Current Trends in Biotechnology and Pharmacy
Vol. 20(4) 3248-3262 October 2026, ISSN 0973-8916 (Print), 2230-7303 (Online)
DOI: 10.5530/ctbp.2026.4.42



# Optimization, purification, and characterization of L-asparaginase from *Aspergillus quadrilineatus*

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**Abstract**

One remedy used to treat lymphoblastic cancer is L-asparaginase. The ability of a new strain to produce extracellular L-asparaginase was investigated in this work. The study examined the production of L-asparaginase utilising MCDB, which exhibited the highest level of L-asparaginase activity. The optimisation, synthesis, purification, and characterisation of the *Aspergillus quadrilineatus* derived L-Asparaginase enzyme are the main objectives of this investigation. After the crude enzyme was removed, it was precipitated with ammonium sulphate, filtered using a Sephadex column, and then further purified using ion exchange chromatography to create L-asparaginase. The enzyme's reaction to different temperatures, pH values, substrate concentrations, incubation times, and SDS-PAGE analyses was evaluated in order to characterise it. To improve the synthesis of L-asparaginase, physical parameters like pH and nutritional parameters like carbon, nitrogen, ionic salts, amino acids, phosphate groups, surfactants, plant oil, and vitamins, as well as their corresponding concentrations, were optimised. The results of the study showed that 37°C and a pH of 8.0 were the most conducive to the synthesis of enzymes. The most effective sources of carbon and nitrogen were L-asparagine (0.5%) and maltose (0.5%), respectively. Vitamins (1%), sodium acetate (1%), citric acid (1%), tryptophan (1%), and sodium dihydrogen phosphate (1%), were found abundant in the yield. With the medium composition described in the paper, continuous fermentation can produce L-asparaginase on a large scale. Protein profiling was conducted using SDS-PAGE on the partly purified enzyme, resulting in a predicted molecular weight of the protein as 47KDa.

**Keywords:** L-asparaginase, *fungi, Characterization*, Optimization, Purification.

## Introduction

A chemotherapy drug called L-asparaginase (EC 3.5.1.1) is used to accelerate the process of turning L-asparagine into L-aspartic acid and ammonia. Lately, there has been a growing focus on the capacity of this chemical to hinder the onset of cancer. (1,2) The enzymatic action of this enzyme is responsible for reducing the levels of L-asparagine. This selective eradication of cancer cells occurs because they are unable to produce this amino acid. The enzyme is made by different bacteria, including Enterobacter cloacae (3), Serratia marcescens (4), and Enterobacter aerogenes (5). Enzymes from Escherichia coli and Erwinia carotovora are used in current treatment to treat acute lymphoblastic leukaemia (6). However, the prolonged usage of L-asparaginase in humans triggers the development of antibodies which might cause an anaphylactic shock or render the medicine ineffective. So, it is important to keep looking at newly found organisms to find strains that can make a lot of L-asparaginase uniquely and importantly. Scientists have looked at different

types of Streptomyces, like S. karnatakensis, S. venezualae, S. longsporus flavus and a marine Streptomyces sp. PDK2, to see if they can make L-asparaginase (7). Around the world, both immersed and solid-state cultures are used to make the enzyme. Extracellular asparaginases are better than intracellular asparaginases in several ways. In normal conditions, they can be made in large amounts in the culture broth and can be cleaned up inexpensively. As drugs are used to treat some types of cancer, amino acid-degrading enzymes are very important. Asparaginase works as a treatment for acute lymphocytic leukemia, as shown by studies by Sahu (8). Tumour cells rely on the uptake of L-asparagine from the bloodstream or bodily fluids due to their inability to produce L-asparagine through synthesis. L-asparaginase enzymes when used as chemotherapeutic drugs can break down the L-asparagine found in the bloodstream. The procedure can indirectly lead to the deprivation of nutrients in tumor cells, ultimately resulting in their demise. Microbial enzymes outperform enzymes produced by plants or animals due to their superior ease of modification, improvement, and purification as well as their lower production costs. Savitri (9) argues that enzymes obtained from microorganisms demonstrate superior stability in comparison to enzymes obtained from plants or mammals. A lot of people are interested in making L-asparaginase using microbial systems because they are environmentally friendly and save money. Verma (10) states that filamentous mushrooms, yeasts, and bacteria are among the microorganisms capable of producing this enzyme. Asparaginase, obtained from Escherichia coli and Erwinia chrysanthemi bacteria, has been used as a therapeutic drug for more than 30 years (11,12). L-asparaginases made by bacteria can sometimes lead to allergy reactions and anaphylaxis (13,14). If scientists look into asparaginase from other places like eukaryotic bacteria, they might find an enzyme that doesn't have as many bad effects. Yeast and filamentous fungi, in particular have been shown in several studies to be able to make L-asparaginase (15,16). Researchers have identified *Aspergillus tamari* and *Aspergillus terreus* as reliable suppliers of this enzyme. When making enzymes for sale, picking a good strain and using a good collecting method are very important steps. For clinical studies having the right amount of enzyme is just as important as having the right source species. It is necessary to look into other sources of enzymes to solve this problem.

## Materials and Methods

### *Used fungal strains*

Fungal samples from the Regional Plant Resource Centre Microbiology Laboratory that showed potential for L-asparaginase production underwent further testing.

### *Identification and characteristics*

The isolates were classified according to their molecular features. The fungal cultures were identified at the Microbial Type Culture Collection (MTCC) in Chandigarh.

### *Preparation of pure cultures and inoculum*

To keep the pure cultures at 4 $^{o}$C, they were grown on Sabouraud Dextrose agar slants and subculture regularly. The inoculum was made up of cultures that had been grown on Sabouraud-Dextrose agar plates for 5 to 6 days.

### *Media used*

A 4.5-pH broth containing glucose and asparaginase was formulated, and 20 ml portions were transferred into 100 ml containers. The containers were sterilized for 15 to 20 minutes at 121$^{o}$C.

### *Inoculation*

A single 5 mm inoculum disc was taken from the culture plates and put into the container that held the soup. Over 10 days, the culture pots were kept at a constant temperature of 30$^{o}$C in an incubator.

### *Assay for Asparaginase*

### *Qualitative analysis*

This is a modified Czapek Dox medium that has glucose (2 grams per liter), L-asparaginase, L-glutaminase, KH2PO41.52 grams per li-

ter, KCl 0.52 grams per liter, MgSO4.7H2O 0.52 grams per liter, small amounts of Fe SO4.7H2O, ZnSO4.7H2O, and $CuNO_3.7H_2O$, and agar (18 grams per liter). For the phenol red dye, the pH of the medium was changed to 6.2. Another step was to put the medium in an autoclave. It was used to make a stock solution of 2.5% phenol red dye. Then, 0.009% phenol red dye was added to the MCD medium that had been cleaned. The mixture was then put onto plates that had already been sterilized. There was a pink zone around the fungal colonies that showed that L-asparaginase-producing fungi were growing (Ashok *et al*.,2019).

***Gene sequence***

> *Aspergillus quadrilineatus* DO11 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence.

GCTCCGGGCGCCAACCTCCCACCCGTGACTACCTAACACTGTTGCTTCGGC-
GGGGAGCCCCCTAGGGGCGAGCCGCCGGGGACCACTGAACTTCATGCCTGA-
GAGTGATGCAGTCTGAGCCTGAATACAAATCAGTCAAAACTTTCAACAATG-
GATCTCTTGGTTCCGGGCTTCGATGAAGAACGCAGCGAACTGCGATAAGTAAT-
GTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCT-
GGCATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCTCAAGCCCG-
GCTTGTGTGTTGGGTCGTCGTCCCCCCCGGGGGACGGGCCCGAAAGGCAGCGG-
CGGCACCGTGTCCGGTCCTCGAGCGTATGGGGCTTTGTCACCCGCTCGATTAGG-
GCCGGCCGGGCGCCAGCCGGCGTCTCCAACCTTATTTTTCTCAGGTTGACCTCG-
GATCGGGGGGGGAATCCCCGCTGATCTTAA

***Quantitative analysis***

The ammonia measurement procedure was carried out by conducting an enzyme assay using a Modified Czapek Dox medium broth following the Nesslerization method as described by Imada (17). To make the enzyme test mixture, 0.5ml of 0.04M L-asparagine was mixed with 0.5ml of Tris HCl buffer (pH 8.6). Then 0.5ml of enzyme and 0.5ml of distilled water were added. Subsequently, the combination was subjected to incubation for 30 minutes at a temperature of 37°C degrees Celsius. Following the incubation period, the reaction was halted by introducing 0.5ml of 30% TCA. To estimate the enzyme activity, 7ml of distilled water was combined with 0.5ml of enzyme mixture followed by the addition of 1ml of Nesseler's Reagent. Next, the optical density was measured at a wavelength of 480nm using a spectrophotometer to estimate the enzyme activity.

The quantity of ammonia emitted by the test sample was evaluated by comparing it to the reference graph. The measurement of the enzymatic activity of L-asparaginase was conducted using International Units (IU). An International Unit (IU) of L-asparaginase activity is the amount of enzyme required to produce one micromole of ammonia per milliliter per minute at a temperature of 37°C and a pH of 8.6.

***Optimising process parameters for the production of L-asparaginase.***

The enhancement of medium and cultural conditions for asparaginase production was achieved by gradually adjusting the controlling factors. The organism was cultivated under varying conditions of temperature, pH, incubation period, carbon sources, Nitrogen sources and Effect of amino acids on asparaginase production.

***Effect of carbon sources***

To examine the influence of different carbon sources on the production of asparaginase the following carbon sources were used: fructose, galactose, lactose, maltose, glucose, and mannitol. Various carbon sources were introduced into the medium at a concentration of 1% and incubated for six days, after which the enzyme activity was assessed using the supernatant.

***Effect of nitrogen sources***

To evaluate the impact of nitrogen sources on the production of asparaginase, both organic and inorganic nitrogen sources were added to the fermentation fluid. The nitrogen sources used include urea, ammonium fluoride, ammonium persulphate, ammonium chloride, cobaltous nitrate, ammonium sulphate, and potassium nitrate. The nitrogen source was added to the medium at a concentration of 1% and incubated for six days and the enzyme activity was determined using the supernatant.

***Effect of pH***

To ascertain the ideal pH for L-asparaginase production, the fungal culture was inoculated into a series of varying pH solutions (4.5, 5.5, 6.5). The pH of the solutions was subsequently modified by combining 1N NaoH with 1N HCl. Before undergoing autoclaving, the organism underwent inoculation. The supernatant from the six-day incubation period of the flasks was utilized to quantify the asparaginase activity.

***Effect of ionic salts***

To assess the impact of various ionic salts (C, B1, B6, B2, M) on the formation of asparaginase, the presence of peppermint oil, olive oil and yeast was examined. Tween-20, Tween-40, and Tween-80 are types of surfactants. Some of the chemicals that were used were potassium acetate, sodium nitrate, calcium chloride, tricalcium phosphate, sodium thiosulfate, sodium trisulfate, sodium chloride, ammonium sulfate, ferrous sulfate, zinc sulfate, ferric chloride, glycine, calcium phosphate, sodium dihydrogen phosphate, and dipotassium hydrogen phosphate. Different ionic salts were added to the medium at a concentration of 0.5%. The mixture was then left to grow for 6 days, and the supernatant was used to measure the enzyme activity.

***Effect of amino acids***

The impact of amino acids on the optimization of L-asparaginase production was assessed by introducing a range of amino acids (Threonine, Tryptophan, Proline, Aspartic acid, Phenylalanine, Cysteine, Methionine, Tyrosine, Arginine, Histidine, Citric acid, Oxalic acid, Yeast extract) at a concentration of 0.5% into the medium. The medium was then incubated for 6 days, and the resulting supernatant was utilized to measure the enzyme activity.

***Purification studies***

***Mass production, classification and extraction, purification***

The organisms were introduced into the previously specified broth media and kept in a stationary environment at a temperature of 30ºC for 6 days. The samples were collected following the appropriate incubation period. The biomass was quantified by weighing it and then subjected to treatment with a 0.05 M Tris-HCL buffer at a pH of 8.5. The sample was centrifuged at a speed of 600 rpm for 20 minutes at a temperature of $4^0$C which led to the formation of a liquid. The statement described above was considered to be the basic composition of the enzyme. The enzyme activity and protein content were quantified and thereafter, the sample underwent the initial purification phase using ammonium sulfate precipitation.

***Ammonium sulphate precipitation***

The initial solution underwent precipitation by adding finely powdered ammonium sulfate until it reached a saturation level of 80%. The temperature was kept constant at 4ºC. The material underwent centrifugation at a speed of 6000 rpm for 10 minutes, continuing overnight. Afterward, the pellet that was retrieved was dissolved in a Tris-HCl buffer solution with a pH of 8.5, which had a concentration of 0.05 M.[18]

***Sephadex G-100 gel filtration***

The samples that underwent precipitation with ammonium sulfate were assessed for their enzymatic activity and protein concentration. Afterward, they were subjected to gel filtration using Sephadex G-100.[19]

***Gel-column preparation and sample application***

A glass tubing chromatography column measuring 60 cm in height and 2.2 cm in diameter was utilized. The eluent was 0.05 M Tris-HCL buffer with a pH of 8.5. To inhibit the formation of bubbles within the gel bed. Then the eluent was transferred from securely sealed brown bottles into containers containing the same temperature. The gel slurry was prepared by dissolving 5 grams of Sephadex in 200 ml of 0.05 M Tris-HCL buffer and allowing it to swell at room temperature for twenty-four hours. The column was secured and closed using Sephadex. Ammonium sulfate was used to precipitate the samples which were then continuously poured onto the column. The resulting fractions were collected in containers with a volume of 5ml. A random assortment of the gathered fractions underwent protein and enzyme activity analysis, and the fractions demonstrating exceptional enzyme activity were combined.

***Ion exchange chromatography***

The column was constructed by dissolving the necessary quantity of DEAE-cellulose in Tris-HCl buffer overnight. After the packaging procedure, the column was rinsed with 5M KCl to produce the desired weak ion exchange material. This was followed by rinsing with 5M NaOH to remove ionic charges from the ion exchanger. Afterward, the material was washed with distilled water and a 0.05 M Tris-HCl buffer solution at a pH of 8.5. Following the utilization of Sephadex for fractionating the peak fractions, they were next transferred to the ion exchange column where 5 ml of the fractions were gathered. After assessing the protein and enzyme activity of the samples and the fractions comprising the most active enzymes were consolidated and stored in a deep freezer.

***Separation of L-asparaginase by electrophoresis.***

An evaluation using gel electrophoresis was carried out in order to determine the molecular weight of the recovered enzyme and to evaluate the degree of homogeneity of the enzyme. The resolving gel consisted of the following constituents: The composition of the solution consists of 3.6 milliliters of acrylamide, 3.75 milliliters of distilled water, 2.5 milliliters of resolving buffer, 100 microliters of 10% SDS, 57.1 microliters of 10% APS, and 42.85 microliters of TEMED. We introduced a 100-ml aqueous solution which was saturated with butanol into the gel and allowed it to undergo polymerization for a duration of 30 to 40 minutes. Following the time limit, we transferred the butanol solution into a separate container which we subsequently cleaned with distilled water and absorbed using tissue paper. The stacking gel was prepared by mixing 3.0 ml of distilled water, 0.65 ml of stacking buffer, 1.2 ml of acrylamide solution, 50 µl of 10% SDS, 50 µl of 10% APS, and 6 µl of TEMED. The combination was then added to the gel that was used for resolving. After inserting the comb then allowed to go through the polymerization process for a period of 30-45 minutes. Following the polymerization process, we firmly attached the gel to the electrophoresis apparatus and carefully removed the comb after adding the tank buffer. We filled each well with a conventional marker and a denatured sample. We carried out the electrophoresis method at an initial voltage of 40 volts for 1 hour then raised the voltage to 60 volts and maintained it for 3 hours. Once the dye reached the bottom of the gel, we intensified the power supply and then extracted the gel from the electrophoresis equipment. After carefully dividing the plates with a spatula and proceeded to insert the gel into the staining solution in a methodical manner so that it could be stained overnight. The gel then underwent a destaining process for either 7 hours or overnight. Submerging the protein in the fixative solution led to the emergence of three distinguishable bands. Then the molecular weight of the protein by compared it to the standard marker.

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***Enzyme characterization based on partial purification***

***Substrate specificity***

The assay mixture was enriched with several substrates, such as L-arginine, L-phenylalanine, L-histidine, L-glutamine, and L-aspartic acid, to evaluate the enzyme's inclination towards a range of substrates. The substrates were present at a concentration of 0.04M, with L-asparagine serving as a control.

***PH optima***

The pH of the Tris-HCl buffer in the reaction mixture was methodically varied between 3 and 10 to identify the most favorable pH for enzyme activity. The enzymatic activity was measured at different pH values.

***Temperature tolerance***

The enzyme was maintained at temperatures ranging from 30 to 50ºCbefore its addition to the reaction mixer for the assay.

***Incubation period***

The enzyme was incubated at varying temperatures of 30, 37, and 50ºCbefore its addition to the reaction mixer for the assay.

**Results and Discussion**

***Phylogenetic analyses of fungal taxa by using ITS sequence data***

The fungal taxa used in the Phylogenetic analyses were obtained based on the BLAST search similarity resulting from the ITS sequence data of respective taxa and through published literature. At the MAFFT server (http://mafft.cbrc.jp/alignment/server/), multiple sequence alignments for the ITS gene were performed online (20). Alignments were then manually corrected using BioEdit (21).

***Optimizing the process parameters for the synthesis of L-asparaginase***

The present study aimed to evaluate the impact of substituting glucose in the basal medium with alternative carbon sources, namely Glucose, lactose, Galactose, Maltose, Mannitol, Sorbitol, Raffinose, Inositol, Arabinose, Starch, Xylose, and Fructoseon the equimolecular carbon foundation. The results in (Table 1 and Fig.1) demonstrate that the highest level of L-asparaginase synthesis was observed i.e.1.81 IU/ml when maltose was used as the carbon source followed by other carbon sources. However, Prakasham (22) discovered that lactose is the most effective carbon source for the manufacture of L-asparaginase in aerobic conditions. Venil and Lakshamanpalsmy (23) have documented a decrease in the synthesis of L-asparaginase following the administration of fructose as a supplement. The highest level of active L-asparaginase synthesis was seen in Staphylococcus sp when it was fed with maltose. According to Akilandeswari (24), it has been determined that proline is the most effective carbon source for the formation of L-asparaginase.

The results in (fig.2 and Table 2) show that potassium nitrate was the best nitrogen source for *Aspergillus quadrilineatus* to make L-asparaginase activity of 1.60 IU/ml. Ammonium sulfate came in second respectively. This discovery aligns with the findings of Elizebeth (25) who concluded that sodium nitrate is the most effective source of nitrogen. In contrast, Narayana (26) discovered that yeast extract exhibited the highest level of activity and whilst Neelima (27) determined that peptone served as the most effective nitrogen source.

The presence of ionic salts also changes the growth of microorganisms. These salts either speed up or slow down growth depending on how they affect the microorganism's development and work with other metabolic enzymes (28). In this case, vitamin C, vitamin B1, peppermint oil, sodium dihydrogen phosphate, glycine, ferric chloride, zinc sulfate sodium chloride, and sodium acetate all helped the growth and had the highest activity level of 2.12 IU mL−1 at pH 6.5, 6 days of incubation and a concentration of 0.5%. The media used was maltose (6.0 g/l) and Potassium Nitrate (1.0 g/l). The ions in the prepared media are different, and their effects have been studied and reported, as shown in (table 3

and Fig 3). When MCD media was mixed with ionic salt, the enzyme activity was the lowest of all the metal ions that were studied.

The presence of amino acids, which act as co-factors for several biosynthetic enzymes and enhance or inhibit growth depending on their effect on the development of the microorganism (Hosamani and Kaliwal 2011). The present study demonstrates that citric acid has a beneficial impact on growth with its maximal activity at a pH of 6.5 over a six-day incubation period with 0.5% citric acid concentration and maltose-6.0g/l and Kno3-1.0g/l media. However, the prepared media contain a variety of amino acids and the impacts of these ions have been examined and documented as illustrated in (Table 4 and Fig 4). The enzyme activity exhibited the lowest value among the amino acids that were tested when MCD media was supplemented with amino acids.

Optimal pH levels in the growing medium are crucial for maximizing L-asparaginase synthesis. It looked at how three different starting pH levels (4.5, 5.5, and 6.5) affected the production of L-asparaginase. The data for the fungi(Table 5 and Fig 5) showed that pH 6.5 was best for L-asparaginase activity (2.50 IU/ml). At different pH levels, the test fungus can grow and make intracellular L-asparaginase. Thirunavukkarasu (29) conducted a study on the impact of pH on asparaginase. They found that a pH of 6.2 was the most favorable for the formation of asparaginase by Fusarium sp.

***Purification studies***

The partial purification of the crude preparation of Lasparaginases was achieved using ammonium sulfate precipitation (80%). The enzyme activity of L-asparaginase synthesis from D0-11 was measured to be 2.56IU/ml whereas the specific activity was found to be 5.6IU/mg. These measurements were obtained after precipitating the enzyme with ammonium sulfate. The purification yield was increased from 100 to 234 IU/mg with the application of Sephadex gel filtration and ion-exchange chromatography as shown in Table 6. Debaryomyces sp. CECT 11815 was acquired by employing ammonium sulphate treatment subsequently followed by anion exchange chromatography and gel filtration as recorded by Dura (30). Dura (30) found that the asparaginase showed notable instability especially during the gel-filtration phase.

***Characterization of purified Enzyme***

The investigation of the enzyme's pH optimization involved the use of a Tris-HCl buffer with a concentration of 0.05 M. The pH values ranged from 3 to 10. Table 7 indicates that the enzyme exhibited its highest level of activity at a pH of 7. The enzyme exhibited its maximum activity at a temperature of 37°C. The examination of substrate specificity revealed that the enzyme functions as a catalyst for L-asparagine, specifically acting on it as its substrate.

Our findings were similar to those of Prakasham (22), for *Staphylococcus sp*, *Pseudomonas stutzeri*, and *Erwinia carotovora*. Sangita (31) produced L-asparaginase at a temperature of 30°C using Serratia marcescens. Narayana et al. (2008) utilized *Streptomyces albidoflavus* to produce L-asparaginase at a temperature of 35°C. Roberts (32) employed Achromobacteriacae to produce L-asparaginase at a temperature of 20°C. The study also investigated the optimal incubation period for an enzyme and substrate to achieve maximum enzyme activity and the most rapid substrate degradation rate. This was done by incubating the enzyme with the substrate for different amounts of time under the best conditions. It was found out what the enzyme activity was after 10, 20, 30, and 40 minutes. The results show that the asparaginase activity peaked after 10 minutes of incubation at 37° C and pH 7.0. The enzyme activity decreased as the incubation time went up above 10 minutes. As Moorthy (33) mentioned, these results were comparable to what was found. However, the highest activity of Streptomyces albidoflavus was observed at pH 8 (34).

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***Electrophoresis***

The molecular weight of L-asparaginase was ascertained using SDS-PAGE which indicated the existence of a solitary peptide chain with a weight of 47kDa (Figure 8).

Several experimental data suggest that the molecular weight of L-Asparaginase ranges from 32 to 150 kDa. Archana Rani and Raja Rao (2015) employed a 12% resolving gel and a 4% stacking gel. The current paper reveals that the pure L-Asparaginase enzyme obtained from the *Aspergillus quadrilineatus* fungus has a molecular weight of 47KDa as determined by the SDS-PAGE examination. Furthermore, Jeon (35) examined the outcome of SDS-PAGE following the completion of the purification process. This analysis showed two prominent bands with molecular masses of around 70 and 50 kDa. This work is an initial investigation of L-asparaginase derived from *Aspergillus quadrilineatus*. Many different types of fungi are associated with the L-asparaginase enzyme as evidenced by literature review (36,37). The *Aspergillus quadrilineatus* exhibited significant enzyme activity within the cellular biomass. The substrate affinity of L-asparaginase varies among various microorganisms and different species have distinct ecophysiological functions concerning the enzyme activity (38). Enzyme manufacturing involves an intricate series of reactions that are facilitated and stimulated by appropriate substrates. *Aspergillus quadrilineatus* showed a preference for L–asparagine as a substrate. Dunlope and Roon (39) confirmed this unique phenomenon by noting an increase in enzyme production when L glutamine or glutamate was added to the fermentation medium. The purification method employed for the L-asparaginase derived from this fungal species yielded a protein consisting of a single peptide chain with a molecular weight of 47KDa. The enzyme exhibited optimal activity at 37°C and showed potential stability at elevated temperatures. The enzymatic activity reached its maximum level at a pH of 8. It is evident that the pH of the fungus species significantly affects the activity of L-asparaginase. This observation is corroborated by the prior study conducted by (40). Additional testing is required to improve the fermentation technique used in batch culture for the goal of large-scale production of this enzyme. Each drug research and development process require the utilization of standardized techniques on a small scale which are subsequently examined and standardized for large-scale manufacturing. Furthermore, both in vitro and in vivo experiments, as well as clinical trials, are carried out. The ongoing endeavor and its result have the potential to be a noteworthy achievement in the medication development initiative.

Table 1: Effect of different Carbon sources utilization on L-asparaginase production by *Aspergillus quadrilineatus.*

| Carbon sources | Enzyme activity (IU/ml) |
|---|---|
| Sorbose | 0.11±0.00 |
| Xylose | 0.22±0.03 |
| Galactose | 0.71±0.01 |
| Lactose | 0.72±0.03 |
| Arabinose | 0.55±0.01 |
| Maltose | 1.22±0.03 |
| Glucose | 0.89±0.04 |
| Raffinose | 0.40±0.01 |
| Starch | 0.51±0.00 |
| Inositol | 0.33±0.00 |
| Mannitol | 0.68±0.06 |
| Fructose | 0.211±0.07 |

Table 2: Effect of different Nitrogen sources utilization on L-asparaginase production by *Aspergillus quadrilineatus.*

| Nitrogen Sources | Enzyme activity (IU/ml) |
|---|---|
| Ammonium Sulphate | 1.52±0.01 |
| Ammonium Fluoride | 0.11±0.00 |
| Ammonium Persulphate | 0.21±0.03 |
| Ammonium Chloride | 0.22±0.04 |
| Sodium Nitrate | 0.98±0.06 |

| | |
|---|---|
| Ammonium Nitrate | 1.01±0.10 |
| urea | 0.13±0.00 |
| potassium Nitrate | 1.60±0.11 |

Table 3: Effect of different Ionic salts utilization on L-asparaginase production by *Aspergillus quadrilineatus.*

| **Ionic salts** | **Enzyme activity (IU/ml)** |
|---|---|
| Vitamin-c | 1.96±0.06 |
| Vitamin-B1 | 2.10±0.03 |
| Vitamin-B6 | 1.80±0.00 |
| Vitamin-B2 | 0.51±0.01 |
| Vitamin-M | 0.52±0.00 |
| Pipermint oil | 1.80±0.00 |
| Olive oil | 1.68±0.17 |
| Tween-20 | 1.64±0.00 |
| Tween-40 | 1.80±0.01 |
| Tween-80 | 1.52±0.00 |
| Calcium Phosphate | 1.96±0.00 |
| Disodium hydrogen ortho-phosphate | 1.52±0.00 |
| Sodium phosphate | 2.10±0.11 |
| Glycine | 2.12±0.15 |
| Ferric chloride | 1.44±0.10 |
| Zinc sulphate | 1.86±0.08 |
| Potassium Nitrate | 1.80±0.00 |
| Sodium Acetate | 2.12±0.00 |
| Ferrous sulphate | 1.64±0.00 |
| Ammonium sulphate | 1.64±0.00 |
| Sodium chloride | 1.78±0.03 |
| Sodium Trisulphate | 1.52±0.00 |
| Sodium Thiosulphate | 1.64±0.00 |
| Tricalcium phosphate | 1.56±0.11 |
| Calcium chloride | 1.64±0.00 |
| Sodium Nitrate | 1.64±0.00 |
| Potassium Acetate | 1.58±0.08 |

Table 4: Effect of different amino acids utilization on L-asparaginase production by *Aspergillus quadrilineatus.*

| **Amino acids** | **Enzyme activity (IU/ml)** |
|---|---|
| Threonine | 1.64±0.00 |
| Tryptophan | 1.94±0.03 |
| Proline | 0.45±0.00 |
| Aspartic acid | 1.58±0.14 |
| Phenylalanine | 1.76±0.06 |
| Cysteine | 0.20±0.01 |
| Methionine | 0.31±0.02 |
| Tyrosine | 0.44±0.02 |
| Arginine | 1.58±0.08 |
| Histidine | 0.43±0.02 |
| Citric acid | 2.46±0.31 |
| Oxalic acid | 0.52±0.01 |
| Yeast Extract | 2.04±0.06 |

Table 5: Effect of different pH on L-asparaginase production by *Aspergillus quadrilineatus.*

| Carbon and Nitrogen sources (%) | Enzyme activity IU/ml (pH 4.5) | Enzyme activity IU/ml (pH 5.5) | Enzyme activity IU/ml (pH 6.5) |
|---|---|---|---|
| Maltose 2.0 + Potassium Nitrate 1.0 | 0.32±0.00 | 0.33±0.00 | 1.52±0.00 |
| Maltose 2.0 + Potassium Nitrate 2.0 | 0.35±0.01 | 0.35±0.01 | 0.52±0.02 |
| Maltose 2.0 + Potassium Nitrate 3.0 | 0.35±0.02 | 0.36±0.01 | 1.36±0.00 |
| Maltose 4.0 + Potassium Nitrate 1.0 | 0.37±0.00 | 0.37±0.00 | 0.49±0.01 |
| Maltose 4.0 + Potassium Nitrate 2.0 | 0.37±0.00 | 0.38±0.00 | 0.52±0.00 |
| Maltose 4.0 + Potassium Nitrate 3.0 | 0.36±0.00 | 0.40±0.00 | 1.24±0.17 |
| Maltose 6.0 + Potassium Nitrate 1.0 | 0.37±0.00 | 1.56±0.11 | 2.50± 0.14 |
| Maltose 6.0 + Potassium Nitrate 2.0 | 0.37±0.000 | 0.49±0.000 | 1.98±0.14142 |
| Maltose 6.0 + Potassium Nitrate 3.0 | 0.43±0.00849 | 0.49±0.01139 | 1.76±0.05657 |

Table 6: Purification profile of L-asparaginase from *Aspergillus quadrilineatus.*

| Steps | Collected volume(ml) | Total activity (IU) | Total protein (mg) | Specific activity (IU/mg) | purification fold | Yield (%) |
|---|---|---|---|---|---|---|
| culture filtrate | 4.451 litres | 1.16 | 100 | 0.0116 | 0 | 100 |
| Ammonium sulfate precipitation | 100 ml | 1.92 | 520 | 0.0036 | 0.3103 | 165.517 |
| Gel filtration | 420 ml | 2.56 | 660 | 0.0038 | 0.3275 | 220.68 |
| Ion exchange chromatography | 6660 ml | 2.72 | 720 | 0.0037 | 0.3189 | 234.48 |
| Ammonium sulfate precipitation | 200 ml | 2.72 | 940 | 0.0028 | 0.2413 | 234.48 |

Table 7: Effect of pH, Temperature, and substrate on partially purified L-asparaginase.

| Parameter | Enzyme activity (IU/ml) |
|---|---|
| Temperature Effect | |
| 30 | 1.06 |
| 37 | 2.78 |
| 50 | 0.78 |
| Incubation Period Effect | |
| 10 min | 1.92 |
| 20 min | 3.25 |
| 30 min | 0.81 |
| 40 min | 0.30 |
| Effect of PH | |
| 3 | 0.70 |
| 4 | 0.80 |
| 5 | 0.83 |
| 6 | 0.90 |
| 7 | 1.62 |
| 8 | 2.94 |
| 9 | 0.4 |
| 10 | 0.1 |
| Effect of Substrate Specificity | |
| L-arginine | 1.65 |
| L-phenylalanine | 1.06 |
| L-Histidine | 2.3 |
| L-Glutamine | 0.73 |
| L-aspartic | 2.23 |
| L-asparagine | 3.11 |

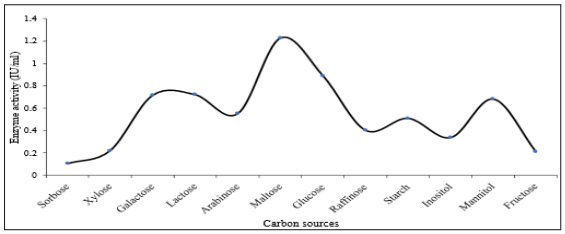



Fig.1: L-asparaginase activity of Do-11 with different carbon sources (1%) 1- Sorbose, 2-Xylose, 3- Galactose, 4-Lactose, 5- Arabinose, 6-Maltose, 7- Glucose, 8- Raffinose, 9- Starch, 10- Inositol, 11- Mannitol, 12- Fructose.

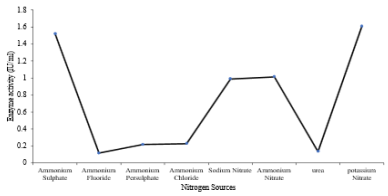



Fig.2: L-asparaginase activity of Do-11 with different Nitrogen sources (1%) 1-Ammonium sulphate, 2-Ammonium fluoride, 3-Ammonium persulphate, 4-Ammonium chloride, 5-Sodium Nitrate, 6- Ammonium Nitrate, 7-Urea, 8- potassium Nitrate

Current Trends in Biotechnology and Pharmacy
Vol. 20(4) 3248-3262 October 2026, ISSN 0973-8916 (Print), 2230-7303 (Online)
DOI: 10.5530/ctbp.2026.4.42

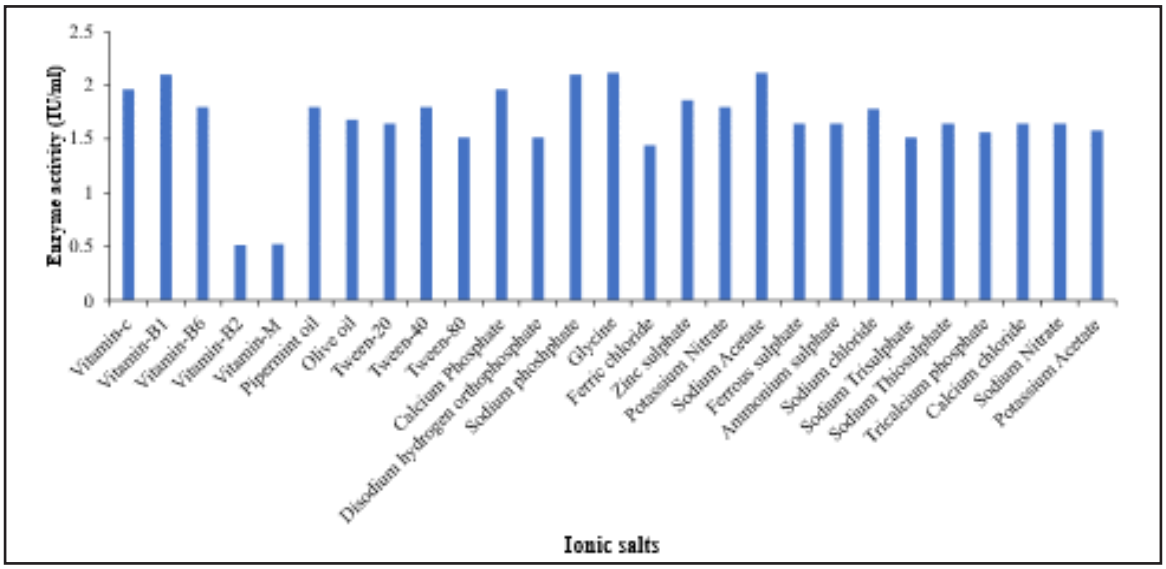




Fig.3: L-asparaginaseactivityofDo-11withdifferent Ionicsalts (0.5%)1-Vitamin-c, 2-Vitamin-B1, 3-Vitamin-B6, 4 Vitamin-B2,5-Vitamin-M,6-pipermintoil,7-oliveoil,8-Tween-20,9-Tween-40,10-Tween-80,11-Calciumphosphate, 12-Disodiumhydrogenorthophosphate, 13-Sodiumphosphate, 14-glycine, 15-Ferric chloride, 16-Zinc sulphate, 17 PotassiumNitrate, 18-SodiumNitrate, 19-Ferrous sulphate, 20-ammoniumsulphate, 21-Sodiumchloride, 22-Sodium Trisulphate, 23-SodiumThiosulphate, 24-Tricalciumphosphate, 25-Calciumchloride, 26-SodiumNitrate, 27-Potassium Acetate.

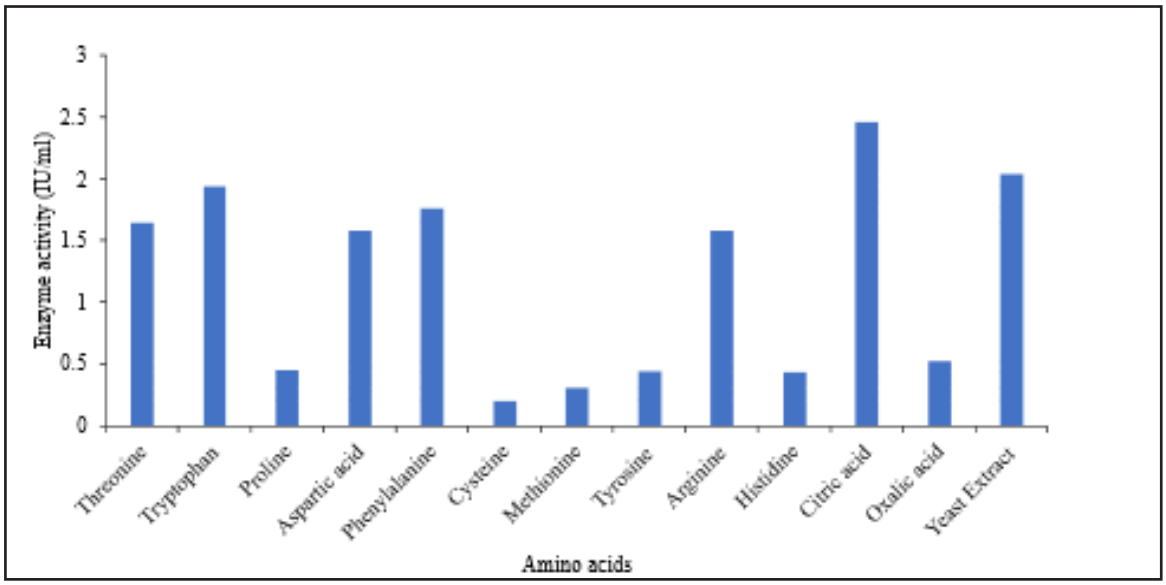



Fig.4: L-asparaginaseactivityofDo-11withdifferentAminoacids (0.5%)1-Threonine,2-Tryptophan,3 Proline, 4-Aspartic acid, 5-Phenyl alanine, 6-Cysteine, 7-Methionine, 8-Tyrosine, 9-Arginine, 10 Histidine,11-Citricacid,12-Oxalicacid,13-Yeastextract.

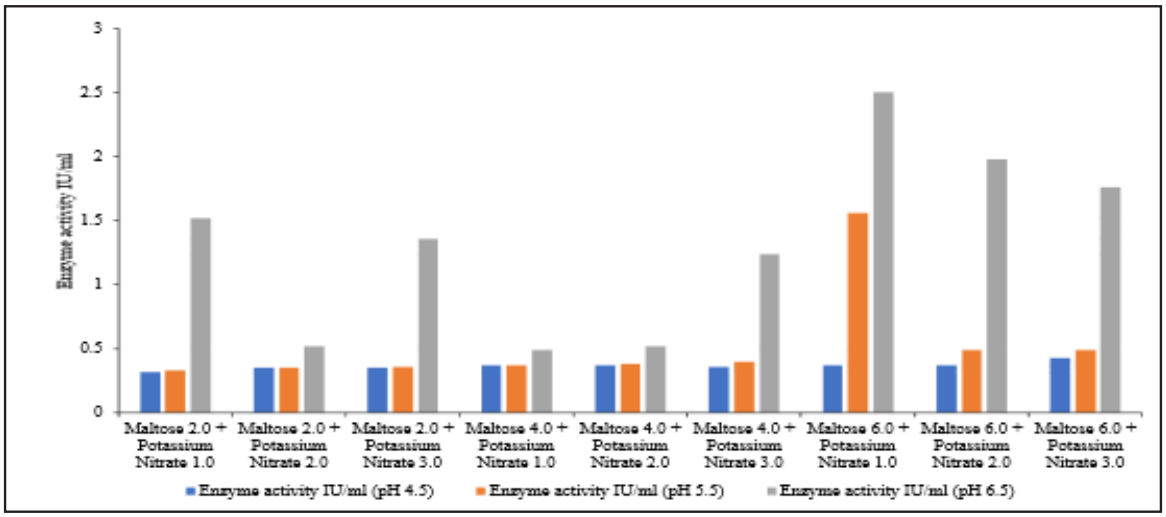



Fig.5: L-asparaginase activity of Do-11 with different initial pH values on L-asparaginase production.

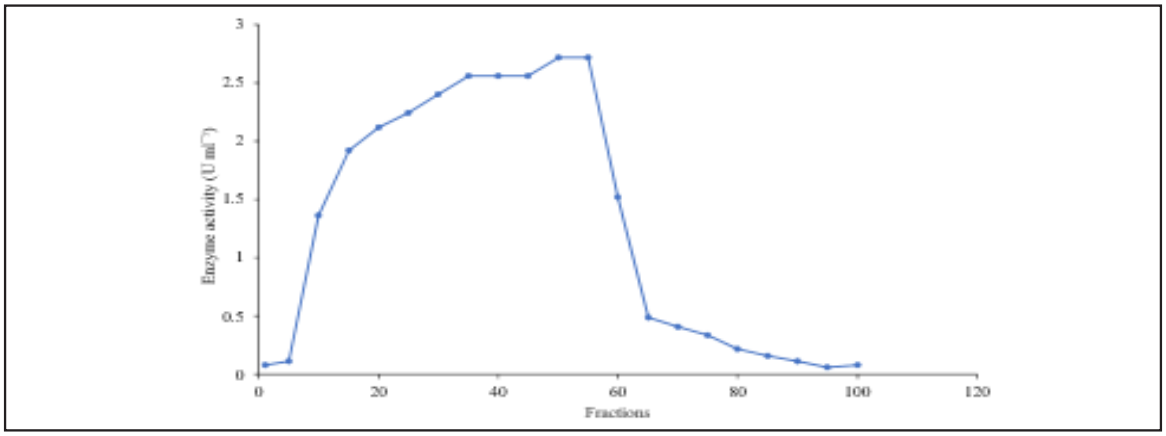



Fig.6 Purification of L-asparaginase from the Aspergillus quadrilineatus Gel filtration columnchromatography(SephadexG-100)ofthefractionscollectedfromammoniumsulfateprecipitationfractions.

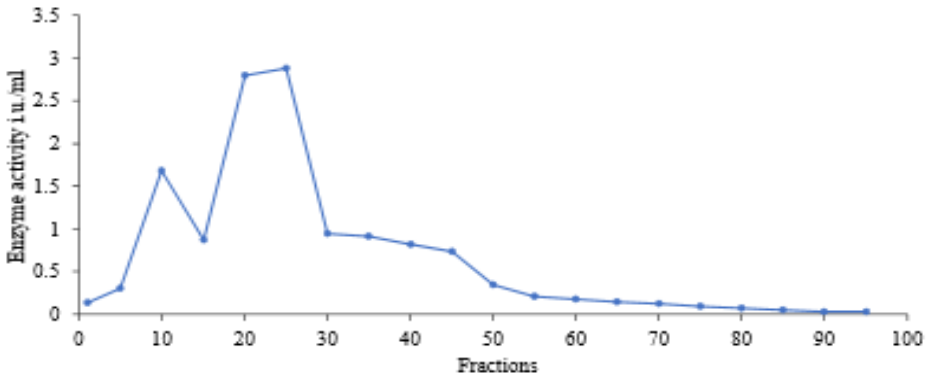



Fig.7 Purification of L-asparaginase from theAspergillusquadrilineatusion exchange column chromatography fractions.

## Conclusion

The microbial-derived L-asparaginase is highly valued due to its importance in the pharmaceutical and food sectors. Thus, in the current investigation, L-asparaginase activity was achieved using the Imada et al assay method. The powerful isolates underwent optimization investigations by submerged fermentation. A comprehensive analysis was conducted to optimize manufacturing factors including carbon, nitrogen, incubation period, amino acids, temperature, pH, and ionic salts. The use of asparagine agar medium is advantageous for achieving optimal enzyme activity. Consequently, the remaining fermentation parameters were conducted using the identical media. According to the experimental data, maltose (0.5%) was determined to be the most efficient carbon source while L-asparagine (0.5%) was identified as the most effective nitrogen source. The concentration of vitamin was 1%, while the concentrations of sodium acetate, citric acid, Tryptophan, and sodium dihydrogen phosphate were also 1% each. The study results showed that the optimal conditions for enzyme production were a pH of 8.0 and a temperature of 37°C. The L-Asparaginase was further purified using gel filtration chromatography with a Sephadex G-100 column. The final overall yield recovery was 165.517%, and the purification resulted in a fold increase of 0.3189. Research on the physical factors affecting the effectiveness of pure L-Asparaginase has shown that it remains functional throughout a wide pH range of 8 and at a temperature of 37ºC. The enzyme demonstrates a high level of selectivity towards its natural substrate, L-asparagine and demonstrates a high level of stability at a pH of 7. The molecular weight of L-Asparaginase was determined to be 47 kilodaltons (kDa) using SDS PAGE analysis.

## Acknowledgment

We express our gratitude for the financial assistance provided by the Forest, Environment, and Climate Change Department of the Government of Odisha, as part of the State plan for the year 2023–24. The authors would like to thank the chief executive of the Regional Plant Resource Centre in Bhubaneswar, Odisha for providing the necessary laboratory and administrative resources for this research endeavor.

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