

# Purification and Cytotoxicity of L-asparaginase from *Streptomyces griseoluteus* GDJ1

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**Abstract:** *L-asparaginase (L-asparagine amidohydrolase E.C.3.5.1.1) is an effective antineoplastic enzyme, used in acute lymphoblastic leukemia (ALL) chemotherapy. L-asparaginase activity of marine actinomycetes in soil samples of Agnitheertham (Rameswaram) and Periyar lake, Kumily were investigated. Totally fifty one actinomycetes were isolated from both the soil samples in different selective media. Out of fifty one isolates, four were found to produce L-asparaginase. Among them, the strain AT-R-S1 produced high amount of L-asparaginase. Hence it was selected for the further identification and based on molecular identification the strain AT-R-S1 was identified as Streptomyces griseoluteus GDJ1. Purification of the enzyme from the strain S.griseoluteus GDJ1 (AT-R-S1) was carried out. The final purification of the enzyme in CM-Sephadex C-50 column chromatography yielded 1.27 U/ml of enzyme in 0.09 mg of protein with a specific activity of 14.11 U/mg with approximately 119 folds purity. Cytotoxicity of different concentration of purified L-asparaginase was determined on VERO cells. The anti proliferative activity of L-asparaginase in MCF-7 and HeLa cells was determined and the EC<sub>50</sub> value was found to be 31.37 and 38.99 µg respectively. The above results were encouraging and worth pursuing for further development of Streptomyces griseoluteus GDJ1 (AT-R-S1) as an alternative resource for therapeutic L-asparaginase.*

**Keywords:** Actinomycetes, L-asparaginase, Purification, Anticancer

## 1. Introduction

All enzymes are proteins composed of amino acids, (in some cases RNA molecules) that catalyse reaction involved in development and maintenance of cell and help in defense against diseases with great specificity[1]. Enzymes act on their targets with higher affinity and specificity and convert multiple target molecules to desired products. Enzymes are being used to treat many diseases like cancer, dermal ulcers, inflammation, cardiac problems, cystic fibrosis and digestive disorders etc [2]. A major potential application of therapeutic enzymes is in the treatment of cancer. Cancer is the most precarious disease in which abnormal cells proliferate rapidly without control and able to invade other tissues by lymph or blood systems [1] and can cause organ failure and death if untreated. Globally, in 2012 nearly 14.1 million new cancer cases and 8.2 million deaths were occurred due to cancer [3] and by 2030 the number is expected to increase to 21 million. In India, 462408 male cancer patients and 517378 female cancer patients were recorded, with a total number of 979786 patients in 2010 with total economic loss of 270.06 million US \$ For treating cancer numerous drugs are available includes, asparaginase, daunorubicin, cyclophosphamide, mercaptopurine, methotrexate etc [4] and new products have been synthesized and tested for their anti cancer activity. However, radiotherapy and chemotherapy has been the mainstay to treat cancer which has deleterious effects to the patients by making them prone to other diseases and weakening their immune system. Hence the exploration of novel therapeutic drug for the treatment of life threatening cancer is in immense need of the hour. L-asparaginase, one of the anti cancer drug used to treat several leukemias, lymphomas, lymphoproliferative disorders, lymphosarcoma, Hodgkin's disease, acute myelogenous leukemia, acute myelomonocytic

leukemia, chronic lymphocytic leukemia, reticulosarcoma, melanosarcoma and acute lymphoblastic leukemia [5]. L-asparagine is an essential amino acid required for the growth of normal cells, L- asparagine can be obtained by either diet or by the synthesis via asparagine synthetase located on the chromosome number 7 (7q21.3) in humans [6]. The starvation of L-asparagine due to lack or less activity of asparagine synthetase tumor cells cannot synthesis their own L-asparagine and depends on the external sources. The breakdown of circulating pool of L-asparagine by L-asparaginase resulted in reduced RNA and protein synthesis, causes cell cycle arrest in the G1-phase and ultimately apoptosis in susceptible leukemic cells [7]. Hence, the normal cells are not affected during the treatment with L-asparaginase since their ability to produce this amino acid [8]. L-asparaginase is a relatively wide spread enzyme found in many micro organisms. Out of the different sources explored, L-asparaginase from *E.coli* gained momentum and after several studies and subsequent clinical trials, it was approved as drug for leukemia by FDA in 1978. Glutamine a co-substrate of L-asparaginases, depreciated by the action of L-asparaginase which has intrinsic glutaminase activity that cause serious side effects such as leucopenia, immunosuppression, acute pancreatitis, thromboembolysis, hyperglycemia and neurological seizures [9]. Protein synthesis was found to be reduced when the glutamine is reduced to a critical level. Further, the multiple administration of such enzyme protein for a long duration produces anti asparaginase antibodies in the tissues, resulting in anaphylactic shock and may also cause neutralization of drug effect by the host immune system. Therefore, glutaminase free and new serologically different L-asparaginase with a similar or higher therapeutic effect than the previously reported ones is urgently required. Considering the above facts, an attempt an attempt was made

to isolate, screen, purify and to determine the anticancer activity of L-asparaginase from the soil sample isolated from Agnitheertham of Rameswaram and Periyar lake.

## 2. Material and Methods

### 2.1 Isolation of Actinomycetes

Soil samples were air dried aseptically. After a week sample was incubated at 55°C- 5 min in order to facilitate the isolation of actinomycetes. Then tenfold serial dilution was prepared with one gram of soil sample using distilled water. Samples were inoculated on Starch Caesin agar (SCA), Actinomycetes Isolation Agar (AIA) and Glycerol Asparagine agar (ISP-5 Medium) in triplicate plates. Streptomycin (20 µg/ml) and cycloheximide (50 µg/ml) were added to the medium in order to retard the growth of bacteria and fungi respectively. All the plates were incubated at 28±2°C for 7 days. Colonies with suspected Actinomycetes morphology (i.e., isolates with grey and white colonies) were purified using yeast-extract malt extract agar medium. The pure cultures of the actinomycetes were streaked on SCA, AIA and ISP-5 Medium plates [12], [13].

### 2.2 Screening of L-asparaginase Producers

The isolated colonies were screened for L-asparaginase production by rapid plate assay technique described by Gulati *et al.*, [10]. Modified agar plates of M-9 and Czapek-Dox agar supplemented with 0.1% of L-asparagine (w/v), 0.009% of phenol red (v/v) were prepared and streaked with the isolated colonies and incubated at 28±2°C for five days. After incubation the plates were observed for the pink colour formation around the colonies and considered as positive L asparaginase producers.

### 2.3 Enzyme Quantification

To 100 µL of the enzyme extract, 200 µL of 0.05 M Tris-HCl buffer (pH 8.2) and 1.7 mL of 0.01 M L-asparagine as substrate were added and incubated at 37°C for 10 min. After incubation, the reaction was stopped by adding 500 µL of 1.5 M TCA. The resulting mixture was centrifuged at 1000 rpm at 4°C. The supernatant was transferred to new tube and 0.5 mL of the supernatant was diluted with 6.5 mL of distilled water and 200 µL of Nessler's reagents and left for 10 min for the development of yellow color. Then the OD was measured at 450 nm using UV-Visible Spectrophotometer (Shimadzu, ). The absorbance was then compared to the standard curve of ammonium sulfate and the µM of ammonia released was calculated. From the ammonia released the L-asparaginase activity (Unit/mL) was calculated using the following formula

$$\text{Units/mL} = \frac{(\mu \text{ mole of NH}_3 \text{ liberated}) \times (\text{Initial volume of enzyme mixture (mL)})}{(\text{Volume of enzyme mixture used in final reaction (mL)}) \times (\text{Incubation time (min)}) \times (\text{Volume of enzyme used (mL)})}$$

One International unit of L-asparaginase was defined as the amount of enzyme which liberates one µ mole of ammonia in one min at 37°C [11].

### 2.4 Identification of Actinomycetes

#### 2.4.1 Morphological and Biochemical Characterization

In order to determine the organism at the genus level, morphological and biochemical tests were carried out based on the International Streptomyces Project (ISP). Cell morphology and motility were determined by microscopic examination (Labomed Asia model No. LB-210). Gram staining was performed according to the manufactures (Himedia Lab, India) instructions. Catalase activity was performed by adding H<sub>2</sub>O<sub>2</sub> to a fresh loop full of culture placed on clean glass slides. The hydrolysis of starch and casein was determined on the plates of SCA containing 0.4% starch and 5 % of skimmed milk powder, respectively. The acid production during utilization of carbohydrate was determined in tryptone broth supplemented with 1 percent of (w/v) any one of the following substrates: Arabinose, Glucose, Raffinose, Sucrose, Xylose, Fructose, Mannitol, Inositol and Lactose. The bromocresol purple (0.001 %) was added to each tube as an indicator of acid production. Growth at different temperatures (4 to 65°C), different pH (5.0-10.0) were tested in SCA broth and salt tolerance was performed at 30°C in SCA broth supplemented with 2 to 10 percent NaCl. Reduction of nitrate, and production of indole, gelatinase, urease, acetoin (Voges-Proskauer-VP), H<sub>2</sub>S and utilization of citrate were also performed.

#### 2.4.2 Genotypic characterization of isolates

##### 2.4.2.1 Isolation of Genomic DNA

Each isolate was inoculated in 100 mL Erlenmeyer flask containing 20 mL of SCA broth and incubated in an orbital shaker incubator (Remi CIS-24 Plus, India) at 150 rpm at 30°C. After 3 days, two milliliters of cultures were transferred into micro centrifuge tube and centrifuged at 10,000g for 2 min. The pellet was resuspended in 2 mL of lysis buffer, vortexed for 1 min and incubated at 37°C for 30 min. SDS (final concentration 1% w/v) and proteinase K (final concentration 100 mg mL<sup>-1</sup>) was added, vortexed for 10s and incubated at 55°C for 2 h. An equal volume of phenol-chloroform-isoamyl alcohol (24:23:1) was added and mixed it by inverting the tubes several times and centrifuged at 10,000g for 15 min. The aqueous layer (upper) was transferred to a new tube and the extraction was repeated once. The obtained aqueous phase was again transferred to a new tube and the DNA was precipitated by adding 1/10<sup>th</sup> volume of 3 M sodium acetate and 0.6 to 1 volume of ice cold 2-propanol and mixed gently. Then the tubes were incubated at -20°C from 1 h to overnight. The mixture was centrifuged at 10,000g for 15 min, the supernatant was discarded and DNA was washed with 500 µL of 70 percent ethanol. Again it was centrifuged at 10,000g for 5 min and finally the ethanol was carefully discarded and air dried until ethanol removed. The precipitated DNA was resuspended in 200–300 µL of double-distilled sterile water. It was allowed to dissolve at 37°C at least 3 h [15].

#### 2.4.2.2 Polymerase Chain Reaction (PCR)

The Polymerase Chain Reaction (PCR) technique was employed to amplify the target gene or region. In brief, a region lying between a forward and reverse primer was amplified in an exponential fashion in the presence of appropriate reagent and cycling condition. The successful amplification of all reactions was assessed on agarose gels prepared with 1X TBE (Tris Boric acid EDTA).

#### 2.4.2.3 Agarose Gel Electrophoresis

Gel tray and comb was prepared by wiping with 70 percent ethanol and air dried for 5 min. The required concentration of agarose was added in 1X TBE and the mixture was left for 10-15 min (to enhance the solubilization). Later, it was heated in a microwave oven for 2-3 min until the solution was clear. This molten agarose solution was left to cool around 60°C without disturbance. Then, ethidium bromide (final concentration 0.5 µg µL<sup>-1</sup>) was added, mixed thoroughly by swirling the container gently and the solution was poured into a prepared gel tray. The agarose gel was allowed to set at room temperature for 20-30 min. The solidified agarose gel (along the tray/comb) was stored in a refrigerator for another 20-30 min before use. Electrophoresis of the gel was performed in a gel tank with 1X TBE at appropriate volt and run time. The presence of specific band was confirmed under ultraviolet (UV) light visualization and documented (UVP- Multidoc-It, USA)

#### 2.4.2.4 PCR amplification of 16S rDNA

The DNA extracted by phenol chloroform method was used as PCR template for 16S rDNA amplification. The polymerase chain reaction was carried out with universal forward (27F) and reverse (1492R) primers. The PCR reaction mixture (50 µL) consisted of 50 ng of genomic DNA in 2 µL, 1X PCR buffer with 2.0 mM MgCl<sub>2</sub>, 200 µM dNTP, specific primers at required concentration and 0.5 units of *T.aq* DNA polymerase (Genetbio Inc, South Korea). The primer concentrations and cycling conditions were given in Table 5 and the PCR component composition was given in appendix 1. Amplified products were separated on 1.5 percent agarose gel containing 0.5 µg/µL ethidium bromide for 20 min at 100V and documented under UV illumination

#### 2.4.2.5 DNA sequencing

Nucleotide sequences were determined by using ABI 3730xl 96 Capillary Genetic Analyzer using big dye terminator v3.1 kit (Applied Biosystems) at Xcelris Labs Ltd., Ahmedabad, India. The sequenced PCR products were analyzed online using BLAST software (<http://www.ncbi.nlm.nih.gov/blast>) and the identity of the sequences were determined (Altschul *et al.*, 1990).

#### 2.4.2.6 Multiple sequence alignment and phylogenetic tree construction

All the nucleotide sequences were converted into FASTA format. Multiple sequence alignment for the assembled nucleotide sequences was done by using the Clustal X program (Thompson *et al.*, 1997) in BIOEDIT software (Hall, 1999). Aligned sequences were imported into an MEGA 6: Molecular Evolutionary Genetics Analysis (MEGA) software version 6.0 (Tamura *et al.*, 2011) software for further analysis. The ends of the alignment were trimmed

to obtain equal lengths for all sequences and the aligned sequences were converted into MEGA format for carrying out phylogenetic analysis. The phylogenetic tree was constructed using the Neighbor-Joining method (Saitou and Nei, 1987) using nucleotide based TN84 evolutionary model for estimating genetic distances based on synonymous and non synonymous nucleotide substitutions. Statistical support for branching was estimated using 1000 bootstrap steps

### 2.5 Purification of L-asparaginase

The purification of L-asparaginase was carried out on the crude extract adapted by Distasio *et al.* [14].

#### 2.5.1 Ammonium sulphate precipitation

Proteins can be concentrated from a culture by salting out method using ammonium sulphate precipitation at 40°C. Finely powdered ammonium sulphate was added to the crude extract pinch by pinch on a magnetic stirrer. The L-asparaginase activity was associated with the fraction precipitated at 75% saturation. The pellet was collected by centrifugation at 10,000 rpm for 10 min to which 5 mL of sodium phosphate buffer (0.05M, pH 7.8) was added. This resuspended pellet in phosphate buffer was further purified by dialysis.

#### 2.5.2 Dialysis

Dialysis was carried out followed by ammonium sulphate precipitation to remove un-desirable molecules and ions of small size from high molecular weight particles like proteins, enzymes etc. The dialysis bag (15 kD cutoff) is previously soaked in 0.05M phosphate buffer and the resuspended pellet in phosphate buffer was poured to the dialysis bag, sealed tightly and placed inside the 0.005M phosphate buffer for 12h at 4°C. After dialysis the samples were collected and treated as partially purified enzyme.

#### 2.5.3 Sephadex G-100 Gel Filtration

The dialyzed ammonium sulphate fraction was applied to a Sephadex G-100 column that was pre-equilibrated with 0.01 M phosphate buffer (pH 8.5). The protein elution was done with the same buffer at a flow rate of 5 mL/min. The fractions were collected using a fraction collector at 4°C. The active fractions were pooled, dialyzed against the 0.01 M phosphate buffer pH 8.5, and concentrated. Then the enzyme was subjected to CM-Sephadex C-50 Ion Exchange Chromatography for further purification

#### 2.5.4 CM-Sephadex C50 Ion-Exchange Chromatography

The concentrated enzyme solution was applied to the column of CM-Sephadex C50 that was pre-equilibrated with a 0.01 M phosphate buffer (pH 8.5). The enzyme solution was then eluted with the NaCl gradient (0.1-0.5 M) and 0.1M borate buffer, pH 7. The active fractions were collected, dialyzed, concentrated, and lyophilized and the enzyme was quantified.

### 2.6 Assessment of cytotoxicity and anti-proliferative activity

Cell cytotoxicity and anti-proliferative effect of L-asparaginase was performed using VERO used as control



cell lines (Kidney Epithelial cells), MCF-7 (Human Breast Adenocarcinoma) and HeLa cell lines (Cervical cancer cell lines). The cells were plated separately in 96 well plates at a concentration of  $1 \times 10^5$  cells/well. After 24 h, cells were washed twice with 100  $\mu$ l of serum-free DMEM medium and starved for an hour at 37°C. After starvation, cells were treated with different concentrations of L-asparaginase (5 - 45  $\mu$ g/ml) for 72 h. At the end of the treatment period the medium was aspirated and serum free medium containing MTT (0.5 mg/ml) was added and incubated for 4 h at 37°C in a CO<sub>2</sub> incubator. The MTT containing medium was then discarded and the cells were washed with 200  $\mu$ l of Phosphate Buffer Saline (PBS). The crystals were then dissolved by adding 100  $\mu$ l of Dimethyl Sulfoxide (DMSO) and this was mixed properly. Spectrophotometrical absorbance of the purple blue formazan dye was measured in a microplate reader at 570 nm using Biorad 680. The data were expressed as the mean percentage of viable cells as compared to the respective control cultures treated with the solvent. The half maximal growth effective concentration EC<sub>50</sub> values were calculated against MCF-7 and HeLa cells by checking its corresponding value in the linear equation of the dose dependent curve as referred by Mosmann, 1983.[16]

### 3. Results and Discussion

In the present study, actinobacteria were isolated from the selected soil samples collected from two study sites such as Rameswaram sea and Periyar lake since soil is considered as one of the richest sources for the potential enzyme producing microorganisms especially actinomycetes.

#### 3.1 Isolation of Actinomycetes

Among the selected three media used, SCA medium showed the highest colonies (CFU/g) in all the study sites as  $16 \times 10^7$ ,  $14 \times 10^6$  observed in Rameswaram sea soil and Periyar Lake soil respectively (Table 1). Whereas, the ISP-5 medium showed the lowest colonies in both sites as  $8 \times 10^7$  and  $3 \times 10^6$  in respective Rameswaram sea and Periyar Lake soils. The results indicated that the SCA could serve as a isolation medium compared to other two media tested. Our results were good agreement with the results of Mohan *et al.*, [17] who found that SCA and glycerol asparagine agar medium could be used as selective media for the isolation and enumeration of actinobacteria.

**Table 1:** Number of Colonies (CFU /g) isolated from soil samples using different medium.

Media	CFU/g	CFU/g
	Rameswaram Soil	Periyar Lake soil
Starch Casein Agar (SCA)	$16 \times 10^7$	$14 \times 10^6$
Actinomycetes Isolation Agar (AIA)	$9 \times 10^7$	$10 \times 10^6$
International <i>Streptomyces</i> Project (ISP-5) medium	$8 \times 10^7$	$3 \times 10^6$

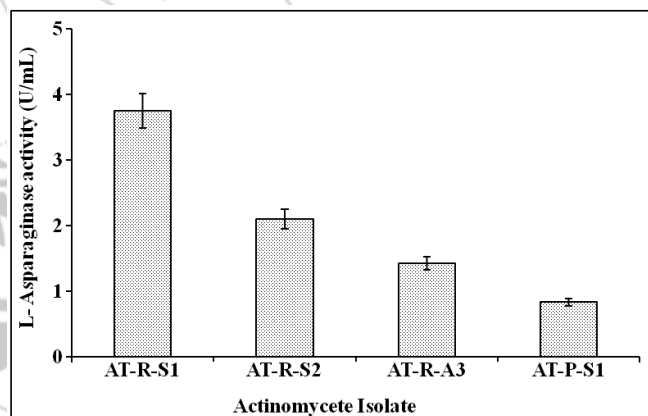
#### 3.2 Screening of L-Asparaginase production

Isolation of L-asparaginase producing actinomycetes was made and screened for their ability to produce L-asparaginase enzyme. Primarily both M9 and Czapekdox

medium used for screening the colonies showed pink colour on plates were considered as positive result for L-asparaginase production. Among the 51 isolates screened, four isolates (three isolates AT-R-S1, AT-R-S2, AT-R-A3 of Rameswaram sea soil and one isolate AT-P-S1 from Periyar lake soil) showed positive results in rapid plate assay

#### 3.3 Quantitative estimation of L-asparaginase

Spectrophotometric analysis of the enzyme production was assessed and the results showed that the L-asparaginase production by the isolates were ranged between 0.83 and 3.75 U/ml. The highest production was observed in isolates of Rameswaram sea soil sample (AT-R-S1) and lowest in the isolate of Periyar lake soil sample (AT- P-S1) (Figure 1). The results revealed that the quantity of L-asparaginase production varied with strains. Similarly, Neelima *et al.*, [18] observed the L-asparaginase activity from *Streptomyces ginsengisoli* at a rate of 3.23 U/ml. *Streptomyces nouresi* produced 3.310 U/ml of L-asparaginase as reported by Dharmaraj [20]. Mohana Priya *et al.*, [19] reported that *Streptomyces sp TA22* showed that the L-asparaginase activity of 2.46 U/ml.



**Figure 1:** L-Asparaginase production by the selected isolates

#### 3.4 Identification of the isolates

##### 3.4.1 Morphological and Biochemical characterization

For the identification of the strains, morphological, physiological and biochemical characterization were done based on the International Streptomyces Project (ISP). The cultural, morphological and biochemical characteristics of the strains AT-R-S1, AT-R-S2, AT-R-A3 and AT-P-S1 were studied and the results were summarized in Table 2 & 3. As summarized in Table 2 all the isolates were found to be gram positive, rod shaped and possessing earthy odour characteristics of actinomycetes. Mycelium was aerial and white in color. Colony elevation was raised with wrinkled and opaque density and tenaciously adhering to the medium. All the isolates were spore forming in nature and spores were non motile, smooth and hairy. The strains exhibited optimum growth under aerobic conditions at temperature 30°C & pH at 7.0. All the strains were mesophilic and alkaliphilic in nature which showed growth at temperature ranging from 25 to 42°C and pH 6 to 10, but no growth was observed at temperature 4, 10, 55 and 65°C & pH 5.0.

All the strains of Rameswaram soil showed optimum growth at 2% (w/v) sodium chloride (NaCl), but maximum tolerance of NaCl concentration was 6% (w/v), indicating all the isolates were indigenous to marine environment and moderate salt tolerance in nature. The isolate (AT-P-S1) from Periyar Lake survived at 2% NaCl and above that concentration no growth was found. All the isolates could utilize arabinose, glucose and mannitol as carbon source. In case of biochemical characterization, all the isolates can have the ability to utilize citrate and the isolates showed negative result to indole test.

**Table 2:** Morphological and Physiological characterization of the isolated strains - 'No growth'; '+' - Normal Growth; '++' - Moderate Growth; '+++'- Optimum Growth

TESTS	AT-R-S1	AT-R-S2	AT-R-A3	AT-P-S1
Elevation	Raised	Flat	Flat	Flat
Surface	Wrinkled	Wrinkled	Wrinkled	Wrinkled
Density	Opaque	Opaque	Opaque	Opaque
Pigment	Brown	White	Yellowish Brown	Black
Grams Reaction	+ ve	+ ve	+ ve	+ ve
Spore Chains	Spiral	Spiral	Spiral	Spiral
Mycelium	Aerial	Aerial	Aerial	Aerial
Spore Motility	Non motile	Non motile	Non motile	Non motile
Spore surface	Smooth & hairy	Smooth & hairy	Smooth & hairy	Smooth & hairy
<b>Growth at Temperature</b>				
4	-	-	-	-
10	-	-	-	-
25	+	+	+	+
30	+++ (optimum)	+++ (optimum)	+++ (optimum)	+++ (optimum)
37	++	++	++	++
42	+	++	++	+
55	-	±	-	-
65	-	-	-	-
<b>Growth at pH</b>				
5.0	-	-	-	-
6.0	+	+	+	+
7.0	+++ (optimum)	+++ (optimum)	+++ (optimum)	+++ (optimum)
8.0	++	++	++	++
9.0	+	-	+	+
10	+	-	+	+
<b>Growth on NaCl (%)</b>				
2.0	+++ (optimum)	+++ (optimum)	+++ (optimum)	+
4.0	++	++	++	-
6.0	+	-	+	-
8.0	-	-	-	-
10.0	-	-	-	-

**Table 3:** Biochemical test profile of the isolated isolates

Test	AT-R-S1	AT-R-S2	AT-R-A3	AT-P-S1
<b>Biochemical Characteristics</b>				
Indole	-	-	-	-
Methy Red	+	+	-	-
Voges Prousker	-	-	+	+
Citrate utilization	+	+	+	+
Caesin Hydrolysis	-	-	+	-
Starch Hydrolysis	-	+	+	+
Urea hydrolysis	+	+	-	+
Nitrate reduction	+	+	-	-
H2S production	-	+	-	-
Catalase test	+	-	-	-
Gelatin Hydrolysis	+	+	+	-
<b>Acid Production from Carbohydrates</b>				
Arabinose	+	+	+	+
Glucose	+	+	+	+
Raffinose	+	+	+	-
Sucrose	+	+	+	-
Xylose	+	+	+	+
Fructose	+	+	+	+
Mannitol	+	+	+	+
Inositol	-	+	+	-
Lactose	+	-	+	+

### 3.4.2 Genotypic characterization of isolates

For molecular identification of isolates AT-R-S1, AT-R-S2, AT-R-A3 and AT-P-S1, the partial 16S rDNA gene sequence was used. The extracted genomic DNA was amplified using 27F and 1492R primers with suitable cycling condition. The gel image shown in Fig 2 was the amplified products (~1500bp) electrophoretated in 2 percent agarose gel after PCR amplification. The PCR products were then purified and sequenced followed by homology search analysis. The gene sequences were deposited in the GenBank database and their corresponding accession numbers were given in Table 4

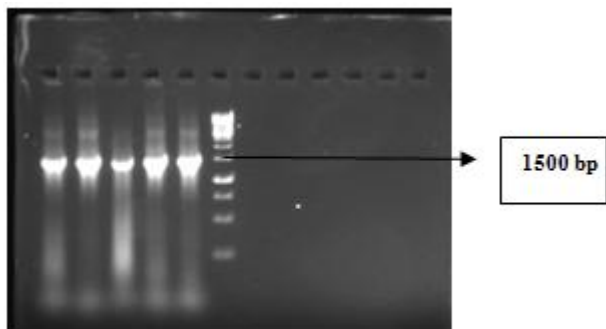
**Table 4:** List of actinomycetes strains identified using the partial sequences of 16S rDNA and their NCBI accession numbers

S. No	Name	Host/ Source	Accession No.
1	<i>Streptomyces griseoluteus</i> GDJ1	Rameswaram soil	KU687334
2	<i>Streptomyces griseoluteus</i> GDJ2	Rameswaram soil	KU687335
3	<i>Streptomyces griseus</i> GDJ3	Rameswaram soil	KU687336
4	<i>Streptomyces Sp</i> GDJ4	Periyar lake soil	KU507504

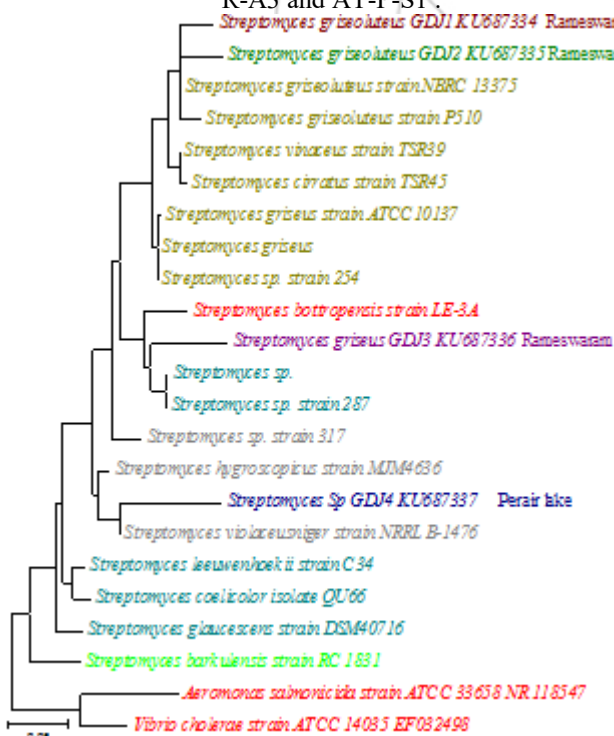
16S rRNA comparisons have been used to investigate the taxonomic status of natural isolates. Among the 4 isolates, 2 isolates (AT-R-S1, AT-R-S2) were identified as *Streptomyces griseoluteus*, AT-R-A1 and AT-P-S1 were identified as *Streptomyces griseus* and *Streptomyces Sp* respectively (Fig 3).

The isolate *Streptomyces griseoluteus* GDJ1 showed 98% sequence similarity with the previously published *Streptomyces sp* INBio\_4508H isolated from the gut of the Costa Rican Beetles. Whereas, the isolate *Streptomyces griseoluteus* GDJ2 showed 99% sequence similarity with the previously published *Streptomyces griseoluteus* strain NBRC 13375 collected from NIH, USA. The isolate *Streptomyces*

*griseus GDJ3* showed 98% sequence similarity with the previously published *Streptomyces griseoluteus strain NBRC 13375* collected from NIH, USA. The isolate *Streptomyces griseus GDJ3* showed 98% sequence similarity with the previously published *Streptomyces griseus strain cfcc3167* collected from Xuzhou, Jiangsu, China. Whereas, the isolate *Streptomyces Sp GDJ4* showed 99% sequence similarity with the previously published *Streptomyces sp. ZZY-2013 strain TRM46796-11* collected from Xinjiang, China. From both biochemical and molecular characters of four isolates of present study were belonging to *Streptomyces*, however they showed dissimilar profiles of biochemical and nutrient utilization.



**Figure 2:** A representative agarose gel showing amplified PCR product of 16S rDNA gene of actinomycetes isolated from Rameswaram and Periyar lake soil samples. M- Marker; Lane 1- Positive control; Lane 2 to 5- amplified 16S rDNA PCR product of isolates AT-R-S1, AT-R-S2, AT-R-A3 and AT-P-S1.



**Figure 3:** Phylogenetic tree of constructed based on 16S rDNA gene sequence of isolates

### 3.5 Purification of L-asparaginase

Based on the enzyme activity, the isolate *Streptomyces griseoluteus GDJI*(AT-R-S1) was taken for further study. In the present study, 63.78 % of yield of protein observed after

precipitation of crude extract with ammonium sulphate with reduced protein content of 152 mg and increased specific activity of 0.104 U/mg (Table 5). This shows that the protein get concentrated and purified than the crude extract.[21] Similarly, Dharmaraj [20] reported that the total protein of *Streptomyces noursei* decreased from 412 to 224 mg and the specific activity increased from 0.803 to 0.933 IU/mg after ammonium sulphate precipitation step. Whereas, the specific activity of 240, 21.21 U/mg was observed after ammonium sulphate precipitation [22]

The specific activity after Sephadex G-100 filtration was 4.613 U/mg with the total activity of 5.49 U/ml (Table 5). The results were comparable with the earlier findings suggested the increasing specific activity and decreased protein content. [20], [23], [24], [25]. After purification with Sephadex G-100 the samples were purified with CM Sephadex C-50 column.

Finally, the specific activity of purified L-asparaginase of *Streptomyces griseoluteus GDJI* (AT-R-S1) in the current study was lower than that of *Erwinia chrysanthemi*, Elspar® and Oncaspar® and their respective specific activities were 908, 270 and 85 u/mg [26], [27]. The degree of purification of the enzyme affects the enzyme activity and the specific activity. The reason may be that the commercially available enzymes are in highly purified form and the enzyme in the current work is partially purified. Further, additional purification steps have to be carried out to realize its full enzymatic potential. The present study suggests that ammonium sulphate precipitation was found to be highly effective. The Sephadex G-100 and Sephadex C-50 chromatography was found as important for the separation and purification of L-asparaginase. By this purification procedure, the strain *Streptomyces griseoluteus GDJI* (AT-R-S1) has produced L-asparaginase with 119 fold purity which is far better than compared to *Streptomyces gulbargensis* which has shown only 82.12 fold purity [28].

**Table 5:** Purification profile of L-asparaginase from *Streptomyces griseoluteus GDJI*

Purification steps	Total activity (U/ml)	Total Protein (mg)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude Extract	24.96	212	0.117	-	-
Ammonium sulphate precipitation	15.92	152	0.104	0.889	63.78
Sephadex G-100 filtration	5.49	1.19	4.613	39.144	21.99
CM Sephadex C- 50 Chromatography	1.27	0.09	14.11	119.85	5.088

### 3.6 Cell line Studies

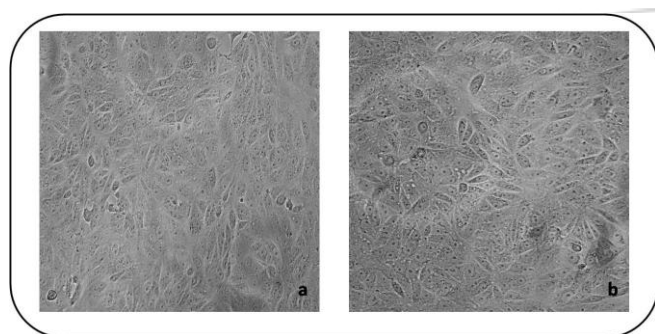
#### 3.6.1 Assessment of cytotoxicity

To use cancer treatment, L-asparaginase must be devoid of toxicity to normal cells. Hence, the purified L-asparaginase was tested for its toxic potential to VERO cell lines and the results showed that the L-asparaginase did not inhibit the growth (Figure 4). Cytotoxicity of purified L-asparaginase was evaluated on VERO cell line at various concentrations



(5µg – 45µg/ml) for 72 hours and the results were shown in Figure 21. From the results it is evident that the L-asparaginase was not toxic to VERO cell lines even after 72 hours of incubation at the higher concentration. The L-asparaginase doesn't show toxicity to the VERO cell lines up to 45µg which shows 100% cell viability (Figure 4). There were no significant changes observed between the control and treated cells.

The morphology and the cell viability in both control and treated cells showed insignificant change. Hence, it can be concluded that the purified L-asparaginase of *Streptomyces griseoluteus GDJI* (AT-R-S1) was non toxic to normal cell line. Similarly, Husain *et al.*, 2015 showed that L-asparaginase of *Enterobacter cloacae* was non-toxic to normal CHO cell line. It was shown that nearly 15–20% of patients treated with L-asparaginase of *E. coli* develop hypersensitivity and toxicity to the drug. [31], [32], [33], [1].



**Figure 4:** Cytotoxicity of purified L-asparaginase on VERO cell lines, a) Control cell without L-asparaginase and b) cells treated with 45µg/ml L-asparaginase after 72 h

### 3.6.2 Assessment of anti-proliferative activity

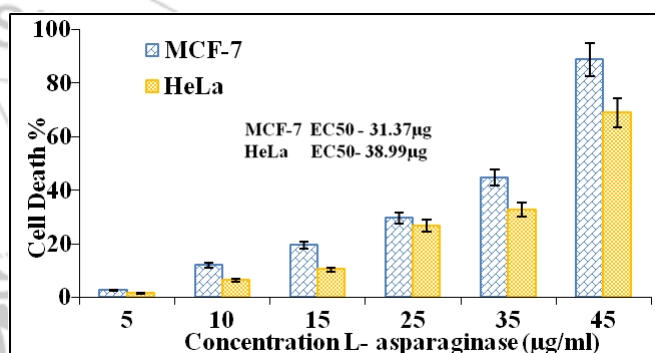
The ultimate aim of the present work is to extract the L-asparaginase with anti proliferative activity. Hence, MCF-7 and HeLa cell line was treated with different concentrations of (5µg – 45µg/ml) purified L-asparaginase for 72 h and the percentage of cell death was shown in Table 6 and Figures 5 a, b, c. It is clear that the percentage of cell death was gradually increased with increasing concentration of purified enzyme. The reduction in cell viability was linear and highly significant ( $r^2 = 0.918$ ; Figure 5 a). The highest cell death % (88.83%) was observed at the highest concentration 45µg/ml of L-asparaginase followed by 44.75% at 25µg/ml whereas, the lowest cell death was observed at 5µg/ml of L-asparaginase which was 2.5 %. The  $EC_{50}$  value was found to be 31.37 µg were 50% of cell death was occurred (Figure 5 a,b).

Whereas, the L-asparaginase was found to be less toxic to HeLa cell lines compared to MCF-7 (Table 6 and Figures 5 a,b,c). The lowest cell death 1.51% was observed at the lowest concentration of L-asparaginase (5 µg/ml) and the highest (68.84%) was at the highest concentration (45 µg/ml) with the  $EC_{50}$  value of 38.99 µg (Table 6). The results of the anti-proliferative suggested that the enzyme was highly potent to MCF-7 cells than HeLa cell lines. The higher  $EC_{50}$  is considered to be less active.

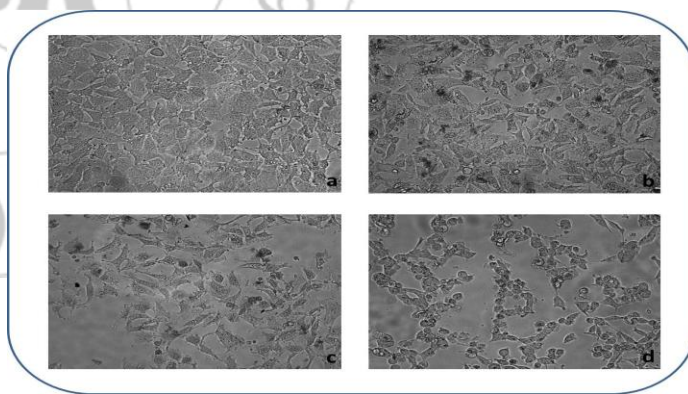
The changes in morphology of treated cells were tracked by inverted phase contrast light microscopy. The results revealed that the control of MCF-7 and HeLa cells which were not treated with L-asparaginase showed characteristic spindle shape morphology resembling healthy fibroblasts. MCF-7 and HeLa cells treated with different concentrations of L-asparaginase showed distorted morphology resembling dead fibroblasts which was found to be more at higher concentrations such as 25 and 45 µg/ml.

**Table 6:** Anti proliferative effect of different concentrations of L-asparaginase and its percentage of cell death

Concentration of L-asparaginase µg/ml	Cell death % of MCF-7	Cell death % of HeLa
5	2.512	1.51
10	11.95	6.45
15	19.38	10.39
25	29.72	26.72
35	44.72	32.72
45	88.84	68.84



**Figure 5: (a)** Effect of different concentrations of purified L-asparaginase of *Streptomyces griseoluteus GDJI* (AT-R-S1) on the cell death of MCF-7 and HeLa cell lines.

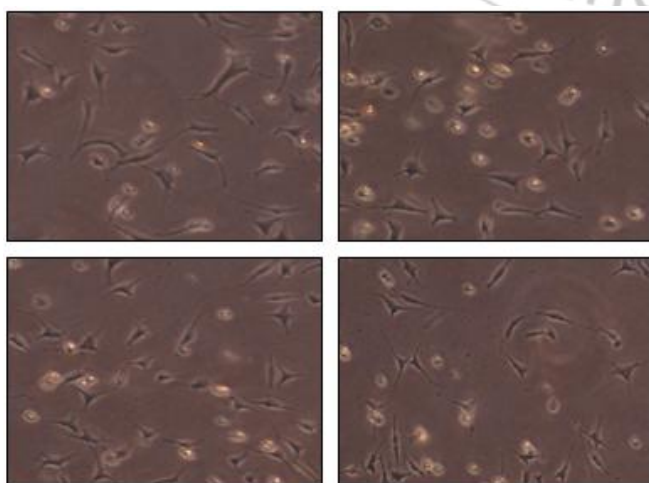


**Figure 5: (b).** Anti cancer activity of L- asparaginase in MCF-7 cell line. a) Control. b-d) 25,35,45 µg/ml concentration of L-asparaginase

Both MCF-7 and HeLa cells were treated with different concentrations of (5µg – 45µg/ml) purified L-asparaginase for 72 h and the cell death % was calculated and presented in Table 17 and Figures 5 a, b, c. The cytotoxicity of L-asparaginase was dose dependent and had effect on both cell lines however the MCF-7 was more susceptible than HeLa cells. It has been shown that the cytotoxicity of L-asparaginase differed with sources and cell lines [47] and the  $IC_{50}$  of L-asparaginase of *Salinicoccus* sp. MKJ997975 against HeLa was 0.171 IU/ml. The study of Oza *et al.*, [34]

showed that L- asparaginase of *Withania somnifera* L. active against acute lymphoblastic leukemia, and *Aspergillus flavus* (KUFS20) against MCF-7 cell line [35]. L-asparaginase interferes with DNA, RNA and protein synthesis, slowdown the growth and thus impedes cancer cells [36], [37] most specifically to the G<sub>1</sub> phase of cell division resulted in cell death. However, it is not necessary that all Asparaginases must possess cytotoxicity [29], [38], [40]. According to Song *et al.*, [39] L-asparaginase induce apoptosis during chronic treatment of K562 and KU812 cells.

Lymphoid origin and solid tumors were prone to L-asparaginase activity and showed resistant to the drugs among them soft tissue sarcoma [41], ovarian cancer [45],  $\beta$ -catenin mutated hepatocellular carcinoma [42], hepatocellular carcinoma with low expression of asparagine synthetase [43], and gastric adenocarcinoma [44] were studied. From the literature surveyed and reviewed L-asparaginase exhibited potent anti neoplastic and anti lymphomatic activity against tumors [1], [46], [29], [32], [40] This enzyme causes selective death of asparagines dependent tumor cells and also induces apoptosis in tumor cells [26]. It has been shown that the glutaminase activity affected the normal cell growth [40], [1]. However, several studies depicted that the L-asparaginase activity was depend on the L-glutaminase activity. The effect of these enzymes on the cell lines showed that the L-asparaginase with glutaminase activity has affected the tumor cells than the glutaminase free enzyme [30]. Their data demonstrated that asparaginase activity alone may not be sufficient for asparaginase cytotoxicity, and that glutaminase activity may be required for full anti-leukemic efficacy. The morphological changes during apoptosis include membrane blebbing, cell shrinkage was also observed with dose depended manner which was similar to the observations of Husain *et al.*, [29]. As a whole, the purified L-asparaginase of *Streptomyces griseoluteus GDJI* (AT-R-S1) did not inhibit the growth of VERO cells upto 45  $\mu$ g/ml whereas at 45  $\mu$ g/ml concentration nearly 88% of cell death of MCF-7 cells was observed. This observation led us to suggest that the purified enzyme has specific antitumor activity to cancer cells and could be safe for normal cells.



**Figure 5: (c).** Anti cancer activity of L- asparaginase in HeLa cell line. a) Control. b-d) 25,35,45  $\mu$ g/ml concentration of L-asparaginase.

## 4. Conclusion

The ultimate aim of the present work is to extract the L-asparaginase with anti proliferative activity. For this, actinomycetes were isolated and screened for L-asparaginase activity from the soil samples of Rameswaram and Periyar lake. Based on the quantification studies, AT-R-S1 was selected for further studies and the strain was identified as *Streptomyces griseoluteus GDJI* based on molecular identification. Purification of the enzyme from the strain *S.griseoluteus GDJI* (AT-R-S1) was carried out. Cytotoxicity of different concentration of purified L-asparaginase was determined on VERO cells. The L-asparaginase doesn't show toxicity to the VERO cell lines up to 45 $\mu$ g which showed 100% cell viability. The anti proliferative activity of L-asparaginase in MCF-7 and HeLa cells was determined and the EC<sub>50</sub> value was found to be 31.37 and 38.99  $\mu$ g respectively. The results of the anti-proliferative suggested that the enzyme was highly potent to MCF-7 cells than HeLa cell lines.

The above results were encouraging and worth pursuing for further development of *Streptomyces griseoluteus GDJI* (AT-R-S1) as an alternative resource for therapeutic L-asparaginase. Protein sequencing, gene identification and other bioinformatics parameters help further in establishing the value of the *Streptomyces griseoluteus GDJI* (AT-R-S1). Finally to conclude, L-asparaginase from *Streptomyces griseoluteus GDJI* (AT-R-S1) is the potential enzyme which has therapeutic and industrial applications.

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