tuLNIin tuLNIinfluence enzyme synthesis and cell yield Martins et al. (2013). The microbial source is very common for L-glutaminase, because of easily cultured, extraction, and purification for the industrial scale production.

Relevant conflicts of interest/financial disclosures: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The most used microorganisms to produce L-glutaminase *E.coli, Actinomycetes, Pseudomonas sp, Erwinia carotovora sp.* Batool et al. (2016). Because of the continuous need to screen newer strains that capable to produce high yield of L-glutaminase, this paper deals with the purification, kinetics properties, and biochemical characterization of L-glutaminase from Bacillus B12.

They work under relatively even extreme conditions of temperature and pH, and offer exquisite products. Enzymes are highly specific in their function as they are programmed to carry out one special task (Sabu *et al.,* 2000). Microbial enzymes play a vital role in the diagnosis, curing, biochemical investigation, and monitoring of many dreaded diseases. Microorganisms represent an excellent source of many therapeutic enzymes owing to their broad biochemical diversity and their availability of genetic manipulation (Kashyap *et al.,* 2002). L-Glutaminase (EC.3.5.1.2) is an amidohydrolase which catalyses the hydrolytical deamination of L-glutamine resulting in the production of L-glutamic acid and ammonia. L-Glutaminase constitutes one of the most biotechnologically and biomedically important group of therapeutic enzymes accounting for about 40% of the total worldwide enzyme sales (Warangkar *et al.,* 2010). It is a pyrimidine derivative and has long been considered as an important compound in the management of childhood acute lymphoblastic leukemia and lymphosarcoma in combination or as an alteLNIative to L-asparaginase (Wriston & Yellin 1973; Capizzi et al., 1984). The mechanism of L-glutaminase as anticarcinogenic enzyme correlates with glutamine depletion due to inability of the lymphatic tumor cell to synthesize glutamine as the normal cells. It belongs to an amidase group that produces glutamic acid and ammonia by glutamine hydrolysis (Marchesi et al., 1998).

L-Glutaminase is known for its use as a enzyme from diverse sources differ greatly in their possessions such as anticancer activity, immunogenicity, cytotoxicity, anti-HIV agent, activity at physiological circumstances, plasma clearance rate, substrate specificity, temperature, pH and salt tolerance etc. Another important application of L-glutaminase is in biosensors for monitoring glutamine levels in mammalian and hybridoma cell cultures without the need of separate measurement of glutamic acid. It is also used as an analytical agent in determination of glutamine and glutamate (Sabu et al., 2000). Commercially available enzymes isolated from different strains are the only sources for the front line therapy but, long term administration of this enzyme in general produces the corresponding antibodies in tissues, resulting in anaphylactic shock or drug neutralization. So there is an urgent need for using new serologically different L-glutaminase with similar therapeutic properties (Pokravskaya et al., 2012). The use of enzymes in industrial processes can often eliminate the use of high temperatures, organic solvents and extreme conditions pH. While at the same time offering increased reaction specificity, product purity and reduced environmental pollution. So L-Glutaminase enzyme as a drug has gained much importance because only a limited number of microbial enzymes deplete essential amino acids so that cancer cells could not survive (Joel et al., 2003). Almost all microbes produce L-glutaminase but only certain microbial strains have the potential for industrial production of this enzyme. Among some well-studied microbial genera, *E. coli, Pseudomonas sp., Brevibacterium sp., Vibrio costicola, Streptomyces rimosus, Streptomyces avermitilis* and *Streptomyces labedae, Streptomyces gresius, Hypocrea jecoLNIea, Zygosaccharomyces sp., Bacillus sp.* and *Micrococcus luteus, Acinetobacter sp., Hansenula, Cryptococcus, Candida, Aspergillus oryzae* and *Beuveria bassiana* etc. are good producers of enzyme. There is a continual need to find out newer microbial sources to obtain high-yielding L-glutaminase producing strains for the therapeutic and industrial applications. Present investigation was aimed to screen the L-glutaminase activity of bacteria isolated from different habitats, to the remarkable abilities shown by bacteria among other microorganisms.

2.0 Materials and methods

2.1 Study sites description:

The study area is characterized by a sedimentary plain with diverse natural and agricultural features. The region supports agricultural activities to varying degrees depending on local soil conditions, topography, and the availability of natural resources. Agricultural practices in the area primarily include the cultivation of fodder crops such as maize, wheat, and barley, along with vegetable crops such as cucumber and tomato. These crops are mainly cultivated in agricul-

tural fields surrounding the Ghazi River. The vegetation in the surrounding areas consists predominantly of palm trees, with a limited distribution of other tree species, including Acacia and Eucalyptus. For the present investigation, a total of 25 pooled soil samples were collected from two different transects across Najaf Province (image-01). The sampling sites were selected to obtain representative soil samples for the objectives of the study and to evaluate the characteristics of the collected soil samples.

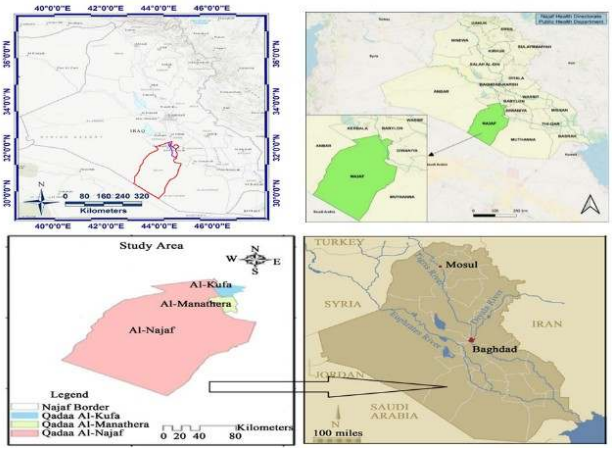


Image 01: Geographical Location Map of the Study Area Showing the Soil Sampling Sites in Najaf Province, Iraq.

2.2 Collection of Samples:

Soil samples were obtained from various forms of land use (transects were ac-cording to land use), using hand auger from a depth ranging up to 20 cm and stored in clean sealing plastic. Collected soils were air-dried in the laboratory, and passed through a 2mm mesh sieve, after they had been disaggregated with a porcelain pestle and mortar. Subsequently, samples were examined in sealed plastic bags (McCauley, 2005). Samples were then powdered by grinding soil in mortar and pestle and finally filtered through sieve. The samples were stored at 4 °C and were used for further studies.

2.3 Isolation of Bacteria

Isolation of bacteria was performed by the serial dilution plate technique using Nutrient Agar Medium containing (Peptone, 5.0 g; Beef-extract, 3.0 g; Sodium chloride, 5.0 g; agar-agar, 20.0 g per liter of distilled water). About 50µg/ml of Nystatin is used to retard the growth of fungal colonies. The sterilization of the medium components was carried out in an autoclave at 121°C at 15 lbs, pressure for 30 minutes. Approximately 20 ml of medium was poured into sterilized petri dishes and allowed to solidify. One gram of soil sample was serially diluted up to 10 folds. 0.1 ml of 10^{-6} dilution was aseptically spread over the surface of nutrient agar medium. The plates were then incubated at 37°C for 48 hours. After incubation, the colonies appeared were isolated and purified using nutrient agar medium. All the isolated bacteria were maintained on nutrient agar slants and stored at 4°C for further use.

2.4 Identification of Bacterial Isolates

Different morphological and biochemical characteristics of the bacterial isolates were studied for identification of the strain and compared with standard description of Bergey's Manual of Determinative Bacteriology (Holt et al., 2013). Morphological characters such as shape and color of both bacterial colonies were determined. All microscopic examinations were carried out using a Bright–Field microscope, with the oil–immersion (100X) for staining studies and the high–power (45X) objective for motility detection by hanging drop method was also performed.

2.5 Screening of the L-Glutaminase enzyme

Minimal glutamine agar (MGA) medium was prepared and used to screen bacteria for L-glutaminase production. The medium contained (g/L) 0.5 KCl, 1.0 $MgSO_4$, 0.1 KH_2PO_4, 0.1 $FeSO_4$, 0.1 $ZnSO_4$, 0.5 NaCl, 3.0 glucose, and 10.0 L-glutamine. Phenol red (0.012 g) was added as a pH indicator, and the pH of the medium was adjusted to 6.8. L-glutamine served as the substrate for enzyme production. Bacterial colonies producing extracellular L-glutaminase were identified by a colour change in the medium from yellow to pink. This colour change occurs due to the release of ammonia during the hydrolysis of the amide bond in L-glutamine by L-glutaminase, resulting in an increase in the pH of the medium (Gulati et al., 1997).

2.6 Biochemical Tests

Biochemical tests like Indole test, Methyl red test, Voges proskauer test, Citrate utilization test, Catalase test, Hydrogen sulphide, Oxidase test, hydrolysis of starch, casein, urea and gelatin tests were performed with isolated bacterial strains (Shirling EB, & Gottlieb D 1998).

2.7 Assay of L-Glutaminase

Quantitative determination of l-glutaminase was carried out by quantifying the amount of ammonia released (Drainas et al., 1977). As per MashbuLNIand Wriston (1964), 1.7 ml of 0.01 M glutamine was taken in a test tube, 0.2 ml of 0.05 M buffer (Tris – HCl pH 8.6) and 0.1 ml of culture filtrate were added to a final volume of 2.0 ml and the reaction mixture and was incubated for 10 min at 37ºC. After the incubation period the reaction was stopped by adding 0.5 ml of 1.5 M trichloroacetic acid (TCA). To 0.5 ml of the above reaction mixture, 6.5 ml of distilled water and 1.0 ml of Nessler's reagent were added and incubated for 10 min. The OD was measured at 480 nm. The blank without culture filtrate was taken for comparison. One unit (U) of l-glutaminase is defined as the amount of enzyme which liberated 1 µmol of ammonia/min/ml under the above assay conditions. All the reactions were performed in triplicates and mean value was recorded (Drainas et al., 1977).

2.8 Determination of L-Glutaminase activity

The active isolate was cultured on M9 broth supplemented with L-glutamine and phenol red as an indicator, pH 6.8 was incubated at 37 ºC with shaking at 125 rpm for 72 h. L-glutaminase activity was measured, following the method of (Drainas et al., 1977). The culture broth was centrifuged at 10000 rpm for 15 min. This method utilizes the determination of ammonia liberated from L-glutamine in the enzyme reaction by the Nessler's reaction. The reaction was started by adding 0.1 ml supeLNIatant into 1.7 ml of 0.01 M L-glutamine and 0.2ml of 0.05M tris-HCl buffer, pH 8.6 and was incubated at 37ºC for 10 min. The reaction was stopped by the addition of 0.5ml of 1.5 M trichloroacetic acid (TCA). The ammonia released in the supeLNIatant was determined calorimetrically by adding 6.5 ml of distilled water and 1.0 ml Nessler's reagent into tubes and was incubated at room temperature for 10 min. The samples were shaked well and the enzymatic activity was estimated spectrophotometrically at 480nm. The blank was run by adding enzyme preparation after the addition of TCA. One unit (U) of L-Glutaminase is defined as the amount of enzyme which liberated 1 µmol of ammonia/min/ml under the above assay conditions. A standard curve was drawn with various concentrations of ammonia and was used as reference to measure enzyme production.

2.9 Optimization of medium and cultural conditions for L–Glutaminase Production

Optimization of different cultural conditions such as incubation period, pH, temperature, carbon, and nitrogen sources on the production of L-glutaminase was determined.

2.9.1 Effect of incubation period on L-Glutaminase production:

To study the effect of incubation period on L-Glutaminase production, the enzyme production by LNI-7 was studied for every 24 hours up to 96 hours. For this study, the LNI-7 culture was inoculated into 500 ml conical flask, containing sterile M9 broth and was incubated from 24 hours up to 96 hours at 37°C and triplicates were maintained. The optimum time, showing maximum production was taken for further experiments.

2.9.2 Effect of pH on L-Glutaminase Production

To study the effect of different pH on L-Glutaminase production, the isolate LNI-7 was grown in sterile M9 broth with various pH levels ranging from 5.0 to 9.0. and was maintained by using phosphate buffer. 0.5 ml of the 24-hour old inoculum was transferred into the sterile M9 broth at different pH and incubated at 37°C for 48 hrs and assayed for enzyme activity. The most favorable pH achieved at this step was used for further study.

2.9.3 Effect of Temperature on L-Glutaminase Production

To study the effect of temperature on L-Glutaminase production, the isolate LNI-7 was grown in sterile M9 broth at different temperatures, ranging from 30 to 55OC for 48 hrs of incubation and temperature was maintained by using respective incubators. 0.5 ml of the 24-hour old inoculum was transferred into a sterile M9 broth and was incubated and further assayed for enzyme activity.

2.9.4 Effect of Different Carbon Sources on L-Glutaminase production:

To study the effect of carbon sources on L-Glutaminase production by growing the bacterial strain LNI-7 on M9 broth, the broth was supplemented with different carbon sources such as Galactose, Maltose, Fructose, Starch, Sucrose, and Cellulose, each at a concentration of 1% (w/v). 0.5 ml of the inoculum

was added to a sterile M9 broth with different carbon sources, incubated at 37°C for 72 hrs and assayed for L-Glutaminase activity using the method of Shah et al 2020.

2.9.5 Effect of Different Nitrogen sources on L-Glutaminase production:

Different nitrogen sources like Yeast extract, Tryptone, Ammonium sulphate, Urea, Peptone and Malt extract were added to the M9 broth separately at a rate of 1% (w/v), and 0.5 ml of the inoculum from the bacterial isolate LNI-7 was added to sterile M9 broth, incubated at 37°C for 72 hrs. and was assayed for enzymatic activity.

2.9.6 Effect of different metal ions on L-Glutaminase production:

Metal ions like Magnesium chloride, Manganese chloride, Ferrous sulphate, Zinc chloride, Nickel carbonate, Lithium sulphate, Copper sulphate, Cobalt chloride and Barium hydroxide were added to the M9 broth separately at the rate of 0.5%. 0.5ml of the inoculum from the bacterial isolate LNI-7 was added to the sterile basal broth, incubated at 37°C for 72 hrs and assayed for enzymatic activity.

2.10 Statistical Analysis:

All measurements were carried out in triplicate. Statistical analyses were performed using one-way analysis of variance (ANOVA), and the significance of the difference between means was determined by Duncan's multiple range tests. Differences at $P < 0.05$ were considered statistically significant. The results were presented as mean values (±SD, standard deviations).

3.0 Result and Discussion

3.1 Isolation and Identification of Bacterial Strains

A total of 36 bacterial isolates were obtained from ten different regions of forest soil samples, collected from Najaf Province, Iraq. Out of 36 only 10 isolates were identified as *Bacillus cereus, Bacillus endophyticus* and *Bacillus substilis*, based on morphological, cultural, physiological, biochemical analysis. *Bacillus cereus* and *Bacillus subtilis* were gram positive, rod shaped bacteria by observing under microscope and these are motile and spore forming bacteria. Whereas, *Bacillus endophyticus* was gram positive, rod shaped but non- motile bacteria (Kashyap et al., 2002).

Table 1: Colony Morphological & Microscopic Characteristics of Isolates

S. No.	Isolation Bacteria	Result	Morphology and Microscopic Characteristics
1	NPI-4	Negative (-)	Circular, cream-coloured colonies with smooth surface and entire margins; rod-shaped bacterial cells.
2	NPI-6	Positive (+)	Irregular, creamy-white colonies with rough surface and undulate margins; Gram-positive, rod-shaped cells arranged singly or in chains; endospore-forming.
3	NPI-7	Positive (+)	Circular, off-white colonies with smooth surface and entire margins; Gram-positive, rod-shaped cells arranged singly or in short chains; endospore-forming.
4	NPI-8	Positive (+)	Irregular, dull-white colonies with rough surface and lobate margins; Gram-positive, rod-shaped cells arranged in chains; endospore-forming.
5	NPI-13	Negative (-)	Circular, cream-coloured colonies with smooth surface and entire margins; rod-shaped bacterial cells.
6	NPI-15	Negative (-)	Irregular, white colonies with rough surface and undulate margins; rod-shaped bacterial cells.
7	NPI-18	Negative (-)	Circular, pale-yellow colonies with smooth surface and entire margins; rod-shaped bacterial cells.
8	NPI-24	Negative (-)	Irregular, cream-coloured colonies with flat elevation and undulate margins; rod-shaped bacterial cells.
9	NPI-28	Negative (-)	Circular, off-white colonies with convex elevation and entire margins; rod-shaped bacterial cells.
10	NPI-35	Negative (-)	Irregular, dull-white colonies with rough surface and lobate margins; rod-shaped bacterial cells.

3.2 Screening of L-glutaminase producing bacterial isolates

Ten sample tested only eight isolate showed color change on the MGA medium plate. The change of color from yellow to pink color was observed (Image-02). It shows that L-glutaminase was produced by the bacteria, which takes up the glutamic acid present in the medium as its substrate and produces ammonia which in turn changes the media alkaline and gives pink color in the presence of the indicator phenol red.

3.3 Biochemical characterization of bacterial strains

Total isolates were tested and out of that three isolates are shows positive responses in biochemical tests NPI-6 exhibited a positive response to catalase production, voges-proskauer, urease activity, casein, starch hydrolysis and gelatin liquefaction but negative to indole production, methyl red, citrate utilization, Hydrogen sulphide and oxidase production. The strain NPI-7 exhibited a positive response to methyl red, oxidase, catalase, starch, indole production, and catalase activity but negative to urease activity, gelatin liquefaction, Hydrogen Sulphide production, voges-proskauer, citrate utilization, casein hydrolysis. The strain NPI-8 was positive to voges-proskauer, citrate utilization, catalase activity, and oxidase and starch hydrolysis and showed negative to indole production, gelatin liquefaction, urease, methyl red and casein hydrolysis. Biochemical tests were conducted for identifying isolates the results are represented in the (Table 1).

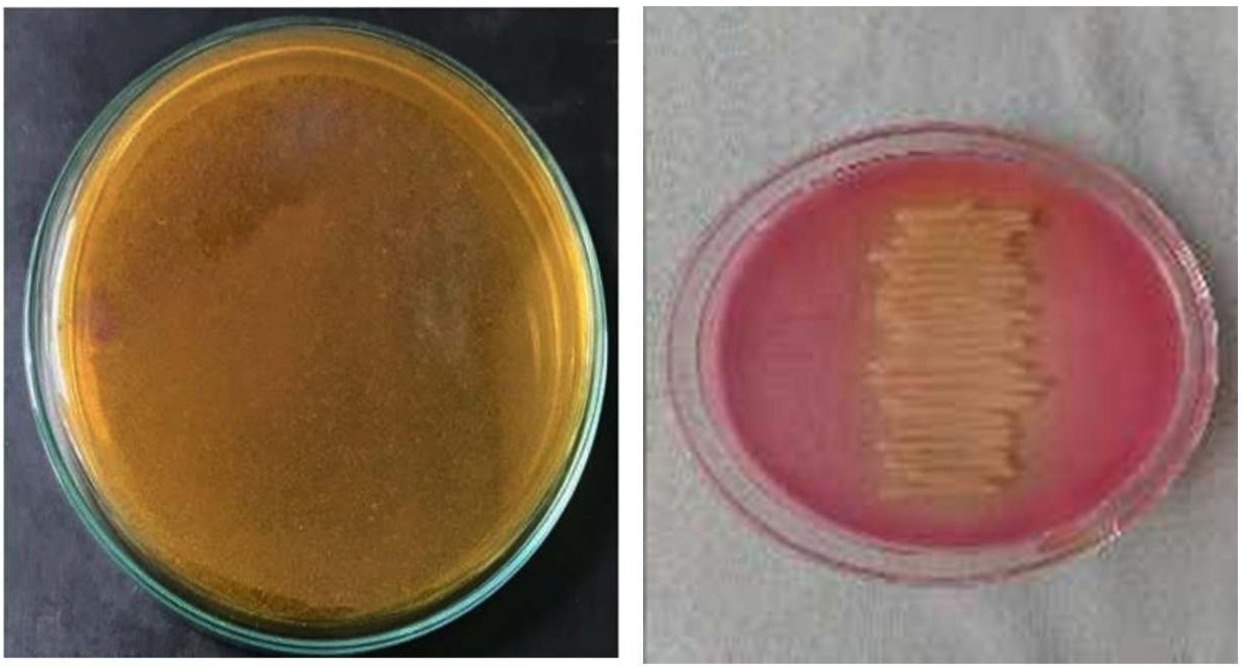


Image 02: Change of Colour of Plate by L-Glutaminase

Table None: 1 Biochemical Tests

S.No	Biochemical characters	Response		
		NPI-6	NPI-7	NPI-8
1	Indole production	-	+	-
2	Methyl red test	-	+	-
3	Voges-proskauer test	+	-	+
4	Citrate utilization	-	-	+
5	Catalase test	+	+	+
6	Hydrogen sulphide	-	-	-
7	Oxidase	-	+	+

S.No	Biochemical characters	Response		
8	Starch hydrolysis	+	+	+
9	Casein hydrolysis	+	-	-
10	Urease test	+	-	-
11	Gelatin liquefaction	+	-	-

(+) Indicates positive; (-) Indicates negative.

3.4 Production profile of L-Glutaminase

A total of ten bacterial isolates were subjected to preliminary screening for L-glutaminase production. The results revealed considerable variation in enzyme activity among the tested isolates, with values ranging from 6.8 to 26.7 U/mL. Among the ten isolates, NPI-7 exhibited the highest L-glutaminase activity (26.7 U/mL), followed by NPI-6 (21.1 U/mL) and NPI-8 (17.9 U/mL). Moderate enzyme production was observed in NPI-13 (13.2 U/mL), NPI-15 (12.9 U/mL), NPI-28 (11.9 U/mL), and NPI-4 (11.8 U/mL), whereas NPI-24 showed the lowest enzyme activity (6.8 U/mL). Based on the preliminary screening results, three bacterial isolates, namely NPI-7, NPI-6, and NPI-8, were selected for further optimization studies and characterization due to their comparatively higher L-glutaminase production (Figure-01).

These isolates were considered promising candidates for subsequent investigations aimed at enhancing enzyme production and evaluating their potential for L-glutaminase applications. The present study demonstrated considerable L-glutaminase-producing potential among the selected bacterial isolates. Among the ten isolates screened, NPI-7 exhibited the highest enzyme activity (26.7 U/mL), followed by NPI-6 and NPI-8. These isolates were selected for further optimization. Similar approaches were reported by Wardah et al. (2023); Hassan et al. (2025), who identified promising *Streptomyces* isolates for L-glutaminase production.

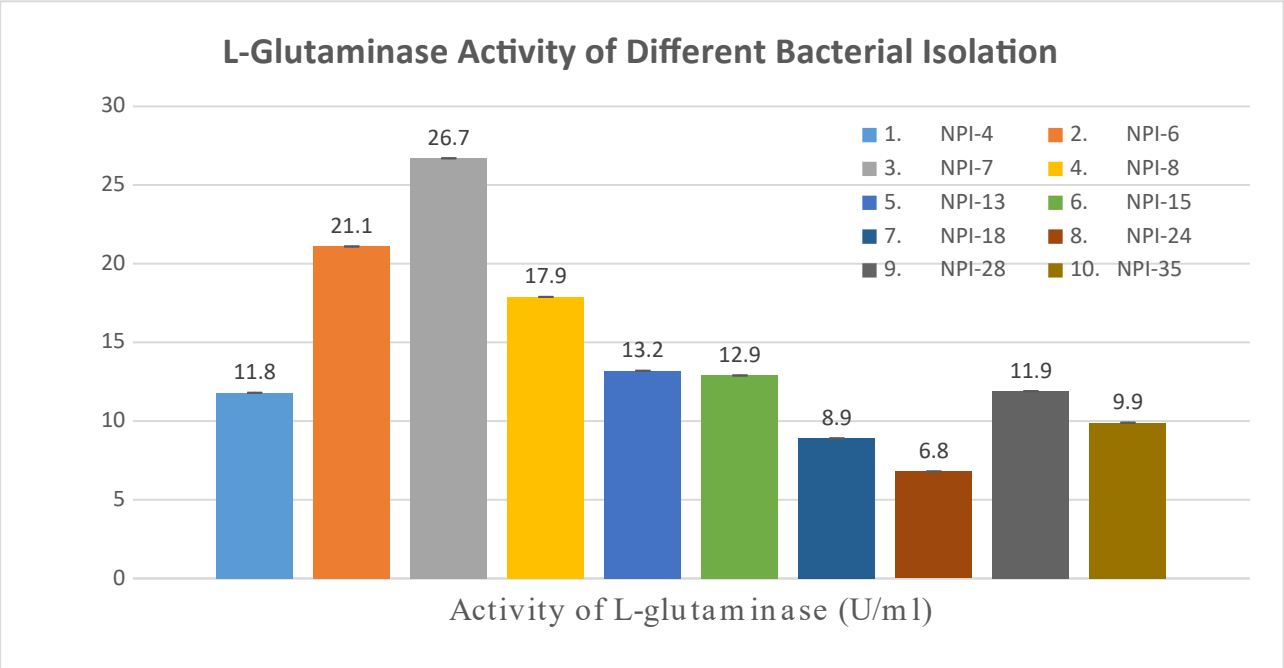


Figure 01: Production of L-Glutaminase Activity Isolates

3.4 Optimization of medium and cultural conditions for L–Glutaminase Production

Optimization of different cultural conditions such as incubation period, pH, temperature, carbon, and nitrogen sources on the production of L-glutaminase were determined.

3.4.1 Effect of incubation period on L-glutaminase production:

The maximum L-glutaminase activity was recorded after 72 hours of incubation, with NPI-7 exhibiting the highest enzyme activity of 28.7 U/mL, followed by NPI-8 (24.3 U/mL) and NPI-6 (21.6 U/mL). The mean enzyme activity of the three isolates was highest at 72 hours (24.87 U/mL), indicating that this

incubation period was optimal for L-glutaminase production under the tested conditions. However, prolonged incubation up to 96 hours resulted in a considerable reduction in enzyme activity, with values decreasing to 18.9, 17.9, and 16.7 U/mL for NPI-7, NPI-6, and NPI-8, respectively. The decline in enzyme production after 72 hours may be attributed to the depletion of essential nutrients, reduced microbial growth, and possible accumulation of metabolic waste products in the culture medium. These factors may adversely affect microbial metabolism and subsequent enzyme production. Therefore, an incubation period of 72 hours was considered optimal for maximum L-glutaminase production by the selected bacterial isolates, particularly *Bacillus endophyticus* NPI-7 (Figure 02). Similar observations have been reported by Prakash et al. (2009) in Bacillus sp. SFL-15 and Poorani et al. (2009) in Escherichia coli SFL-1, supporting the influence of incubation time on microbial L-glutaminase production. Maximum enzyme production was observed at 72 hours, particularly in NPI-7 (28.7 U/mL). The subsequent decline at 96 hours may be associated with nutrient depletion and changes in culture conditions. Similar incubation-dependent variations were reported by Hassan et al. (2025).

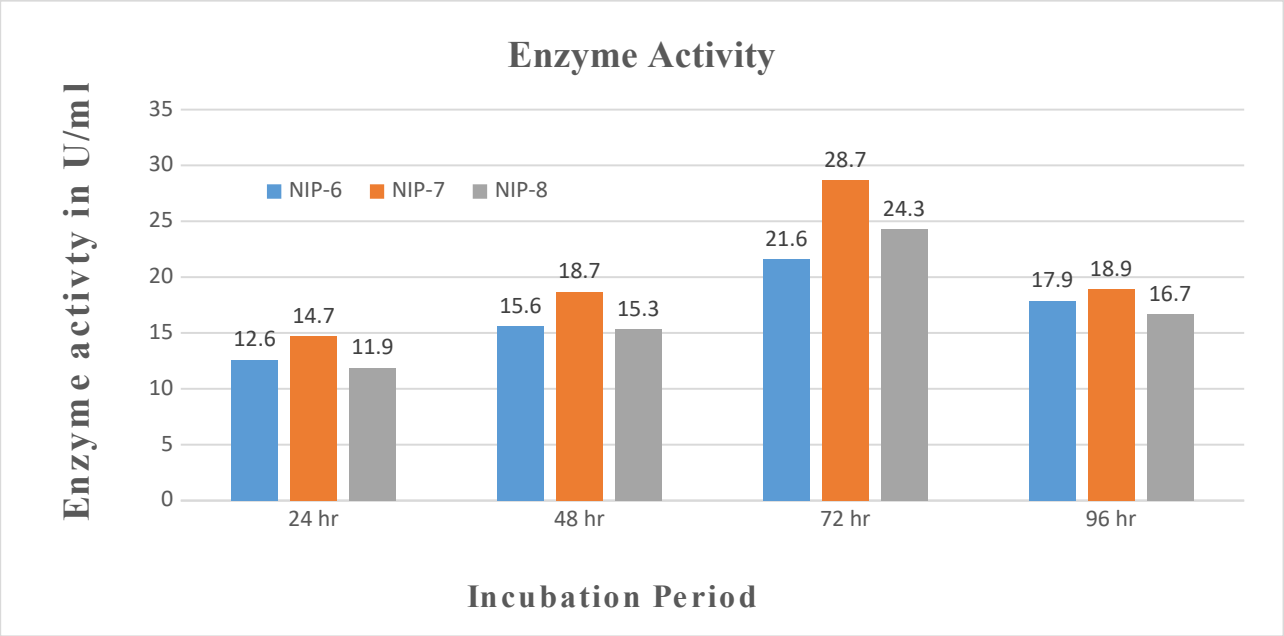


Figure None: 02 Effect of Incubation Period for L-Glutaminase Production

3.4.2 Effect of pH on the L-Glutaminase production:

The results revealed that all three isolates exhibited maximum L-glutaminase production at pH 7.0. Among the tested isolates, NPI-7 showed the highest enzyme activity (29.7 U/mL), followed by NPI-8 (24.3 U/mL) and NPI-6 (23.6 U/mL). The mean enzyme activity at pH 7.0 was 25.87 U/mL, indicating that neutral pH was the most favourable condition for L-glutaminase production. At pH 55.0, enzyme activity ranged from 13.7 to 14.8 U/mL, whereas at pH 6.0, it increased to 17.3–18.7 U/mL. However, a further increase in pH to 8.0 and 9.0 resulted in a reduction in enzyme production. The lowest enzyme activity was recorded at pH 9.0, with values ranging from 12.6 to 13.5 U/mL. These findings indicate that both acidic and alkaline conditions negatively influenced L-glutaminase production in the selected isolates.

The reduction in enzyme production under highly acidic or alkaline conditions may be attributed to changes in microbial growth, cellular metabolism, nutrient uptake, and enzyme synthesis. Therefore, pH 7.0 was considered the optimum initial pH for maximum L-glutaminase production, particularly by *Bacillus endophyticus* NPI-7 (Figure 03).

Similar findings were reported by Sinha et al. (2016) in Bacillus sp., Lee et al. (2008) in *Bacillus amyloliquefaciens* DL3, Durai et al. (2014) in Vibrio sp., and Pandian et al. (2014) in *Alcaligenes faecalis* KLU102, where maximum L-glutaminase production was also observed at pH 7.0. These findings support

the importance of neutral pH for optimum microbial L-glutaminase production. Neutral pH (7.0) supported maximum enzyme production, with NPI-7 recording 29.7 U/mL. This may be attributed to favourable conditions for microbial growth and enzyme biosynthesis. Comparable findings were reported by Kulasekaran et al. (2024), who investigated L-glutaminase from marine *Bacillus australimaris* NIOT30.

3.4.3 Effect of temperature on the L-Glutaminase production:

The results revealed that L-glutaminase production increased with an increase in temperature up to 40°C in all three isolates. The maximum enzyme activity was recorded at 40°C, with NPI-7 exhibiting the highest activity of 28.7 U/mL, followed by NPI-8 (22.3 U/mL) and NPI-6 (19.6 U/mL). The mean enzyme activity at this temperature was 23.53 U/mL, indicating that 40°C was the most favorable temperature for L-glutaminase production among the tested conditions. At 45°C, a reduction in enzyme activity was observed in NPI-7 and NPI-8, whereas NPI-6 showed a slight increase to 19.9 U/mL. Further increases in temperature to 50°C and 55°C resulted in a considerable decline in enzyme production. These findings suggest that temperatures above 40°C were less favorable for L-glutaminase production by the selected bacterial isolates.

The reduction in enzyme production at higher temperatures may be attributed to temperature-induced changes in microbial growth, cellular metabolism, and protein synthesis. Elevated temperatures may also affect cellular integrity and metabolic processes, thereby reducing enzyme production. Therefore, 40°C was considered the optimum temperature for maximum L-glutaminase production, particularly by *Bacillus endophyticus* NPI-7 (Figure 04).

Similar findings were reported by Dutta et al. (2016) in *Pseudomonas stutzeri* ISL B5 and Mesas et al. (1990) in *Corynebacterium glutamicum*, indicating the influence of incubation temperature on microbial L-glutaminase production. he highest enzyme production was recorded at 40°C, with NPI-7 exhibiting 28.7 U/mL. Reduced production at higher temperatures may reflect temperature-induced physiological stress.

Similar optimum temperature conditions were reported by Hassan et al. (2025).

Figure 03: Effect of Initial pH for L-Glutaminase

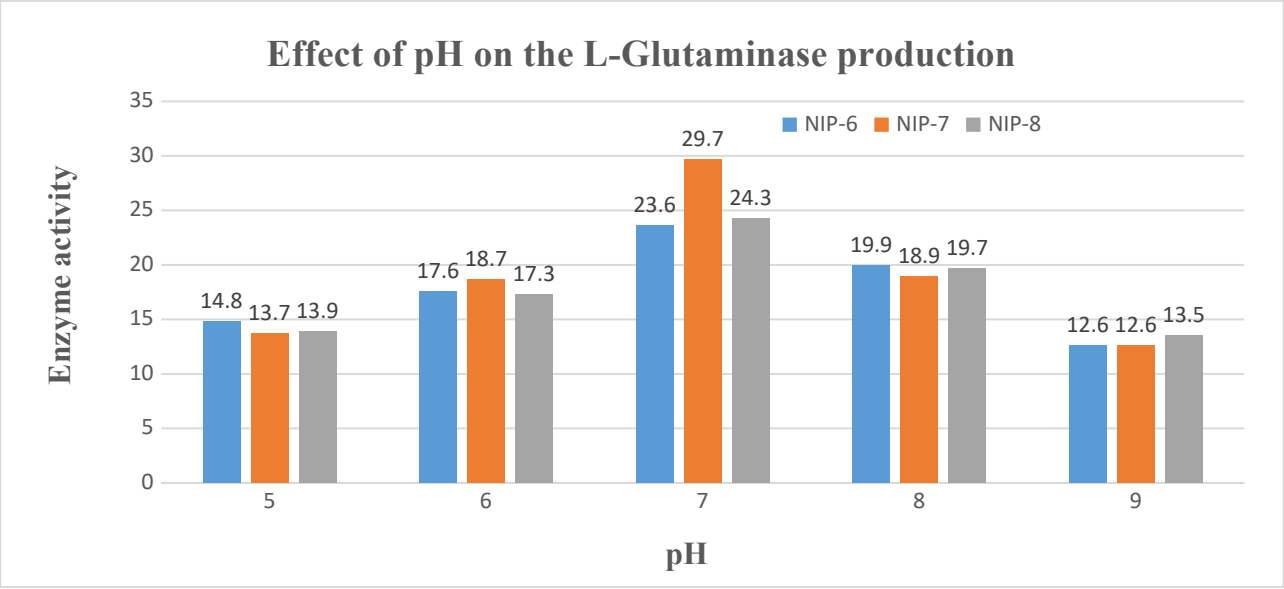
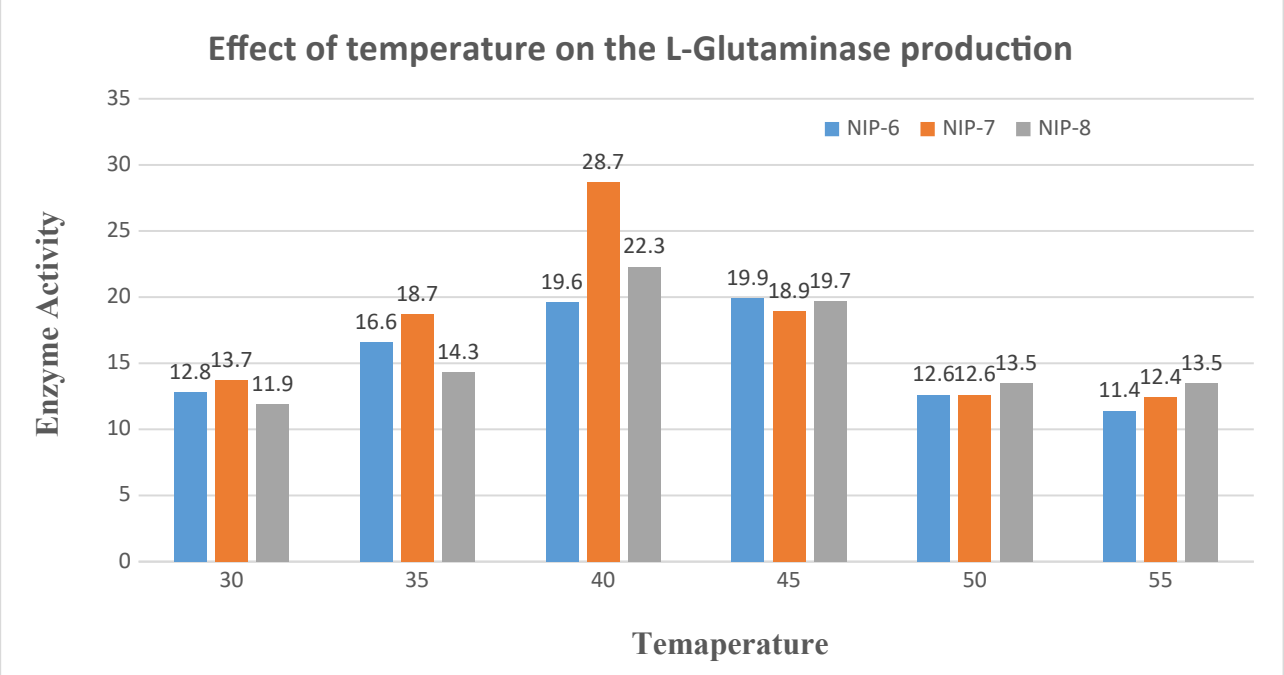


Figure 04: Effect of Temperature for L-Glutaminase Production



3.4.4 Effect of carbon sources on the L-Glutaminase production:

The results revealed considerable variation in L-glutaminase production among the different carbon sources tested. Among the six carbon sources, sucrose supported the maximum enzyme production in all three isolates. The highest enzyme activity was recorded in NPI-7 (28.7 U/mL), followed by NPI-8 (24.3 U/mL) and NPI-6 (23.6 U/mL). The mean enzyme activity with sucrose was 25.53 U/mL, indicating that it was the most favorable carbon source for L-glutaminase production.

Maltose and fructose also supported comparatively higher enzyme production. With maltose, enzyme activities of 23.9, 21.9, and 21.7 U/mL were recorded in NPI-6, NPI-7, and NPI-8, respectively. Similarly, fructose supported enzyme activities of 21.6, 24.7, and 21.3 U/mL in the respective isolates. The mean enzyme activities obtained with fructose and maltose were 22.53 and 22.50 U/mL, respectively. In contrast, galactose and starch resulted in comparatively lower enzyme production, suggesting that the utilization of different carbon sources had a considerable influence on L-glutaminase synthesis.

The variation in enzyme production may be attributed to differences in carbon source utilization, metabolic pathways, and the availability of energy required for microbial growth and enzyme synthesis. Therefore, sucrose was considered the most suitable carbon source for maximum L-glutaminase production by the selected bacterial isolates, particularly Bacillus endophyticus NPI-7 (Figure 05).

Similar observations were reported by Keerthi et al. (1999), who observed enhanced enzyme production in *Beauveria bassiana* BMTF S10. Similarly, Iyer and Singhal (2009, 2010) reported a significant influence of fructose on L-glutaminase production by *Providencia sp*. and *Klebsiella oxytoca*. These findings demonstrate that the selection of an appropriate carbon source is an important factor in optimizing microbial L-glutaminase production. Among the six carbon sources tested, sucrose supported maximum enzyme production (28.7 U/mL in NPI-7). Fructose and maltose also supported considerable production. Differences in carbon utilization may explain the variation among isolates. In contrast, Hassan et al. (2025) and Ibrahim et al. (2024) reported different preferred carbon sources, demonstrating strain-dependent nutritional requirements.

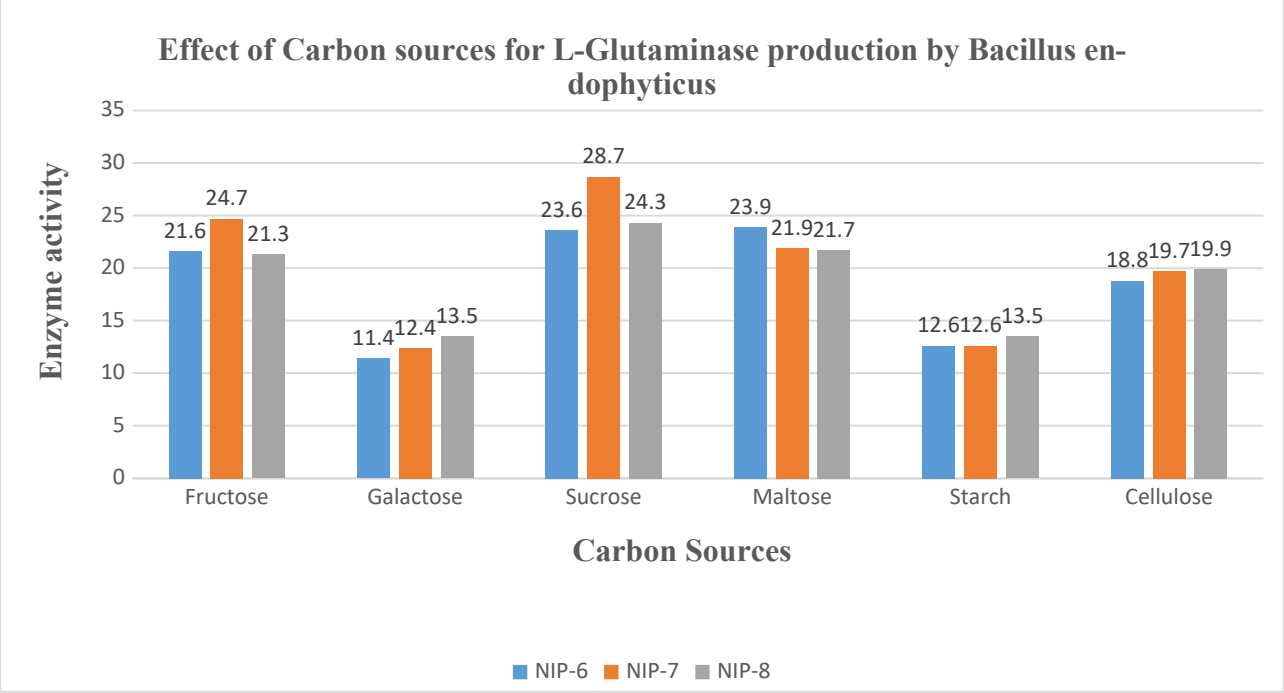


Figure 05: Effect of Carbon Sources for L-Glutaminase Production

3.4.5 Effect of nitrogen sources on the L-Glutaminase production:

Nitrogen sources play an important role in microbial growth, cellular metabolism, and enzyme biosynthesis by providing essential nitrogen required for the synthesis of proteins, enzymes, and other cellular components. In the present study, the influence of six different nitrogen sources, namely yeast extract, tryptone, ammonium sulphate, malt extract, peptone, and urea, on L-glutaminase production was investigated using the three selected bacterial isolates, NPI-6, NPI-7, and NPI-8.

The results revealed considerable variation in enzyme production among the different nitrogen sources tested. Among the six nitrogen sources, yeast extract supported the highest L-glutaminase production. The maximum enzyme activity was recorded in NPI-8 (32.7 U/mL), followed by NPI-6 (31.2 U/mL) and NPI-7 (29.7 U/mL). The mean enzyme activity with yeast extract was 31.20 U/mL, indicating that it was the most favorable nitrogen source for L-glutaminase production.

Malt extract also supported considerable enzyme production, with activities of 29.9, 31.5, and 28.9 U/mL in NPI-6, NPI-7, and NPI-8, respectively. The mean enzyme activity obtained with malt extract was 30.10 U/mL. Ammonium sulphate supported moderate enzyme production, whereas tryptone and urea resulted in comparatively lower enzyme activities. Peptone exhibited the least favorable effect on enzyme production, with activities ranging from 12.6 to 13.5 U/mL (Figure-06). Similar findings were reported by Sivakumar et al. (2005) in Streptomyces rimosus, demonstrating the influence of nitrogen sources on microbial enzyme production. These observations highlight the importance of selecting an appropriate nitrogen source for optimizing L-glutaminase production.

Yeast extract was the most favorable nitrogen source, producing maximum activity of 32.7 U/mL in NPI-8. Its rich nutritional composition may support microbial growth and enzyme biosynthesis. Similar nutritional effects were reported by Wardah et al. (2023) and Hassan et al. (2025).

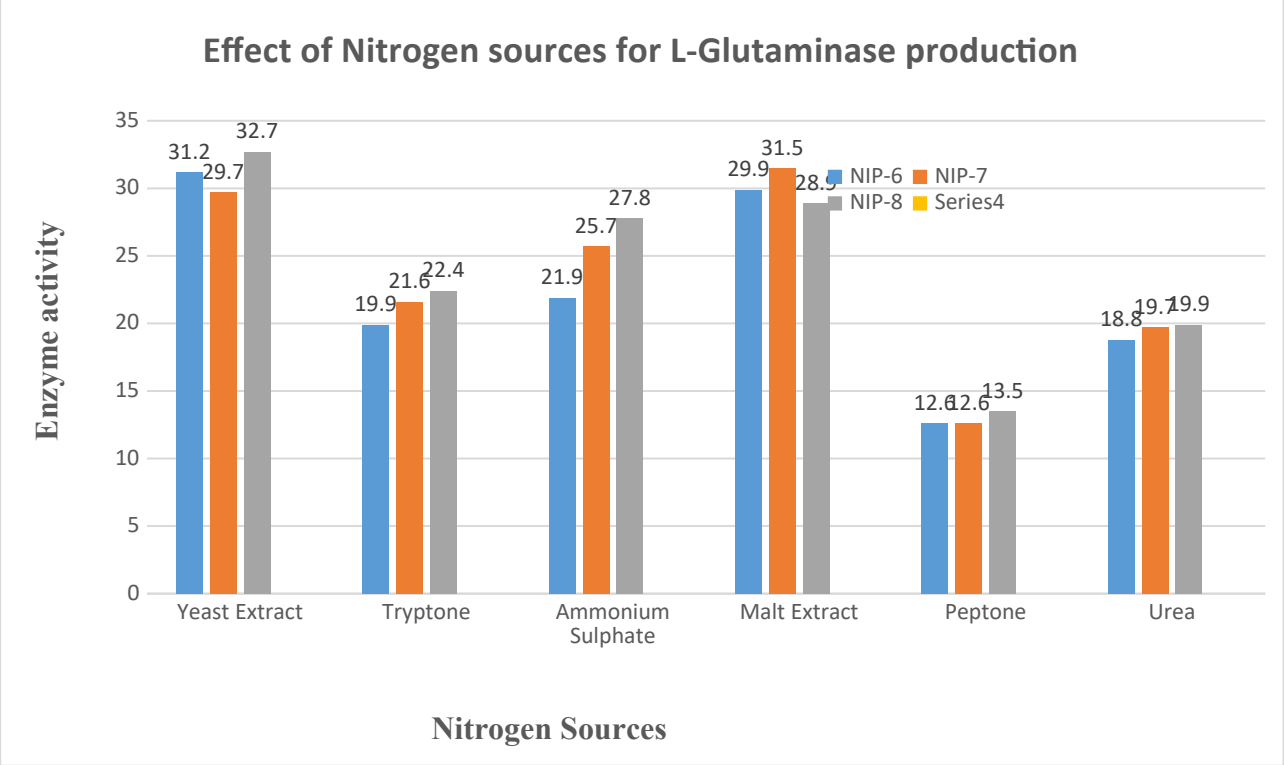


Figure 06: Effect of Nitrogen Sources for L-Glutaminase

3.4.6 Effect of metal ions on the L-Glutaminase production:

Mineral salts are essential inorganic nutrients required for microbial growth, metabolism, and enzyme production. These nutrients play important roles in maintaining cellular functions and regulating various metabolic activities. In the present study, the influence of different mineral salts, namely $MgCl_2$, $CuSO_4$, $FeSO_4$, $MnCl_2$, $NiCo_4$, Li_2SO_4, $ZnCl_2$, $CaCl_2$, and Ba $(OH)_2$, on L-glutaminase production was investigated using the three selected bacterial isolates, NPI-6, NPI-7, and NPI-8. The results revealed considerable variation in enzyme production in the presence of different mineral salts. Among the tested compounds, $MgCl_2$ supported the highest L-glutaminase production. The maximum enzyme activity was recorded in NPI-8 (32.7 U/mL), followed by NPI-6 (31.2 U/mL) and NPI-7 (29.7 U/mL). The mean enzyme activity in the presence of $MgCl_2$ was 31.20 U/mL, indicating that it was the most favorable mineral salt for L-glutaminase production.

$MnCl_2$ also supported comparatively higher enzyme production, with activities of 29.9, 31.5, and 28.9 U/mL in NPI-6, NPI-7, and NPI-8, respectively. The mean enzyme activity with $MnCl_2$ was 30.10 U/mL. Ba $(OH)_2$ and $FeSO_4$ supported moderate enzyme production, whereas $CuSO_4$ and Li_2SO_4 resulted in comparatively lower enzyme activities. The lowest enzyme production was observed in the presence of $NiCo_4$, with activity values ranging from 12.6 to 13.5 U/mL. The variation in enzyme production may be attributed to the different physiological and metabolic roles of individual metal ions in microbial cells. Certain metal ions may act as cofactors or influence cellular metabolic pathways involved in enzyme biosynthesis. Therefore, $MgCl_2$ was considered the most favorable mineral salt for maximum L-glutaminase production by the selected bacterial isolates (Figure 06). Similar findings were reported by Soda et al. (1972) in *Bacillus sphaericus*. L-glutaminase production was also reported in *Pseudomonas fluorescens* ACMR 171, *Vibrio costicola* ACMR 267, and *Vibrio cholerae* ACMR 347 by Yano et al. (1988). These findings emphasize the importance of mineral salts in optimizing microbial L-glutaminase production. $MgCl_2$ supported the highest enzyme production (32.7 U/mL in NPI-8), followed by $MnCl_2$. Magnesium may contribute to cellular metabolism and enzyme biosynthesis. The influence of metal ions on microbial enzyme production has also been demonstrated by Hassan et al. (2025).

The findings identified 72 hours of incubation, pH 7.0, 40°C, sucrose, yeast extract, and $MgCl_2$ as individually favorable conditions for L-glutaminase production. Recent studies by Wardah et al. (2023) and

Chacko et al. (2026) further emphasize the importance of statistical optimization and response surface methodology for enhancing microbial enzyme production. Molecular identification, enzyme purification, biochemical characterization, and combined optimization are recommended

to establish their potential for industrial applications.

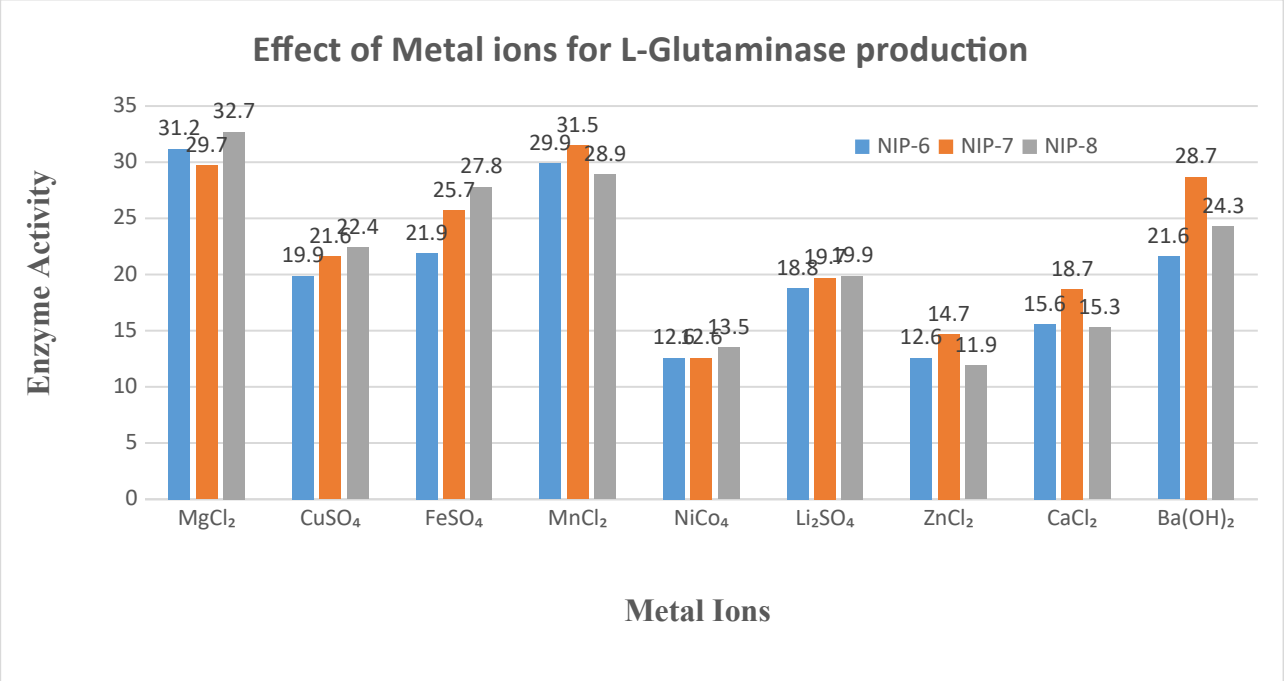


Figure 07: Effect of Metal Ions for L-Glutaminase Production

4.0 Conclusion

The present study demonstrated the potential of bacterial isolates for L-glutaminase production. Among the ten bacterial isolates screened, NPI-7 exhibited the highest initial enzyme activity and was identified as a promising L-glutaminase-producing isolate. Based on preliminary screening, three isolates, namely NPI-6, NPI-7, and NPI-8, were selected for further optimization studies and characterization.

The optimization studies revealed that different cultural and nutritional parameters significantly influenced L-glutaminase production. The optimum conditions identified were an incubation period of 72 hours, pH 7.0, and an incubation temperature of 40°C. Among the different carbon sources evaluated, sucrose supported the highest enzyme production, whereas yeast extract was identified as the most favorable nitrogen source. Among the tested mineral salts, $MgCl_2$ supported maximum enzyme production. The highest recorded enzyme activity was 32.7 U/mL in the presence of yeast extract and $MgCl_2$.

Overall, the findings demonstrated that the selected bacterial isolates, particularly NPI-7, have considerable potential for L-glutaminase production. The results also highlight the importance of optimizing nutritional and environmental parameters to enhance microbial enzyme production. Similar studies have emphasized the significance of statistical optimization approaches, including response surface methodology, for improving enzyme yields (Wardah et al., 2023; Chacko et al., 2026).

Further investigations involving molecular identification, enzyme purification, biochemical characterization, and combined optimization of fermentation parameters are necessary to improve enzyme yield and establish the industrial applicability of these bacterial isolates. The selected isolates may serve as promising candidates for future research and large-scale production of L-glutaminase for industrial and biotechnological applications.

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