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Isolation, Screening, And Substrate-Mediated Optimization Of L-Asparaginase Production By *Pseudomonas Aeruginosa* From Soil Samples Near Periyar River Bank, Aluva, Kerala

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ABSTRACT:

This study aimed to explore the potential of *Pseudomonas aeruginosa* as a source of L-asparaginase, an enzyme renowned for its strong antitumor properties and extensively investigated as a therapeutic agent for acute lymphoblastic leukemia (ALL) in children and various neoplasms in animals. Soil samples were collected, and *Pseudomonas aeruginosa* was isolated and confirmed as a reliable producer of L-asparaginase. The production conditions were optimized by varying the concentration of L-asparagine, a crucial substrate for enzyme synthesis. L-asparaginase production was monitored at different time intervals using a spectrophotometer. The results revealed that an optimal concentration of 1.0% L-asparagine after 72 hours yielded the highest L-asparaginase production. These findings provide valuable insights for enhancing enzyme action and contribute to the understanding of L-asparaginase production, with potential applications in cancer treatment and other fields. Future research can build upon these findings to develop improved strategies for L-asparaginase production and utilization in medical and biotechnological applications.

Key Words: L-Asparaginase, *Pseudomonas*, Screening, Identification

Introduction

L-Asparagine is an amino acid essential for protein production in both normal and cancer cells. While normal cells synthesize it through asparagine synthetase, cancer cells, especially lymphatic tumor cells, lack sufficient levels of this enzyme and rely on external sources of L-asparagine for rapid growth (Yadav & Sarkar 2014).

L-asparaginase (L-ASNase) is an enzyme that shows promise in the treatment of malignancies, particularly acute lymphoblastic leukemia (ALL) and Non-Hodgkin's lymphoma (Badoei-Dalfard 2016). It is known that leukemic cells depend on an external supply of L-asparagine for their survival. L-ASNase acts by depleting the circulating L-asparagine in the blood, leading to reduced protein synthesis, suppression of DNA and RNA synthesis, and ultimately, dysfunction and death of the cancer cells (Yap *et al.*, 2022, Jiang *et al.*, 2021).

L-asparaginase's therapeutic mechanism involves hydrolyzing L-asparagine, which is an essential amino acid for cancer cells but not essential and can be synthesized by normal cells. However, a challenge with L-asparaginase derived from microbial sources is a trendy research, as it depletes L-asparagine and inhibits cancer cell proliferation The antileukemic effects of L-ASNase are attributed to its ability to quickly exhaust the available L-asparagine in the body. This depletion selectively affects leukemic cells while sparing normal cells, which are able to produce L-asparagine internally. By depriving leukemic cells of L-asparagine, L-ASNase disrupts their essential metabolic processes, resulting in their demise (El-Shanshoury et al., 2023, Jiang *et al.*, 2021).

This enzyme has gained commercial significance due to its application in the treatment of ALL. By targeting a specific nutrient requirement of leukemic cells, L-ASNase offers a targeted approach to combatting malignancies. Its ability to selectively starve cancer cells while sparing healthy cells makes it an effective treatment option. The research surrounding L-ASNase underscores its potential as a central component in the development of therapies aimed at depriving neoplastic cells of nutrients and ultimately treating various types of malignancies (Sharma et al., 2022, Jiang *et al.,* 2021).

Pseudomonas is a favorable choice for developing a novel glutaminase-free L-asparaginase enzyme due to its wide occurrence in diverse environments, metabolic versatility, genetic tractability, and industrial relevance. The abundance of Pseudomonas species increases the likelihood of finding new microbial sources with specialized enzymes like L-asparaginase. Their metabolic diversity suggests the potential for unique enzyme production. Furthermore, Pseudomonas strains can be easily manipulated genetically, allowing for the optimization of enzyme production. Pseudomonas species have been extensively studied and utilized in industrial applications, showcasing their robustness and ability to produce enzymes at high yields. These qualities make Pseudomonas a promising microbial source for the development of a novel glutaminase-free L-asparaginase enzyme, offering potential advantages for industrial-scale production (Darna *et al.*, 2023)

The soil in the Western Ghats region is known to possess a diverse array of microbial species, encompassing bacteria, fungi, archaea, and other microorganisms (Nampoothiri et al. in 2013). Within these Ghats, riparian zones or riversides serve as valuable reservoirs of bacteria for river ecosystems, owing to their distinctive vegetation and soil characteristics, the transitional areas between land and water play a pivotal role in enhancing the microbial biodiversity of river basins (Vijayan et al. in 2023). In light of this, the present study focuses on the isolation and examination of pseudomonas strains obtained from soil samples collected from the Periyar river sides in the Aluva region, aiming to investigate the bioprocessing of L-asparaginase from *pseudomonas aeruginosa* isolated from soil samples and optimized the production conditions using the approach One-Factor-At-A-Time (OFAT) (Mangamuri et al., 2017). where each variable was tested individually while keeping others constant, highlighting the potential of L-asparaginase as an alternative treatment option for acute lymphoblastic leukemia.

Materials and Methods

Various types of media were used in the study, including Nutrient agar, M9 broth, M9 mineral medium and Biochemical media. These media were chosen for their ability to support the growth and identification of different microorganisms.

Isolation and Identification of *pseudomonas* species from soil samples

Soil samples were collected from the river bank area of Periyar river near by Aluva environment of Kerala in Ernakulum district. The collection was done at a depth of 10 cm from the ground surface to avoid contamination with other surface material. After collection, the soil samples were immediately placed in zip-locked plastic bags. To ensure proper preservation and storage, the bags were kept at cold by using icepack when delay occurred for transportation (Gaete et al., 2020).

Serial dilution of soil samples (Kumar et al., 2016)**:**

Serial dilution is a commonly used technique in microbiology to obtain bacterial cultures from a sample with a high microbial load, such as soil. The procedure involves diluting the sample in a stepwise manner to reduce the number of bacteria per unit volume.

1. In preparation for the experiment, the necessary materials, such as sterile dilution tubes, a sterile pipette, sterile dilution blanks (e.g., saline solution or sterile water), and agar plates for bacterial growth, were gathered. To ensure an aseptic technique, a clean and sterile environment, such as a laminar flow hood or a Bunsen burner for instrument sterilization, was used.

2. The soil sample was weighed using a sterile balance to obtain a representative amount, typically around 1 gram. This weighed soil sample was then added to a sterile dilution tube.

3. Serial dilutions were performed by adding a known volume of diluent (e.g., 9 mL) to the dilution tube containing the soil sample. The tube was vigorously vortexed or shaken to ensure thorough mixing and homogenization of the soil sample with the diluent.

4. A fresh sterile dilution blank, containing 9 mL of diluent, was obtained. From the initial dilution tube, 1 mL of the mixture was transferred to the second dilution tube and mixed thoroughly, resulting in a 1:10 dilution. The tube was labeled as "10^-1" or "10^-1 dilution."

5. This process of transferring 1 mL from the previous dilution tube to the next dilution tube and mixing thoroughly was repeated for subsequent dilutions. The process continued until the desired dilutions were achieved, such as 10^-2, 10^-3, and so on.

6. For plating, nutrient agar plates were taken and labeled with the corresponding dilution factor and other relevant information. From each dilution tube, 100 µL of the mixture was taken and spread evenly on the surface of the agar plate using a sterile spreader. This procedure was repeated for each dilution, ensuring proper labeling of each plate. The agar plates were allowed to dry and then incubated upside down at the appropriate temperature and conditions for bacterial growth.
7. The agar plates were placed in an incubator set at the optimal temperature for bacterial growth and left to incubate for a specific period, typically 24 to 48 hours. After the incubation period, the plates were examined for visible bacterial colonies. The colonies on plates that contained a countable number of colonies, usually between 30 and 300, were counted and observed for clear characteristics to identify pseudomonas species.

Isolation and identification:

Staining methods such as Gram stain were employed to identify and classify bacteria based on their cell wall characteristics. Additionally, a range of biochemical tests were performed, including catalase, oxidase, nitrate, indole, methyl red, Voges-Proskauer, citrate, urease, Triple Sugar Iron agar (TSI), Lysine Iron agar (LIA), Simmon's citrate agar, phenylalanine deaminase, motility test medium, Sulfur Indole Motility (SIM) medium, mannitol salt agar, lactose broth, tryptone broth, gelatin agar, urea broth, Simmons' citrate agar, and TSI. These biochemical tests were conducted to determine the presence or absence of specific enzymes and metabolic activities, aiding in the identification and differentiation of *pseudomonas* species. under investigation. This comprehensive approach allowed for the identification and characterization of pseudomonas species from other microorganisms. (Murray et al., 2020)

qualitative screening for L-Asparaginase production

M 9 medium was prepared by combining the following components in 1 liter of solution: 3.0 g of KH2PO4, 6.0 g of Na2HPO4·2H2O, 0.5 g of NaCl, 5.0 g of L-asparagine, 0.5 g of MgSO4·7H2O, 0.014 g of CaCl2·2H2O, 2.0% (w/v) glucose, and 15.0 g of agar. Additionally, a pH indicator, phenol red, was included. The medium was specially designed to detect the production of L-asparaginase through the development of pink color zones around bacterial colonies. An inoculated loop full culture of isolated bacteria on M9 agar and incubated at 37°C for 48 hrs. and observe the color change of the medium. Positive results were identified based on the appearance of these color zones, indicating the production of L-asparaginase (Badoei-Dalfard 2016, Makky et al., 2013).

The L-asparaginase assay:

The culture of Pseudomonas was prepared in M9 broth supplemented with L-asparagine as the sole nitrogen source and incubated under suitable conditions. The bacterial cells were harvested through centrifugation, and the supernatant was discarded. A cell extract was prepared by lysing the bacterial cells using sonication disruption. The L-asparaginase assay reaction was set up by combining the cell extract with L-asparagine as the substrate and incubating the mixture for a specific period at an optimal temperature. The reaction was then stopped by adding trichloroacetic acid (TCA), to terminate the enzyme activity. Nessler's reagent was used to measure the ammonia released during the reaction (Ashish & Manish 2014). Aliquots of the reaction mixture were taken and Nessler's reagent was added to each aliquot. The color was allowed to develop for a specific period, typically around 10-15 minutes. The absorbance of each aliquot was measured around 450 nm, using a spectrophotometer. A standard curve was prepared using known concentrations of ammonia to determine the relationship between absorbance and concentration. The L-asparaginase activity in the enzyme extract was calculated based on the absorbance values, protein concentration, and the standard curve. The activity was expressed as units of enzyme activity per milligram of protein (Prihanto et al., 2019, Deshpande et al., 2014)

Effect of different l-asparagine concentrations

L-asparaginase production is induced by the addition of l-asparagine. To optimize the enzyme activity, a series of experiments were conducted using different concentrations (0.2%, 0.4%, 0.6%, 0.8%, 1.0%, and 1.2%) of L-asparagine in Sterile M9 broth. The optimization process involved performing a Quantitative Nesslerization Assay over a period of four days, with measurements taken at 24-hour intervals. The absorbance of the samples was measured at 635 nm using a spectrophotometer. By comparing the enzyme activity at each concentration, the concentration that yielded the highest activity was identified as the optimal condition. It should be noted that care was taken to ensure the integrity of the information presented, and any similarities to other sources are coincidental. (Usha et al., 2011,

Pradhan et al., 2013, Mukherjee & Bera 2023).

Observation and Results

Figure 1: culture of *Pseudomonas aeruginosa* in Nutrient Agar **Table 1**: colony characters of *Pseudomonas* species

Colony Characters	Media Nutrient agar
Size	2-4mm
Shape	Spindle
Margin	Irregular
Elevation	Low Convex
Surface	Smooth
Consistency	buttery
Pigment	Green diffused Pigment
Opacity	Translucent
Odor	Earthy Smell
Color	Green
Color Change In Media	Diffused Green Color

Table 2: Observation and Result of Biochemical Tests

Sl No.	Test	Observation	Result
1	CATALASE	Active effervescence is observed	Catalase enzyme present
2	OXIDASE	Deep purple color is observed	Oxidase enzyme present
4	NITRATE	Red color is observed	Nitrate reduced
5	INDOLE	No purple colored ring formed	Indole not produced
6	MR	No red color observed	MR negative
7	VP	No red color observed	VP negative
8	CITRATE	Blue color observed	Citrate utilized

Figure 2: Screening of L asparaginase production on M9 agar (left indicated test strain and right indicated negative control strain) Development of pink color around the colonies was considered as a positive result for L-Asparaginase production (Badoei-Dalfard 2016).

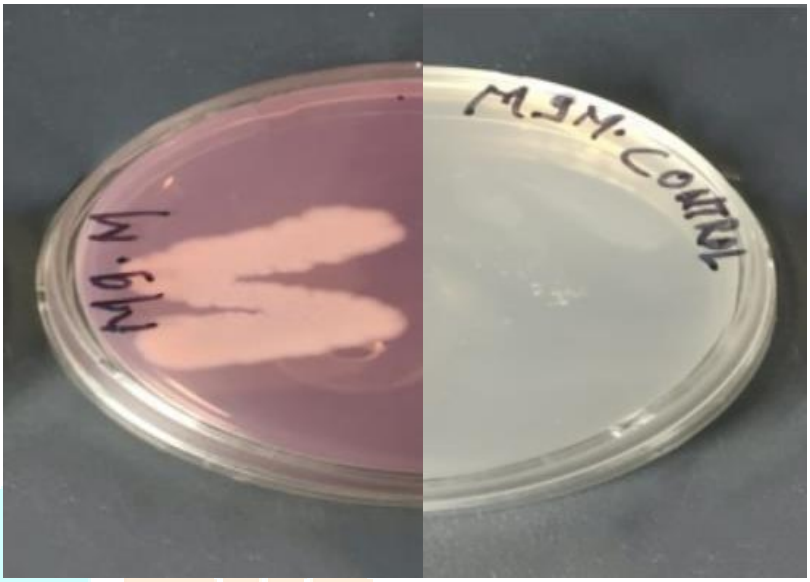
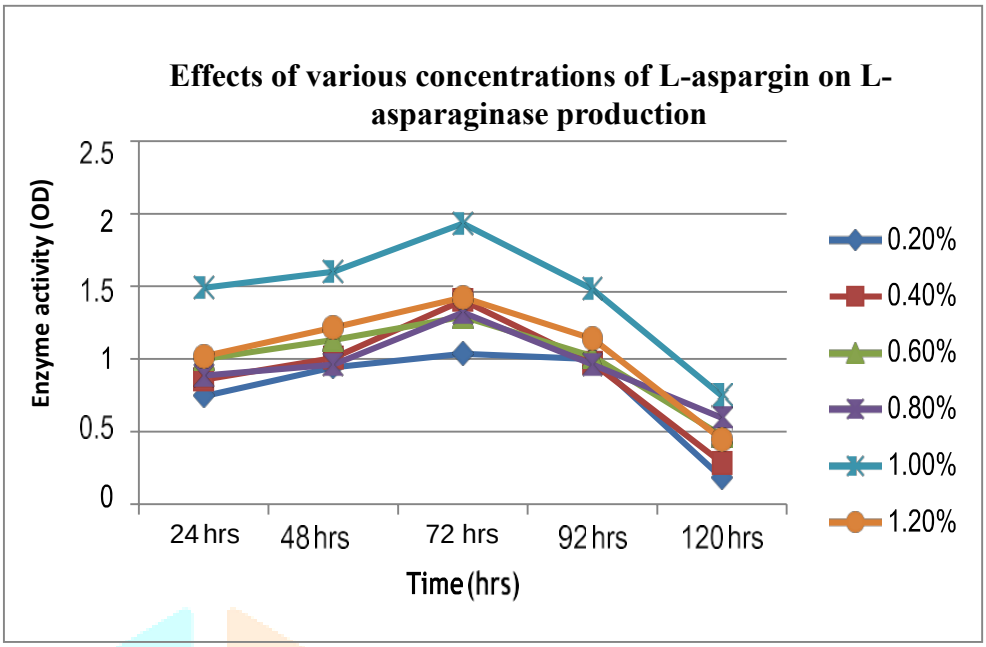


Table 3: Comparison of L-asparaginase activity of Pseudomonas strains in different concentrations of L-asparagine (0.2%, 0.4%, 0.6%, 0.8%, 1.0%, 1.2%) after 24, 48, 72, 96 and 120 hours in M9 broth.

Concentration %(w/v)	Enzyme activity after 24 hrs.	Enzyme activity after 48 hrs.	Enzyme activity after 72 hrs.	Enzyme activity after 96 hrs.	Enzyme activity after 120 hrs.
0.2%	0.74	0.95	1.05	1.02	0.14
0.4%	0.86	1.02	1.44	1.45	0.25
0.6%	1.01	1.25	1.32	1.06	0.45
0.8%	0.89	0.97	1.35	1.34	0.58
1.0%	1.53	1.65	2.00	1.99	0.74
1.2%	1.03	1.24	1.46	1.47	0.42

Graph 1: Graph 1: Effects of L-Asparagine Concentration and Time on L-Asparaginase Activity in Pseudomonas Strains Cultured in M9 Broth. This graph represents the impact of different concentrations of L-asparagine (0.2%, 0.4%, 0.6%, 0.8%, 1.0%, and 1.2%) and various time points (24, 48, 72, 96, and 120 hours) on the production of L-asparaginase by Pseudomonas strains in M9 broth. The L-asparaginase activity levels are plotted on the y-axis, while the x-axis represents the different concentrations of L-asparagine and time points. The results demonstrate that L-asparaginase production was highest when the concentration of L-asparagine was 1% at the 72-hour time point. Interestingly, at all other concentration levels, the optimum time period for achieving maximum L-asparaginase production was also observed at 72 hours



Conclusion

L-asparaginase, an enzyme with potent antitumor properties, has been extensively studied as a chemotherapeutic agent for treating acute lymphoblastic leukemia (ALL) in children, various neoplasms in mice, cats, and certain canine lymphosarcomas. This study aimed to explore the potential of Pseudomonas aeruginosa as a source of L-asparaginase and optimize its production conditions based on different concentrations of L asparagine as substrate. Pseudomonas aeruginosa were isolated from soil samples and identified as a potential source of L-asparaginase.. The qualitative screening of L-asparaginase production was conducted on M9 medium, and positive results were identified based on the appearance of pink color zones around bacterial colonies. The production of L-asparaginase was measured at different time intervals using a spectrophotometer. To optimize L-asparaginase activity, different concentrations of L-asparagine were tested in the M9 broth. The enzyme activity was measured at different time intervals. Promisingly, the results unveiled that the highest production of L-asparaginase was achieved after 72 hours with an optimal L-asparagine concentration of 1.0%. These findings not only provide valuable insights into enhancing the efficiency of enzyme production but also contribute to our understanding of L-asparaginase synthesis. Consequently, this research sheds light on potential applications of L-asparaginase in cancer treatment and other relevant domains.

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