

Isolation and Characterization of L- Asparaginase Producing Endophytic Bacteria from *Simarouba Gluaca*

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Abstract: Cancer cells differentiate themselves from normal cells in diminished expression of L-asparaginase. It is the enzyme that catalyzes the hydrolysis of L-Asparagine to L-aspartic and ammonia, because of these it is used as a medication and in food manufacturing. As a medication L-asparaginase is used to treat various types of leukemia such as, acute lymphoblastic leukemia, acute myeloma leukemia and non Hodgkin's lymphoma. Hence they are not capable of producing L-asparaginase and mainly depend on the L-asparagine from circulating plasma pools. The clinical action of this enzyme is attributed to the reduction of L-asparaginase, since tumor cells unable to synthesis these amino acids are selectively killed by L-asparaginase depravation. This enzyme is widely distributed, being found in as it is widely distributed bacteria as well as found in animals, microbes and plant sources. In the present study L-asparaginase producing bacteria was isolated from *Simarouba glauca*. It was grown on the modified M9 medium in which L- asparagine was the major source of L- asparaginase production was detected by the formation of pink colored zones on the medium. After the partial purification of the L-Asparaginase enzyme, the enzyme activity was found to be 155.83 Units/ml and specific activity of 779.15. The optimum pH was to be found at pH 8 at a temperature of 37°C in the presence of 10mM Mg²⁺. The molecular identification was done by 16S rDNA, PCR and sequence analysis by BLAST further confirmed that *Bacillus cereus*.

Keywords: L-Asparaginase, *Simarouba Glauca*, *Bacillus Cereus*, Enzyme Specific Activity, Cancer Cells

1. Introduction

Cancer is characterized by uncontrolled and invasive growth of cells. These cells may spread to other parts of the body and this is called metastasis. It can be controlled by using many enzyme drugs. Now a day's many enzymes used as drugs and L-Asparaginase is one among them. L-Asparaginase belongs to the family of amidohydrolase. It is the first enzyme with anti-leukemic activity to be intensively studied in human beings [1]. The enzyme is present in many animal tissues, bacteria, plants and in small mammals. L-asparaginase is widely used in the treatment of leukemia and other lymphoproliferative disorders. Cancer cells differentiate themselves from normal cells in diminished expression of L-asparaginase. It has established itself to be an indispensable component. Normal cells can synthesize asparagine by themselves whereas cancer cells cannot. Thus

they need an additional supply of asparagine in plasma and tissues especially the leukemic cells. L-asparaginase, the enzyme that catalyzes the hydrolysis of L-Asparagine to L-Aspartate and ammonia Table 1 the conversion of asparagine to L-aspartic acid and ammonia. Because of these it is used as a medication and in food manufacturing. As a medication L-asparaginase is used to treat various types of leukemia such as, acute lymphoblastic leukemia, acute myeloma leukemia and non Hodgkin's lymphoma. Hence they are not capable of producing L-asparaginase and mainly depend on the L-asparagine from circulating plasma pools. This catalytic reaction is essentially irreversible under physiological conditions. The clinical action of this enzyme is attributed to the reduction of L-asparaginase, since tumor cells are unable to synthesis these amino acids are selectively killed by L-asparaginase depravation. The L-asparaginase destructs the free source of asparagine and resulting in killing of cancerous

cells.

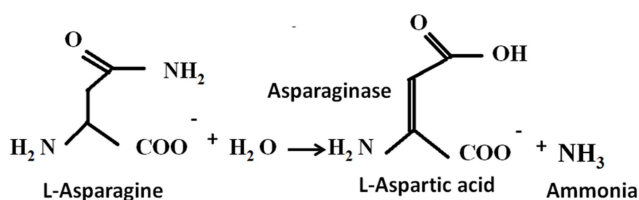


Figure 1. Schematic representation of L-asparaginase reaction.

The present study, L-asparaginase enzyme is produced from *Bacillus* species which sustain as endophytes in certain species of plants which have anticancer activity. The main example coming under this group is that *Simarouba gluaca*, which is also known as “Lakshmi taru”, “paradise tree” or dysentery-bark”. Recent researches showed that the leaf of this particular plant showing anticancerous activity along with antioxidant activity. The leaf extract of these plants showing asparaginase activity. Hence it could be a great source material for isolating endophytic bacteria with bioactive metabolites. *Simarouba gluaca* leaf decoction when taken in limited amounts can raise the immunity, reduce appetite and increase quality of life in cancer patients. There are different sources of materials were used for the isolation L-asparaginase. But the enzyme isolated from different species will have different physiological, pharmacological and serological properties. Common species that produces L-asparaginase are *Erwinia corotovora* and *E.coli*. L-Asparaginase is also produced from the *Bacillus* species [2].

Based on the previous reports, L-asparaginase is remarkably effective in cancer therapy, such as acute lymphoblastic leukemia. The enzyme cuts off the supply of asparagine in the blood stream and the cell death occurs due to the scarceness of the cell's proteins. In this study, we isolated an endophytic bacterium which is capable of producing L-asparaginase, an enzyme showing anticancer activity from *Simarouba gluaca* leaves.

2. Materials and Methods

2.1. Sample Collection

The leaf samples were collected in a sterile polythene bag from the local areas of Thrissur District and transferred immediately to the laboratory for the isolation of bacteria.

2.2. Isolation and Screening of Organism

The bacteriological studies were carried out by serial dilutions followed by plating on Modified M9 medium and later this culture were used for further studies based on their morphological and biochemical characteristics as out lined in the Bergey's Manual of determinative bacteriology [3].

2.3. Submerged Fermentation of Enzyme

The bacterium, which is capable of producing enzyme, was inoculated into 50 (ml) of the production media

containing substrate L-asparagine-1%, Glucose -0.2%, KH_2PO_4 -0.0152%, KCl -0.052%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ -0.052%, $\text{CuNO}_3 \cdot 3\text{H}_2\text{O}$ -0.003%, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ -0.005%, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ -0.003% and pH should be adjusted to 6.2 and sterilize the medium by autoclaving at 121°C for 15 min. Inoculate 100µl of overnight culture (OD of 0.23) incubate at 37°C for 72 hs. in orbital shaker at 120 rpm. Centrifuge the culture at 12,000 rpm for 10 minutes at 4°C in order to obtain the enzyme. The supernatant was collected in vials and estimated enzyme activity and enzyme solution was stored at 4°C.

2.4. Quantification of Total Protein and Enzyme Assay

Total protein can be estimated Lowry's method which was read at 660 nm and BSA used as a standard protein. [4]. The enzyme assay involves the estimation of amount of ammonia released during the enzymatic reaction. Reagent A: 50mM Tris Buffer, pH 8.6, Reagent B: 189 mM L-Asparagine, Reagent C: 6 mM $(\text{NH}_4)_2\text{SO}_4$, Reagent D: 1.5 M TCA, Reagent E: Nessler's reagent, Reagent F: Enzyme Solution

2.5. Partial Purification by Ammonium Sulfate

For the partial purification of enzyme, 75 ml of enzyme was taken and 36.6g ammonium sulfate was added to attain 80% saturation. The precipitate was collected by centrifugation and dissolved in 3ml of 0.5 M sodium acetate buffer at pH4.7. It was dialyzed against 0.005M sodium acetate buffer at pH4.7.

2.6. Enzyme Characterization

The enzyme characterization is done by estimating different factors influencing the enzyme activity. The activity of L-asparaginase depends on the various factors such as pH, temperature, substrate concentration and metal concentration.

2.7. Effect of pH in Enzyme Activity

The activity of majority of enzymes is often limited within a narrow pH range. The pH at which an enzyme exhibits maximum activity is referred as the optimum pH. The effect of pH on enzyme activity could be ascribed to a number of factors i) the pH may alter the ionization state of the reactants ii) enzyme might inactivated iii) H^+ may be involved in the reaction and thus alter the equilibrium position of the reaction iv) pH might alter the ionization state of the side chains of amino acids essential for catalysts. Dependency from pH was analyzed by incubation of substrate and enzyme with various pH buffers from pH 4 to 10. Assay was conducted at 37°C and reaction was stopped by heating at high temperature for few minutes.

2.8. Effect of Temperature

Rate of chemical reaction is increased by temperature and also the rate of enzyme catalyzed reactions increase with increasing temperature and extend of enhancement depends upon the energy of activation. The effect of temperature can

be used to determine the energy of enzymes catalyzed reaction. However, since enzymes are proteins and their typical tertiary structure of enzymes very much affected by temperature, a majority of enzyme proteins are denatured by temperature. Besides temperature, the loss of enzymes activity of enzyme depends upon the experimental conditions, constituents present in the solution and the length of incubation time.

To verify the thermal stability, the concentrated proteins were incubated at different temperature conditions for 15 min. various temperature conditions for the assay are 20°C to 60°C. The reactions were inactivated at high temperature for few minutes and the product formation was monitored by enzyme assay.

2.9. Effect of Cofactors in Enzyme Activity

The enzyme assay was carried out at different monovalent and bivalent cations such as Na^+ , K^+ , Zn^{++} , Mg^{++} respectively with concentration of 10 mM.

3. Molecular Characterization of Bacterial Isolates

The genomic DNA was isolated and visualized by 0.8% of agarose gel electrophoresis. Polymerase chain reaction was carried out for amplifying the DNA. The genomic DNA was amplified using the primers; 16S Forward Primer: 5'-GAG TTT GAT CCT GGC TCA G-3' and 16S Reverse Primer: 5'-G(AT)A TTA CCG CGG CGG CTG-3'.

Initial denaturation was done at 94°C for 3 minutes and followed by 30 cycles of denaturation at 94°C, annealing at 48°C for 30 seconds, primer extension at 72°C for 30 seconds and final extension at 72°C for 7 minutes. The PCR product was then visualized on 0.8% agarose gel. The PCR a product was cleaned up using ethanol precipitation and the cleaned up products was sequenced using Big dye Terminator sequencing kit. The cleaned up PCR products was sequenced in Applied Biosystems AB 3730XLcapillary sequencer at the sequencing facility. The obtained sequences were then used for similarity searching using Basic Local Alignment Search Tool (BLAST) from National Centre for Biotechnology Information.

4. Results and Discussions

4.1. Isolation and Identification of Organism

L-asparaginase producing bacteria were isolated from *Simarouba gluaca* leaves by using modified M9 medium. The bacteria were able to produce pink colored colonies were selected as potent organism. L-asparaginase enzyme can be produced from the *Bacillus* species which sustain as endophytes in certain plants which have anticancer activity. Certain endophytic bacteria are present in the *Simarouba gluaca* leaves and the decoction of *Simarouba gluaca* were also used in traditional medicine in the treatment of cancer

and tumors. It can also be used to complement in modern cancer treatment. *Simarouba gluaca* is very effective in reducing the size of tumors and secondary infections in cancer patients. Thus the medicinal plant could be a great source of material for the isolation of endophytes with bioactive metabolites. [4]. Figure 2 shows the typical plant of *Simarouba gluaca*.

Bacteriological identification were carried out by gram staining as well as biochemical tests and the potent organism were identified as *Bacillus cereus* and the molecular characterization were carried out by blast analysis were confirmed that *Bacillus cereus*. Figure 3 shows the gram staining of *Bacillus cereus*.



Figure 2. Shows the typical plant of *Simarouba gluaca*.

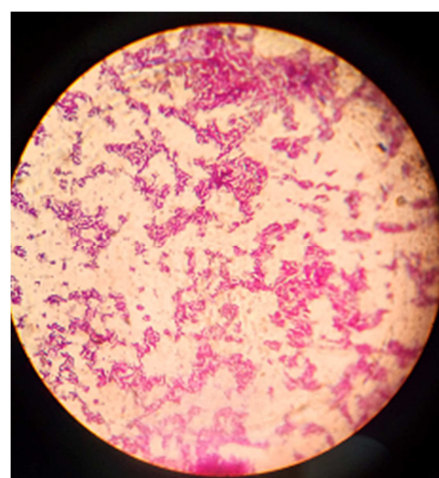


Figure 3. Shows the gram staining of *Bacillus cereus*.

4.2. Enzyme Quantification

The enzyme assay were carried out by using asparagine as a substrate, the enzyme activity was determined by the amount of ammonia released when react with asparagine this result compared with based on the standard graph of ammonia. The enzyme activity was calculated using according to standard procedure explained in the previous references. [5]. The enzyme activity was found to be 155.83 U/ml, the specific activity was 779.15. Table 1 shows the list of total protein, enzyme activity and specific activity of the purified asparaginase. Common bacterial species were produces L-asparaginase are *Erwinia corotovora* and *E.coli*.

Varieties of asparaginase are produced from fungi, *Basidiomycetes* and small mammals like hedgehogs. However, L-asparaginase produced from *Erwinia corotovora* has been very much useful in clinical trials. L-asparaginases from *Erwinia* and *E. coli* have been reported as effective drugs in the treatment of acute lymphoblastic leukemia. [6,7]. L-asparaginase is also produced from the *Bacillus* species [8]. It is a genus of gram-positive, rod-shaped bacteria and a member of the phylum *Firmicutes*. *Bacillus* species can be obligate aerobes (oxygen reliant) or facultative anaerobes. It includes both free-living (non-parasitic) and parasitic pathogenic species.

Table 1. Enzyme activity, total protein and specific activity.

Total protein	Enzyme activity	Specific activity
0.2 mg/ml	155.83U/ml	779.15

5. Enzyme Characterization

5.1. pH

The enzyme assay was carried out at different pH by changing the pH of the buffer in order to find the maximum enzyme activity. The maximum enzyme activity was observed at an optimum pH of pH 8. Figure 4 shows the optimum pH of the purified enzyme. A novel and semi-microorganism can produce asparaginase and having medicinal activity. A pH and dye-based fast procedure used for screening L-asparaginase producing micro-organisms. The procedure is suitable for bacterial and fungal screening. [9]. It is generally observed that L-asparaginase production is accompanied by an increase in pH of the culture filtrates. The plate assay was devised using this principle by incorporating the pH indicator phenol red in the medium containing asparaginase. Phenol red at acidic pH is yellow and alkaline pH turns pink, thus a pink zone is formed around the microbial colonies producing L-Asparaginase. [9]. L-asparaginase activity was studied as a function of pH in range between 4.5–10.5. L-asparaginase was active over broad pH ranges (4.5–10.5). The enzyme activity increase gradually till pH 8.5 with maximum activity 20.932 IU (relative activity, 100%). At higher pH's, enzyme. [10].

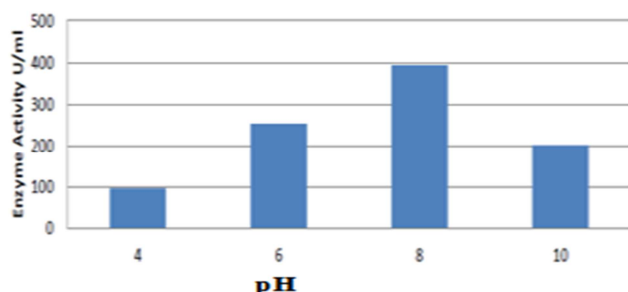


Figure 4. Optimum pH.

5.2. Temperature

The temperature assay was carried out at different incubation temperatures to find out the optimum temperature.

The temperature influences the rate of chemical reaction thus affecting the rate of enzyme activity.

The enzyme has showed maximum activity at 50°C (265 U/ml). Figure 5 shows optimum temperature. A slight decrease in enzyme activity was observed at 40°C (249 U/ml). [11]. The bacterial flora that associated with an intertidal marine alga (*Sargassum* sp.), by the presence of extracellular L-Asparaginase; one out of five *Bacillus* strains was found positive and confirmed that it can produce the enzyme asparaginase. The maximum asparaginase activity was found at 37°C and pH 8.0. The optimum NaCl concentration for enzyme activity was found to be 2% (w/v). The enzyme activity was not affected by the addition of different metal ions (Ca^{2+} , Co^{2+} , Fe^{2+} and Mg^{2+}) but strongly inhibited by EDTA. [12].

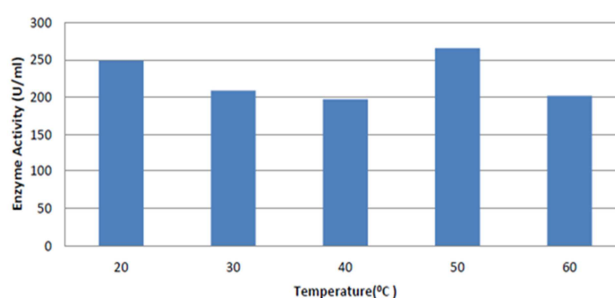


Figure 5. optimum temperature.

5.3. Substrate Concentration

The assay was carried out at different substrate concentration (L-asparagine) such as 0.5%, 1%, 1.5% and 2% respectively.

The maximum activity of asparaginase enzyme was found at a substrate concentration of 1% (485.83 U/ml). Figure 6 shows effect of substrate in enzyme activity. The activity of L-asparaginase of *Streptomyces fradiae* was evaluated at different levels of pH, temperature, effect of substrate concentration and incubation time. [10].

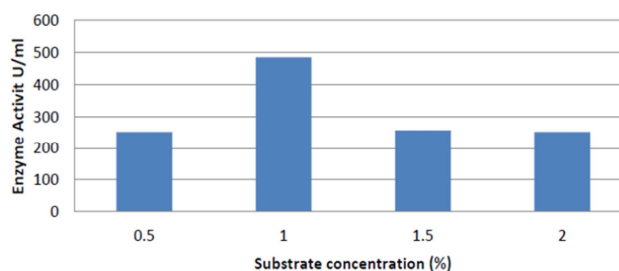


Figure 6. effect of substrate in enzyme activity.

5.4. Metal ion Concentration

The assay was carried out at different metal ion concentrations. Monovalent metal ions such as Na^+ and K^+ and divalent ions such as Zn^{2+} and Mg^{2+} were used.

The enzyme shows maximum activity with Mg^{2+} ions (467.5 U/ml) Figure 7 shows effect of metal ion in enzyme activity. While it shows minimum activity with Na^+ ions

(270.4 U/ml) [13]. Screening of the bacterial flora associated with an intertidal marine alga (*Sargassum* sp.) for the presence of extracellular L-Asparaginase out of five *Bacillus* strains was found positive. The maximum Asparaginase activity was found at 37 °C and pH 8.0. The optimum NaCl concentration for enzyme activity was found to be 2% (w/v). The enzyme activity was not affected by the addition of different metal ions (Ca^{2+} , Co^{2+} , Fe^{2+} and Mg^{2+}); but strongly inhibited by EDTA.

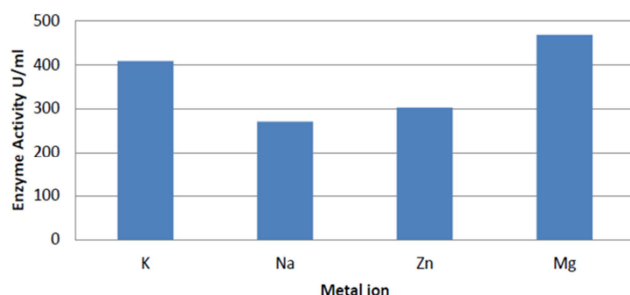


Figure 7. Effects of metal ions in enzyme activity.

6. Discussion

L-asparaginase has grown considerably since this enzyme was found to have antitumor activity. L-asparaginase is an enzyme that catalysis the hydrolysis of asparagine to aspartic acid and finally to ammonia. The therapeutic use of this enzyme was responsible for emission in most patients suffering from acute lymphoblastic leukemia. L-asparaginase is the first enzyme with anti-leukemic activity to be intensively studied in human beings since the extraction from human cell is difficult and microorganism have proved to be a better alternative for L-asparaginase extraction. Endophytes are present ubiquitously in all plants species and get importance for their ability to produce various bioactive compounds. It has been reported earlier that some of the endophytic bacteria belongs to members of diverse genera namely *Pseudomonas* and *Bacillus*. In recent years there are constant researches on *Simarouba glauca* in inhibiting the metastasis and invasion of breast cancer tumors according to local Ayurveda practitioners. Maybe because of the reason the leaf of this plant is having anticancerous activity. Detailed studies require proving this data.

7. Conclusion

The endophytic bacteria were isolated from *Simarouba glauca* and it was identified as *Bacillus* species which have anti-cancerous activity as well as antioxidant activity. Hence it could be great source for isolating such endophytic bacteria. Studies of various parameters such as pH, temperature, substrate and metal ion concentration were found to be the enzyme has the maximum enzyme activity at pH 8 at a temperature of 50°C in the presence of bivalent cation such as Mg^{++} . Hence the present study can concluded that the *Bacillus cereus* isolated from *Simarouba glauca* producing L-asparaginase and having the activity of

155.83 U/ml and specific activity of 779.15. The leaf extracts of *Simarouba glauca* showing L-asparaginase activity data not published. This showed that the plant may carry the gene for the production L-asparaginase (Parakkottil Choth Madhu *et al* data not published). It can also be used to complement in modern cancer treatment. The leaf extract of this plant is very effective in reducing the size of tumors and secondary infections in cancer patients. Thus the medicinal plant could be a great source of material for the isolation of endophytes with bioactive metabolites. It can use for the large scale production L-asparaginase and purified enzyme can be sued for the anticancer activity as well as antioxidant activity. The overall results were provided evidence of antioxidant and anticancer activities of L-asparaginase isolated from endophytic *Bacillus*. The selectivity of this organism proved to be a promising anticancer potential producing by L asparaginase. However, the research for new lead compounds from bacterial source with more effective and less toxic compounds constitute interesting alternatives for the development of anticancer drugs in cancer treatment.

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