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# Isolation and Identification of L-asparaginase Producing Bacteria from Soils of Different Agroclimatic Zones of Jammu (J&K), India

**Sweeta Manhas [a*], Vikas Sharma [a] and Asha Chaubey [b]**

*[a] Department of Biochemistry, Sher-e-Kashmir University of Agricultural Sciences and Technology, Chatha, Jammu, India.*
*[b] Fermentation Technology Division, Indian Institute of Integrrative Medicines, Canal Road, Jammu, India.*

***Authors' contributions***

*This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.*

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## ABSTRACT

A milestone was set in the field of medicine with the discovery of L-asparaginase present in the serum of guinea pig responsible for action against the acute lymphoblastic leukemia. Since then, the use of L-asparaginase as a potential anti-cancer drug has been a great success in the field of modern oncology. Despite its varied sources, new sources are continuously being explored to obtain a high therapeutic index drug. The study aims to isolate potential L-asparaginase producing microorganisms from environmental niches of Northwestern Himalayas. The climatic conditions of the region and ancient agricultural practices which are still being followed in these areas, add up to the diverse microbial repository and a potential habitat which can be explored to obtain a potent asparaginase producing microbial source. In the study, soil samples from different regions of Jammu were collected aseptically. From the isolation studies, a total of 44 bacterial isolates were

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**Corresponding author: E-mail: Sweetamanhas1@gmail.com;*

obtained. Pure cultures were then screened for asparaginase activity both qualitatively as well as quantitatively using agar plate and nesselerization method. Screening studies resulted in the isolation of a potent L-asparaginase producer isolated from black gram soil sample of Rajouri, Jammu showing an enzyme activity of 9.14 U/ml. Based on biochemical and molecular approaches, the isolate was identified as *Enterobacter aesburiae* strain R16C1 / No. MT93543. The study resulted in the isolation of a pure bacterial culture from the soils of Northwestern Himalayas possessing anti-cancer asparaginase activity. Further, optimization studies can help in considerably increasing the enzyme activity which can be tested against different human cancer lines for its anti-cancer efficacy.

*Keywords: L-Asparaginase; anti-cancer; Enterobacter aesburiae; Northwestern Himalayas; isolate.*

## 1. INTRODUCTION

L-asparaginase (EC 3.5.1.1) catalyses the amido hydrolytic breakdown of L-asparagine into L-aspartate and ammonia by attacking the side chain amide group in L-asparagine. The enzyme is produced by the cells to carryout deamination reaction of converting L-asparagine into ammonia and aspartic acid [1]. This catalytic action of the enzyme makes it a desirable anti-cancer drug which is potentially used in chemotherapy treatment of certain lymphoblastic malignancies, majorly in the treatment of Acute Lymphoblastic Leukaemia (ALL) and lymphosarcoma over the years [2]. Since, its discovery in guinea pig serum, L-asparaginase has shown anticancer activity which depends on the ability of asparaginase to hydrolyse asparagine [3, 4]. The enzyme uses in leukaemia treatment and other lympho proliferative disorders has expanded greatly due to its growth inhibiting properties by causing nutritional deprivation to the growing cancer cells. Leukaemia cells are different from normal cells as they lack asparagine synthetase and fail to produce asparagine making them completely dependent on the host to supply asparagine required for protein synthesis [5]. By causing the breakdown of asparagine in serum, L-asparaginase can create an environment which lacks asparagine in turn affecting synthesis of protein in leukemic cells. This leads to cancer cell growth inhibition or death [6]. Non-toxicity, biodegradability and its easy administration at local sites can be referred as the main properties of enzyme, preferred for cancer therapies [7].

A variety of microorganisms are known for their L-Asparaginase producing potency along with some plant and animal species. Amongst, its variety of sources, a high level of L-Asparaginase is produced using *Escherichia coli*, and *Erwinia chrysanthemum* species for anticancer therapy [8]. The multidisciplinary enzyme has wide applications in several other fields also including its role as a biosensor, mineralization of soil nitrogen, amino acids biosynthesis and its role in food industry to reduce acrylamide content of baked foods [9]. The enzyme grabbed attention due to its effective therapeutic effects against lymphocytic leukaemia various human cancers including breast, colorectal, lung *etc.* [10]. In recent studies, contribution of asparaginase to the reduction of cancer metastasis. has also been reported. Amongst other leukaemia treatments such as steroids, radiation therapy, severe combined treatments which includes stem cell or bone marrow transplants, chemotherapy is of great success and highly preferential [11]. Despite its success in the field, there is still need to look for new sources of asparaginase due to adverse reactions of the drug such as bronchospasm, hypotension, and urticaria along with hepatitis, altered production of coagulation factors and pancreatitis [12]. Other side effects include chills, vomiting, pancreatitis, fever, weight loss caused by hepatocellular dysfunction during treatment of Lymphoblastic Leukaemia [13]. Side effects are due to its inherent toxicity because of L-glutaminase activity. L-glutamine is essential for transporting nitrogen in the blood, while its long-term depletion during the treatment causes acute biochemical disorders in the body [14]. Presently, glutaminase-free bacterial production of asparaginase is in focus due to adverse toxicity effects of asparaginase [15]. There are varied sources of asparaginase but bacterial L-asparaginases have drawn much attention because of their eco-friendly nature, anticarcinogenic activities and cost effectiveness [16]. The enzyme obtained from different sources have different properties therefore looking for new asparaginase sources can lead to the possibility of obtaining a high therapeutic index drug which shows less or no adverse effects during treatment. The enzyme is found not only in microorganisms but other sources like many plants, animal tissues, bacteria, and in the serum of certain rodents [17].

The activity of asparaginase is dependent on its multimeric state and sources [18]. L-asparaginase from E. coli (Kidrolase) was natively a tetramer, but under storage conditions at -80°C, its oligomeric state was reportedly changed to a monomer and other higher-order state Both the states were less active compared to its native state [19]. L-asparaginase obtained from *Pyrococcus furiosus* was a fully functional dimer, while, under varied conditions it converted into a monomer that showed no or low activity [20]. Several other studies suggested that asparaginase from varied sources had different oligomeric states like dimer, hexamer or tetramer [21]. The variation in genetic makeup of enzyme when obtained from different sources suggests that exploring new sources of L-asparaginases could provide us with a serologically different enzyme having similar therapeutic effects. This study therefore aimed to explore the diverse microbial repository of Northwestern Himalayas and obtain a pure asparaginase producing isolate.

## 2. MATERIALS AND METHODS

Four different rhizospheric soil samples were collected from Janglote in Kathua, Jammu (black gram), Snetar in Udhampur, Jammu (Mung bean), Kangri in Rajouri, Jammu (black gram) and Loran in Poonch, Jammu (Rajmash).

**Collection of samples:** Plants were uprooted, and the soil was collected in sterile air tight polythene bags. Soil samples were air dried at 30 $^{0}$C in a hot air oven and kept for isolation in air tight containers.

**Isolation of pure cultures:** Using serial dilution method, isolation of L-asparaginase producing cultures from collected soil samples was carried out. 1 gm of soil from each sample was taken separately and suspended in 9 ml distilled water and left on a shaker for 1 h at room temperature. Soil samples were then serially diluted up to 10 ml with distilled water. Up to $10^{-8}$ dilutions were taken for all the samples [22]. Alternate dilutions from each soil sample were taken and 80 µl of the dilution was spread over nutrient agar plates. To control fungal growth, Fluconazole 75 µg/ml was added to the nutrient agar medium. All the plates were then incubated at 37°C for 24 - 48 h. Bacterial colonies of different size, morphology and colour were picked and streaked again on nutrient agar plates to isolate pure cultures. Pure cultures thus obtained were then used for the screening of L-asparaginase activity using qualitative and quantitative studies.

**Screening of asparaginase positive cultures:** Qualitative screening of L-asparaginase producing bacteria was done by using rapid plate assay method. Isolated cultures were inoculated on czapekdox agar media plates containing 1 % L-asparagine along with 0.1% phenol red indicator dye, inoculated plates were then incubated overnight at 37$^{0}$C along with control plates. The colonies showing formation of pinkish red colour were considered as L-asparaginase positive. The isolates showing L-asparaginase activity were then quantitatively estimated for enzyme activity using Nesslerization method [23]. The activity of enzyme was determined in culture broth by using asparaginase enzyme assay (Imada et al. 1973). For asparaginase assay, 0.1 ml of substrate solution (L-asparagine 189 mM) was used and mixed with 1 ml of buffer (Tris 50 mM, pH 8.6) followed by an icubation at 37 $^{0}$ C for 10 - 15 mins., To the incubated mixrure, 0.1 ml of enzyme solution was then added except blank and was further kept at 37 $^{0}$ C for 30 mins. 1.5M, TCA (0.1ml) was added to stop the reaction. The contents were then centrifuged and clarified. To 0.2 ml of supernatant, 4.3 ml of water (de-ionized) was added. Nessler's reagent (0.5 ml) was then added to the mixture. OD for the samples was taken at 436 nm. Ammonium sulphate standard curve was used to calculate the amount of ammonia released.

1 unit of L-asparaginase produced in the reaction mixture is the amount of enzyme which catalyses the formation of 1 µm of ammonia in 1 minute [24].

### 2.1 Characterization Studies

The isolates were smeared on the slide and heat fixed. The smear was air dried and observed under the microscope using gram stain. Biochemical characterization was done by performing different biochemical tests (Table 1). For molecular characterization, 16s rRNA sequencing was done. As per bacterial identification report, DNA of the isolated culture was extracted and amplification of 16S rDNA region was performed using Emerald Amp GT PCR master using 27F and 1492R primers, amplification of 1500 bp was performed and the test amplicon was purified using Gel elution/SAP.

The sequencing results were then assembled and compared with NCBI data base. 16S rRNA sequence obtained was aligned in the GenBank database using BLAST and sequences that were highly similar were used for the study of molecular taxonomy. Real Time method was used for generating the time. Divergence times were calculated for all the branching points in topology using Maximum Likelihood method [25] and evolutionary analysis was done in MEGA6 [26] (Fig. 1).

## 3. RESULTS AND DISCUSSION

The extracellular secretion of the enzyme is dependent on available nitrogen and carbon sources present in the medium and cultural parameters like medium pH, temperature at which the culture is incubated, size of the inoculum used and time of fermentation. All the factors are different for different types of organisms (Bascomb et al. 1975). Several microorganisms are known to produce asparaginase such as *Saccharomyces cerevisiae, Dickeya chrysanthemi, Escherichia coli*, *Serratia marcescens, Aspergillus sp*ecies, *Proteus vulgaris* among others and screening work still continues to find new asparaginase producers [27]. However, only from *E. coli* and *D. chrysanthemi* asparaginases are produced on an industrial scale for pharmaceutical use. In this study, we explored the possibility of isolating a

**Table 1. Biochemical characterization of bacterial isolate R16C1**

| **Morphological tests** | **Culture code R16C1** | **Results** |
|---|---|---|
| | Gram staining | -ve |
| | Shape | Rod shaped |
| | Color | Cream |
| **Biochemical tests** | Indole Test | +ve |
| | MR test | - ve |
| | VP test | +ve |
| | Citrate slant test | +ve |
| | Nitrate reduction test | +ve |
| | Arginine test | +ve |
| | Amylase test | -ve |
| | Protease test | -ve |
| | Asparaginase test | +ve |
| | **Carbohydrate fermentation tests** | |
| | Dextrose test | +ve |
| | Sucrose test | +ve |
| | Galactose test | +ve |
| | Fructose test | -ve |
| | Mannitol test | -ve |
| **Culture identified as per Bergey's Manual of Systematic Bacteriology Vol. 2** | Enterobacter | |

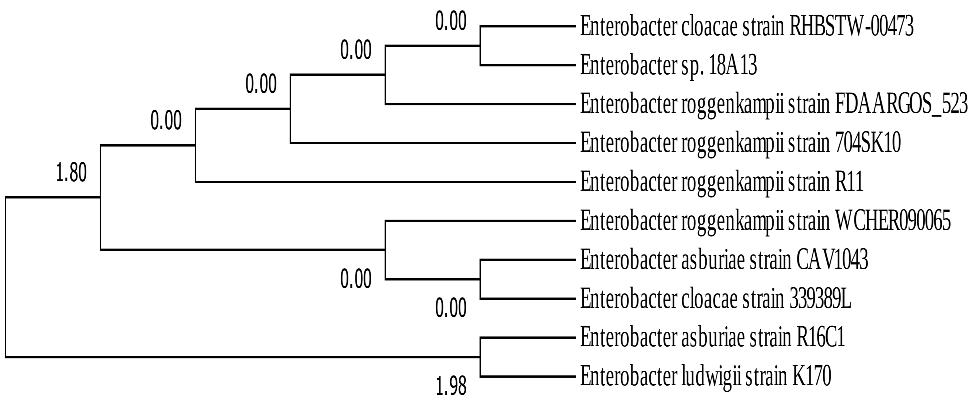


**Fig. 1. Phylogenetic tree of the results obtained**

new asparaginase producer from soil samples of different regions of J&K. 44 different bacterial isolates were obtained from the isolation studies conducted on 4 different rhizospheric soil samples collected from 4 different regions of J&K - black gram (Kathua), black gram (Rajouri), mung bean (Udhampur) and rajma (Poonch). Of all the cultures showing pink colour formation on nutrient plates containing 1% L-asparagine, those showing high intensity of pink colour formation were considered as L-asparaginase positive and picked up for further quantitative determination of enzyme activity using nesselerization enzyme assay Fig. 2.

On qualitative screening 2 out of 44 isolates showed L-asparaginase activity in rapid plate assay. L-asparaginase production accompanies pH increase of the growth medium due to the breakdown of asparagine into aspartic acid and ammonia. The release of ammonia causes alkalinity of medium which leads to change in colour. The plate assay was thus based on the incorporation of pH indicator (phenolred) in a 1% L- asparagine containing growth medium. A pink-colored zone was formed in the plates which indicated the production of enzyme [28]. Recently, plate assay method has become the most commonly used screening method due its sensitivity, efficiency, quick and reproducible results for the screening of a large number of microorganisms [29,30]. Based on primary screening, a quantitative study was performed to estimate asparaginase activity for both the cultures by using nessler's method and the culture isolated from black gram (Rajouri) soil sample exhibited maximum enzyme activity of 9.14 U/ml under un-optimized conditions. The observed enzyme activities for both the cultures were plotted. Bacterial strain R16C1 showed highest activity of 9.14 U/ml as compared to RA8C1 showing 4.22 U/ml of activity after an incubation of 72 h at 37$^0$C (Fig. 3). In both the strains maximum enzyme activity was observed after an incubation of 72 h.

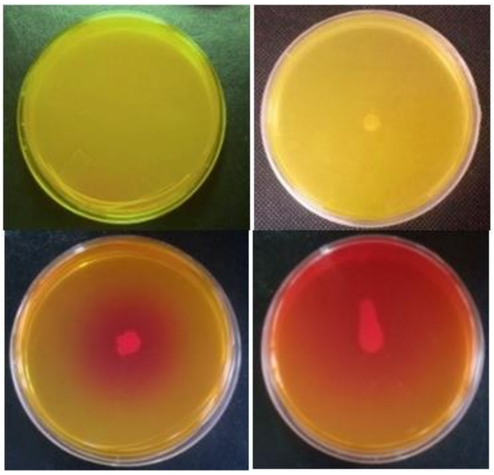

**Fig. 2. Plates showing (a) Control plate (b) L-Asparaginase –ve culture (c) & (d) L-Asparaginase +ve cultures**

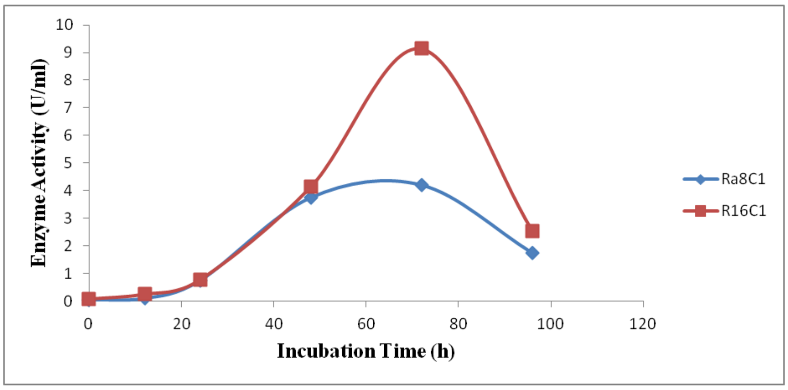


**Fig. 3. Comparative enzyme activity study of isolated bacterial cultures -Ra8C1 and R16C1**

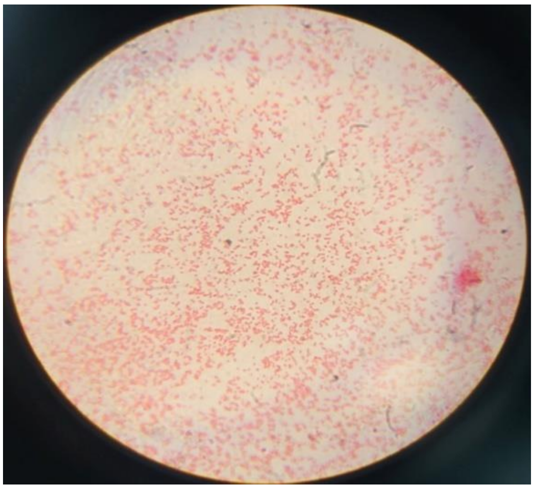

**Fig. 4. 100X view of gram stained culture R16C1**

## Enterobacter asburiae strain R16C1 16S ribosomal RNA gene, partial seq

GenBank: MT936543.1

FASTA   Graphics

```
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DEFINITION  Enterobacter asburiae strain R16C1 16S ribosomal RNA gene, partial
            sequence.
ACCESSION   MT936543
VERSION     MT936543.1
KEYWORDS    .
SOURCE      Enterobacter asburiae
  ORGANISM  Enterobacter asburiae
            Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacterales;
            Enterobacteriaceae; Enterobacter; Enterobacter cloacae complex.
REFERENCE   1  (bases 1 to 1566)
  AUTHORS   Manhas,S., Sharma,V., Chaubey,A. and Mansoor,S.
  TITLE     Sequence characterization of R16C1-strain isolated from black gram
            rhizospheric soil sample of Rajouri region (J&K)
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1566)
  AUTHORS   Manhas,S., Sharma,V., Chaubey,A. and Mansoor,S.
  TITLE     Direct Submission
  JOURNAL   Submitted (28-AUG-2020) Biochemistry, SKUAST Jammu, Chatha, Jammu,
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            ##Assembly-Data-END##
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                     /country="India"
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                     /product="16S ribosomal RNA"
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      181 cgagcgscag cggamagtag cytgctactt tgccggcgag cggcggacgg gtgagtaatg
      241 tctggsaaac tgcctgatgg agggggataa ctactggaaa cggtagctaa taccgcataa
      301 cgtcgcaaga ccaaagaggg ggacctcggg cctcttgcca tcagatgtgc ccagatggga
      361 ttagctagta ggtggggtaa cggctcacct aggcsacgat ccctagctgg tctgagagga
      421 tgaccagcca cactggaact gagacacggt ccasactcct acgggaggca gcagtgggga
      481 atattgcaca atgggcgcaa gcctgatgca sccatgccgc gtgtatgaag aaggccttcg
      541 ggttgtaata ctttcagcgg ggaggaaggc gttgaggtta atawmctcag cgantgacgn
      601 tacccgcara nnragcanck gctaactccg tgccagcagc cgcggtaata cggagggtgc
      661 wagcgtnnat cggaattact gggcgtaaag cgcacgcngg cggtctgtca agtcrgatgt
      721 gaaatcccyg ggctcannct gggaactgca ttcgaaactg gcrggctaga gtcntgtaga
      781 gggggtaga attccrggtg tagcngtaaa tgcgtagaga tctggaggaa taccggtggc
      841 garsgcggnc ccctggacaa asactgacgc tcaggtgcga agcgtgggga gcaaacrgga
      901 ttasataccc yggtagtcca cgccgtaaac gatgtcsact tggaggttgy kccctgaggc
      961 gtsgctyccg gagctmacgc gtyaagtcga ccgcctgggg agtacgsycs smaggttram
     1021 actmawawrw attgacgggr gcscgcacaa gcggtggagc rtgtggttkw attygatgcw
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https://www.ncbi.nlm.nih.gov/nuccore/MT936543

**Fig. 5. *Enetrobaxter aesburiae*16rRNA sequence derived from isolated cultureR16C1**

Further, characterization studies for the culture showing highest activity were carried out. Morphological, biochemical as well as molecular studies were used to characterize the isolated bacterial strain (R16C1). Gram staining of bacterial isolate R16C1 showed gram negative bacteria, round edged, non-elevated colonies, short rods in shape (Fig. 4). Different biochemical tests were performed for the isolate R16C1. Biochemical characterization results showed that R16C1 was able to utilize galactose, dextrose and sucrose with positive test results for arginine decarboxylation, indole, citrate slant, nitrate reduction and VP test whereas the culture gave negative test results for amylase, protease, fructose, mannitol and MR test (Table 1). Characterization results showed a close relationship of the culture obtained to Eenterobacteriacae bacterial family. Molecular studies on the culture using 16s rDNA showed that the culture belongs to Enterobacteriacae family. Both the studies indicated that the culture is a member of Enterobacteriacae bacterial family. Based on the results, the strain was identified as *Enterobacter aesburiae* and the sequence product of the culture was submitted in Genbank database, Accession number MT93543 (Fig. 5). Of all other asparaginase producing bacterial species, members of bacterial family Enterobacteriaceae are known to be the best producers of asparaginase [28].

## 4. CONCLUSION

Using first asparaginase therapy was breaking medical innovation and its success in the field helped in extending the lives of millions of people but the asparaginase products that are being currently used in the market do not possess desirable pharmaceutical characteristics. Therefore, different sources of the enzyme sources are being looked for in order to produce enzyme with high chemotherapeutic index to be used as frontline therapy thus, reducing the risks associated with the line of treatment. Considering the need, this work deals with the bioprospection of microorganisms from different soil samples of north-western Himalayas for L- asparaginase enzyme. Different samples of rhizospheric soil were collected from Jammu Division for the isolation of a potential L-asparaginase producer. Of all the isolates, two bacterial strains Ra8C1 and R16C1 were found positive for enzyme production. The strains were then tested quantitatively using nessler's assay for the production of enzyme. A comparative study revealed that the culture from black gram soil sample of Rajouri (R16C1) showed highest asparaginase production of 9.14 U/ml Isolation and screening studies thus gave a potential L-asparaginase producer from black gram soil sample of Rajouri (Jammu). Further, biochemical and molecular studies revealed that the isolated culture was *Enterobacter aesburiae*. Conclusively, a new bacterial species-*Enterobacter* has been isolated and identified in the present study. By inducing optimal assay conditions and optimizing different parameters that affect the enzyme activity, asparaginase activity can be further increased and produced on a large scale under optimal conditions for further purification and anti-cancer studies on different human cancer cell lines.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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