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BIOCHEMICAL CHARACTERIZATION, ANTI-PROLIFERATIVE AND CYTOTOXICITY EFFECT OF PURIFIED L-ASPARAGINASE, AN ANTI-LEUKEMIA ENZYME ISOLATED FROM NEW BACTERIA *Myroides Gitamensis*

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ABSTRACT

L-asparaginase (L-asp) is used in the treatment of acute lymphocytic leukemia (ALL). L-asp catalyzes the L-asp into ammonia and L-aspartic acid through deamination. The formed ammonia finds to be toxic to the organism. Therefore, the search for less cytotoxic anti-proliferative L-asp is of great importance. Because of its cost-effectiveness, consistency, and easiness of process control, microbial L-asparaginase is chosen over plant or animal enzymes. L-asp enzyme which was studied in the article was isolated from a newly discovered bacteria *Myroides gitamensis*. The biochemical characteristics and antiproliferative, cytotoxic effect of purified L-asp were studied in order to develop safer biochemically stable anti-leukemia medication. To provide comprehensive information on the characteristics of the isolated L-asparaginase enzyme analytical tools were utilized, including cancer cell lines, brine shrimps, enzyme reactions etc. MOLT-4 and K-562 cell lines showed an IC50 value of 91.41 IU/mL and 77.42 IU/mL, respectively. Cytotoxicity activity of L-asp on *Artemia salina* showed that at 24 hours (LC50 value of 217.0 µg/mL) and toxic at 48 hours (LC50 value of 126.4µg/ml).

Keywords: Enzyme, L-asparaginase, Cytotoxic Activity, Leukemia, Biochemical Characterization, Anti-proliferative Effect

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INTRODUCTION

Humanity every day faces serious problems associated with various diseases, and the search for the best remedies/drugs that can solve these problems is endless. The drugs/agents currently in use have many disadvantages. Many of them cause unwanted reactions in the body.

For example, several different treatments are available to treat human cancer. Surgery, radiation, drugs, or a combination of both, may be used. Cytotoxic drug therapy, known as chemotherapy, is the most critical component of treatment for many forms of cancer. Chemical substances are employed to inhibit DNA formation and transcription in cancer cells, which often results in apoptosis.

However, chemotherapy has a non-specific effect, and there is every chance of interfering with other metabolic pathways. In addition, cancer cells tend to develop resistance properties to chemotherapy, which causes severe problems in the treatment's later stages. As a result, there is an urgent need to create new safe anti-cancer medications or to change existing therapy approaches.[1] Enzyme is one of these drugs

that exhibit less toxic effects compared to chemotherapy. Enzymes are more specific and act in a targeted manner, especially L-asparaginase (L-ASP). Since the 1950s L-ASP is used in the treatment of acute lymphoblastic leukemia (ALL) and has become a critical enzyme for treating patients (especially children). L-ASP, isolated from various sources, is commonly used in combination with other chemotherapeutic agents to treat ALL.[2] Bacterial enzymes are currently used to treat certain types of blood cancers. To date, the most active L-ASP is obtained from the bacteria *E. coli* and *Erwinia carotovora*. Nowadays they are actively used for the treatment of ALL and lymphosarcoma leukemia.[3]
However, the commercially available L-ASP has some side effects. For example, it can lead to diabetes, anaphylaxis, pancreatitis, low cholesterol, leukopenia, and blood clotting disorders that can cause bleeding.[4] These side effects are thought to be due to allergic reactions. Because the body recognizes these bacterial enzymes as foreign, they trigger complicating immune responses. The use of L-asp from various sources could solve this challenge.[5] In this regard, attempts are being made to find L-asp from new bacterial sources with less cytotoxic effect and more stable for the treatment of ALL.
Because of its cost-effectiveness, consistency, and easiness of process control, microbial L-asp is preferable compared to plant or animal L-asp.[6] Bacteria are easy to grow in any geographic location, easy to manipulate genetically and extract compounds, unlike plants. Bacterial L-ASP is relatively more stable than the corresponding plant-derived enzymes.
The L-ASP to be studied in this research was extracted from a new source of bacteria, *Myroides gitamensis*, which was discovered in the soil of a slaughterhouse in India by our co-authors at GITAM University. They discovered bacteria *Myroides gitamensis* is included in the National Center for Biotechnological Information (NCBI), as well as in various banks of microbial culture collections.[7,8]
Previously, L-asparaginase was isolated from novel bacterial species *Myroides gitamensis* from different soil samples of Visakhapatnam, India; the bacteria *Myroides gitamensis* was characterized from morphological, biochemical, phenotypic points of view; potentially low-cost production of L-asp by solid-state fermentation via response surface methodology (RSM) and purification was carried out.[9-11]
The goal of this research was to develop a less toxic biochemically stable anti-leukemia drug. For this purpose, the purified L-asp used was extracted from the *Myroides gitamensis* to study biochemical characteristics and the anti-proliferative, cytotoxic effect of L-asp. Thus, this research will develop improved treatments for blood cancers and assess the toxic effect of purified L-asp from new novel bacteria.

EXPERIMENTAL

In the process of work, to study the biochemical stability and activity of L-asp isolated from *Myroides gitamensis* bacteria, the effect of different parameters such as the influence of pH, temperature, various metal ions, surfactants, and chelators on the main characteristics of L-asp enzyme were studied; enzyme kinetics were studied using the enzymatic reactions, i.e. Michaelis-Menten constant (Km) and the maximum reaction rate (Vmax), using the Lineweaver-Berk graph by studying the effect of L-asparagine amino acid concentration (substrate) on L-asp activity. The antiproliferative activity of L-ASP enzyme was studied using two different cancer cell lines of human acute T-lymphoblastic leukemia (MOLT-4) and myelogenous leukemia (K-562); also, the lethal concentration (LC50) of L-asp was determined using *Artemia Salina* (Brine shrimp) to study the cytotoxic effect of L-asp enzyme. A detailed description of the methods used is given in the Supplementary Materials.

RESULTS AND DISCUSSION

Biochemical Characterization of L-asp

Determination of Optimum pH and Stability of the Purified L-asp

Figure 1 shows that the optimum activity of the enzyme was at pH 8.0. The enzyme showed stability over a pH range of 6 to 8 at both 3 and 5 h of the incubation period (Fig.-2). Similar results were observed by Krishnan and Chandra with an optimal pH of 8.0 for the L-asp produced by *Bacillus licheniformis* CUMC305.[12]

Optimum Temperature for the Purified Enzyme

Figure-3 shows the dependence activity of L-asp on temperature The L-asp exhibited maximum activity in the temperature range from 30°C to 50°C and an optimum activity was of 40°C.

The thermo-stability of the purified L-asp is presented in Fig.-4. The purified L-asp remained 100% of its activity at 40°C for 2-3 hrs. On incubation of enzyme at 60°C and 70°C, the activity was decreased to 50% and 40% respectively within 5 min and on further incubation, a significant decrease in activity was observed.

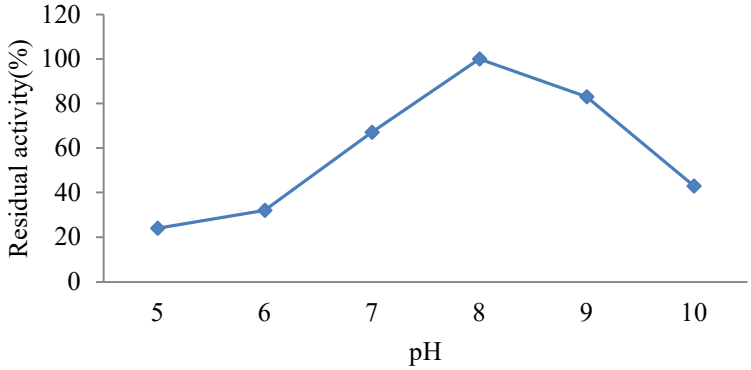

Fig.-1: Effect of pH on purified on the Activity of L-asp

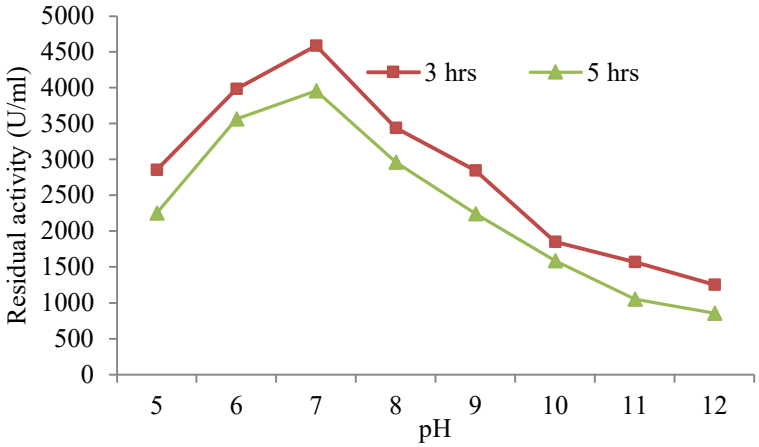

Fig.-2: Effect of pH Stability on L-asparaginase

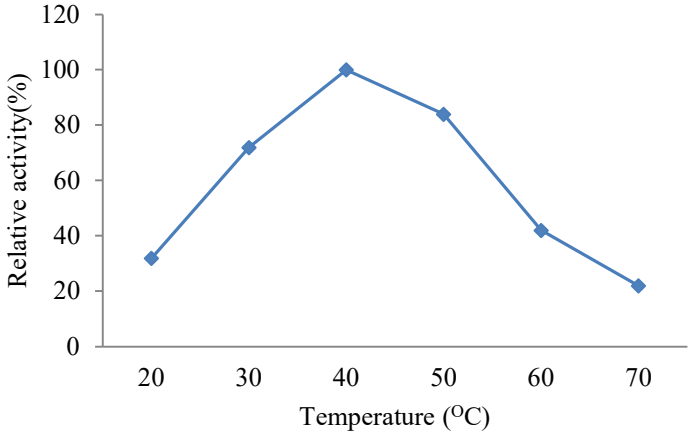

Fig.-3: Effect of Temperature on purified L-asparaginase

Metal ions Effect on the Purified L-asp

The influence of metal ions on the L-asp activity was depicted in Fig.-5. It was observed that the activity of L-asp was increased by divalent metal ions. Na^{+}, Ca^{+2} and Fe^{+2} by 38%, 26% and 24% respectively, whereas Hg^{+2},Mg^{+2}, Zn^{+2} inhibited the activity of L-asp by 68%, 48%, 26%, 24% and 6% respectively. A similar effect of ions of metal on the activity of L-asp from *Staphylococcus* MGM1 was reported.[13,14]

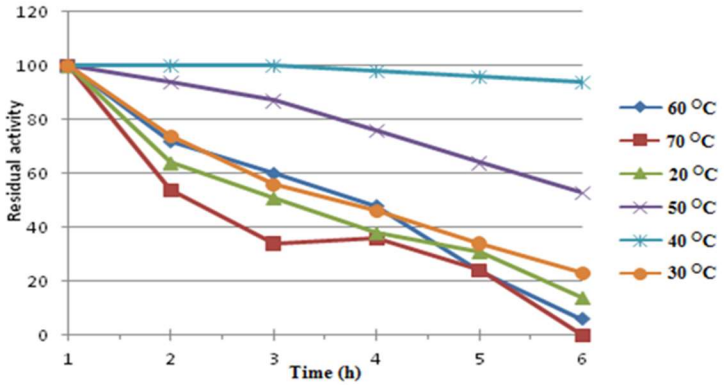

Fig.-4: Effect of Temperature on the Stability of purified L-asp

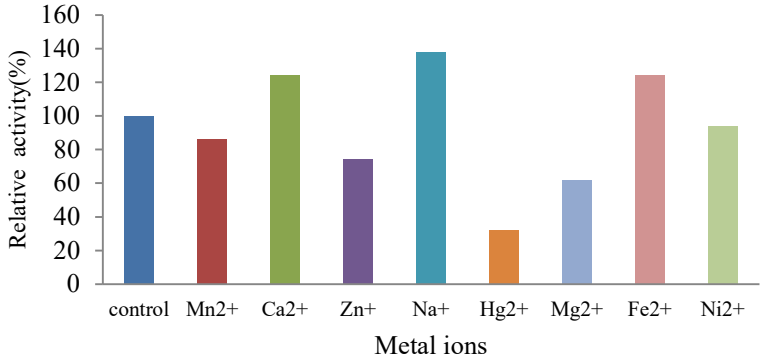

Fig.-5: Effect of Metal Ions on L-asp activity

The Chelators and Surfactants Effect on the Purified L-asp

The chelators' influence on the L-asp activity is shown in Fig.-6. L-asp activity was enhanced by Tween-20 and β-ME by 36% and 18% respectively. Whereas urea, EDTA, SDS has shown to decrease the L-asparaginase activity by 68%, 66% and 44% respectively. Authors reported similar results, where it was shown that EDTA and SDS repressed the production of L-asparaginase in *Fusarium brachygibbosum.*[15]

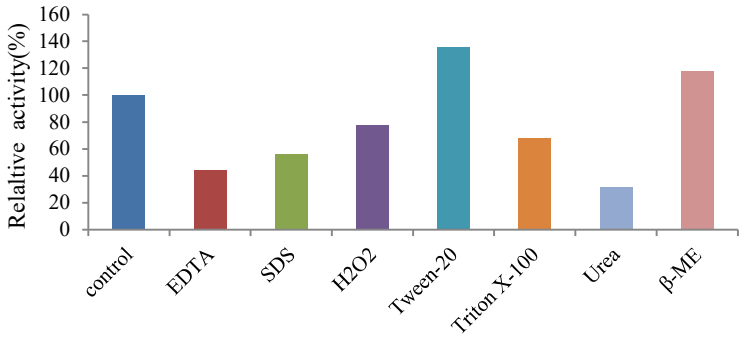

Fig.-6: Chelators and Surfactants Effect on L-asp Activity

Study of L-asp Enzyme Kinetics

Effect of Substrate Concentration on Purified L-asp

The effect of varying concentrations of L-asparagine on the velocity of L-asp demonstrated a typical hyperbolic saturation curve. The values of K_m and V_{max} of the L-asp from *Myroides gitamensis* BSH-3^T using L-asparagine as substrate were found to be 5.26 mg and 12.5 IU/mg respectively (Fig.-7). Senthil and Selvam reported that K_m and V_{max} of L-asp of *Streptomyces* sps were 0.0598 and 3.5478 IU/ µg respectively.[16]

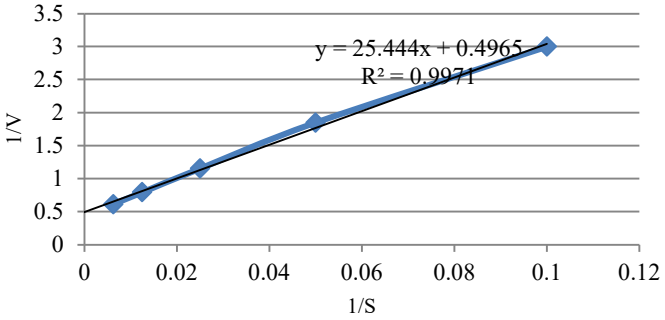

Fig.-7: Substrate Concentration Effect on L-asparaginase Activity to determine Km by Lineweaver-Burk

Anti-proliferating Activity of L-asparaginase from *Myroides gitamensis* BSH-3^T

Both MOLT-4 and K-562 cell growth inhibition increases with the increased dosage of L-asparaginase. MOLT-4 and K-562 cell lines demonstrated an IC50 value of 91.41 IU/mL and 77.42 IU/mL, respectively (Fig.-8). The growth inhibition rate of K-562 cells was slightly higher than MOLT-4 cells at the same dosage of the enzyme. At 100 IU/mL of L-ASP the growth of inhibition for MOLT-4 was 64.4% and for K-562 was 79.4%. These results show that L-asparaginase can be utilized as a potential anti-proliferative drug.

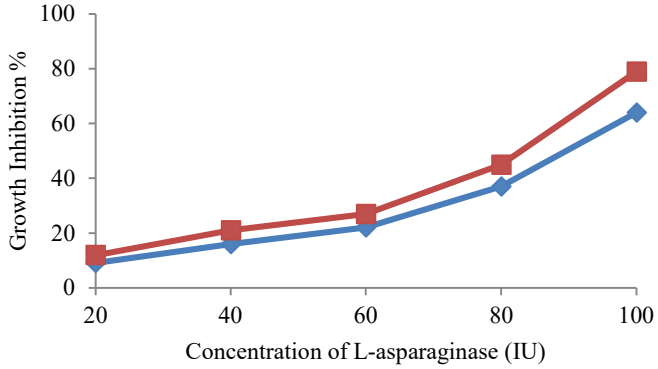

Fig.-8: L-asparaginase Effect on MOLT-4 (blue) and K-562 (red) Cell Lines

Cytotoxicity Activity of L-asparaginase purified from *Myroides gitamensis* BSH-3^T

The data presented in Table-1 shows that L-asparaginase has cytotoxicity on brine shrimp nauplii. L-asparaginase exhibited a cytotoxic effect when the concertation increases from 50µg/mL to 300µg/mL. LC50 value of L- asparaginase was 217.0µg/mL at 24 h is moderately cytotoxic, whereas, at 48 h, LC 50 is 126.5 µg/mL.

Table-1: Mortality Percentage of Brine shrimp naupilli at 24 hr and 48 hrs incubation with L-asparaginase

#	µg/mL	Mortality, %	
		24 h	48 h
1	50	6	24
2	100	13	31
3	150	18	45
4	200	27	64
5	250	52	81
6	300	93	93

The effect of cyclophosphosphamide (positive control) on the growth of brine shrimp nauplii at 24 and 48 h incubation is shown in Table-2. Cyclophosphamide is a cytostatic anti-tumor chemotherapy drug. The LC50 value of cyclophosphomide was 78.2µg/ml at 24 h and 69.8µg/ml at 48 h. It clearly shows that cyclophosphosphamide was more toxic than L-asparaginase at both incubation periods.

Thus, the experimental results show that the cytotoxicity activity of L-asparaginase on Brine shrimp (*Artemia salina*) shows that the enzyme was moderately toxic at 24 hours (LC 50 value of 217.0µg/mL) and toxic at 48 hours (LC 50 value of 126.4µg/ml).

Table-2: Mortality Percentage of Brine Shrimp naupilli at 24 hr and 48 hr Incubation with Cyclophosphamide

No.	µg/mL	Mortality, %	
		24 h	48 h
1	20	10	10
2	40	16	19
3	60	36	44
4	80	53	61
5	100	65	76
6	120	83	91

CONCLUSION

In the present research, the purified L-asp used was extracted from the *Myroides gitamensis* to study biochemical characteristics and anti-proliferative, the cytotoxic effect of L-asp to develop a less toxic biochemically stable anti-leukemia drug. Thus, this research will develop improved treatments for blood cancers and assess the toxic effect of purified L-asp from new novel bacteria.

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