Isolation and identification of L-asparaginase producing bacteria from soils of different agroclimatic zones of Jammu (J&K)

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 North Western 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 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 soil s of North Western 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.

Key words: L-Asparaginase, anti-cancer, *enterobacter aesburiae*, North Western Himalayas

Introduction:

L-asparaginase (EC 3.5.1.1) catalyszis the amidohydrolytic breakdown of L-asparagine in to L-aspartate and ammoia by attacking the side chain amide group in L-asparagine. 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 lymphosarcomas over the years (Egler et al.,

2016). Since, its discovery in guinea pig serum, L-asparaginase has shown anticancer activity which depends on the ability of asparaginase to hydrolyse asparagine (Kidd, 1953; Yellin and Writson, 1966). The enzyme use in leukemia 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 asparagines making them completely dependent on the host to supply asparagines required for protein synthesis (Rizzari et al., 2014). By causing the breakdown of asparagine in serum, L-asparaginase can create an environment which lacks asparagine in turn affecting synthesis of protein in leukaemic cells. This leads to cancer cell growth inhibition or death (Choi and Coloff, 2019). Non-toxicity, bio-degradability and its easy administration at local sites can be referred as the main properties of enzyme, preferred for cancer therapies (Kamble et al., 2012).

A variety of microorganisms are known for their L-Asparaginase producing potency alongwith 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 (Mohammed M Kassab., 2023). 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 (Jia et al., 2021). The enzyme grabbed attention due to its effective therapeutic effects against lymphocytic leukemia various human cancers including breast, colorectal, lung *etc.* (Kumar and Sobha, 2012). In recent studies, contribution of asparaginase to the reduction of cancer metastasis. has also been reported. Amongst other leukemia 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 (Agarwal and Kango, 2019). 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 (Sanawar et al., 2017). Other side effects include chills, vomiting, pancreatitis, fever, weight loss caused by hepatocellular dysfunction during treatment of Lymphoblastic Leukemia (Lim et al., 2021). 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 (Kravchenko et al., 2008). Presently, glutaminase-free bacterial

production of asparaginase is in focus due to adverse toxicity affects of asparaginase (Prihnato et al., 2020). There are varied sources of asparaginase but bacterial L-asparaginases have drawn much attention because of their eco-friendly nature, anticarcinogenic activities and costeffectiveness (Ashok et al., 2019). 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 the course of 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 (Lopes *et al*., 2015). The activity of asparaginase is dependent on its multimeric state and sources (Bansal et al., 2010). 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 (Charbonnaeu et al., 2017). 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 (Garg et al., 2017). Several other studies suggested that asparaginase from varied sources had different oligomeric states like dimer, hexamer or tetramer (Einsfeldt et al., 2016). 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 North Western Himalayas and obtain a pure asparaginase producing isolate.

Materials and Methods

Four different rhizospheric soil samples were collected from Kathua (black gram), Udhampur (Mung bean), Rajouri (black gram) and Poonch (Rajmash)**.** Plants were uprooted and the soil was collected in sterile air tight polythene bags. Soil samples were air dried at 30 ^{0}C in an hot air even and kept for isolation in air tight containers. 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. (Devi and Ramanjeneyulu, 2016). 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.

Qualitative screening of L-asparaginase producing bacterias was done by using rapid plate assay method. Isolated cultures were inoculated on czapekdox agar media plates containing 1 % L-asparagine alongwith 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 (Gulati *et al*., 1997).

Characterization studies

The isolates were smeared on the slide and heat fixed. The smear was air dryed 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. RelTime method was used for generating the time. Divergence times were calculated for all the branching points in topology using Maximum Likelihood method (Tamura and Nei, 1993) and evolutionary analysis was done in MEGA6 (Tamura *et al*., 2013) (Fig. 4).

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 inocculum 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 (Farag *et al*., 2015). 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 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. # 1.

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 in to 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 (Doriya and Kumar, 2016). 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 (Dhale and Mohan-Kumari, 2014; Meghavarnam and Janakiraman, 2015). 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 (Figure # 2). In both the strains maximum enzyme activity was observed after an incubation of 72 h.

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. # 3). 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 (Figure # 5). Of all other asparaginase producing bacterial species, members of bacterial family Enterobacteriaceae are known to be the best producers of asparaginase (Doriya and Kumar, 2016).

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.

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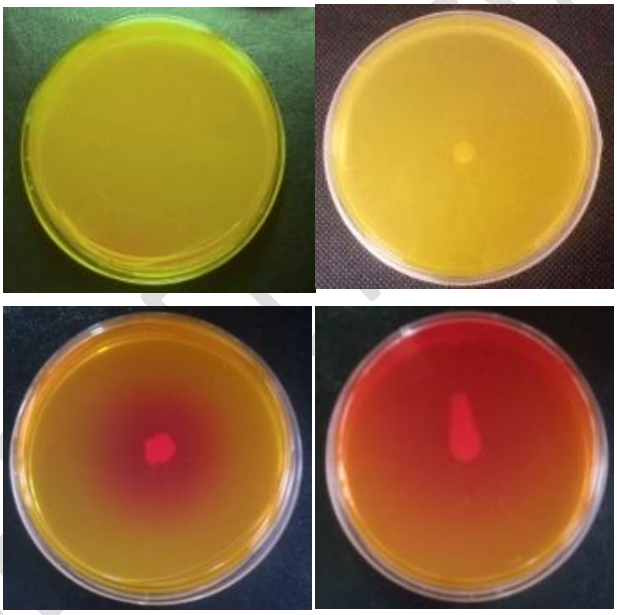

Figure # 1: Plates showing (a)Control plate (b) L-Asparaginase –ve culture (c) & (d) L-Asparaginase +ve cultures

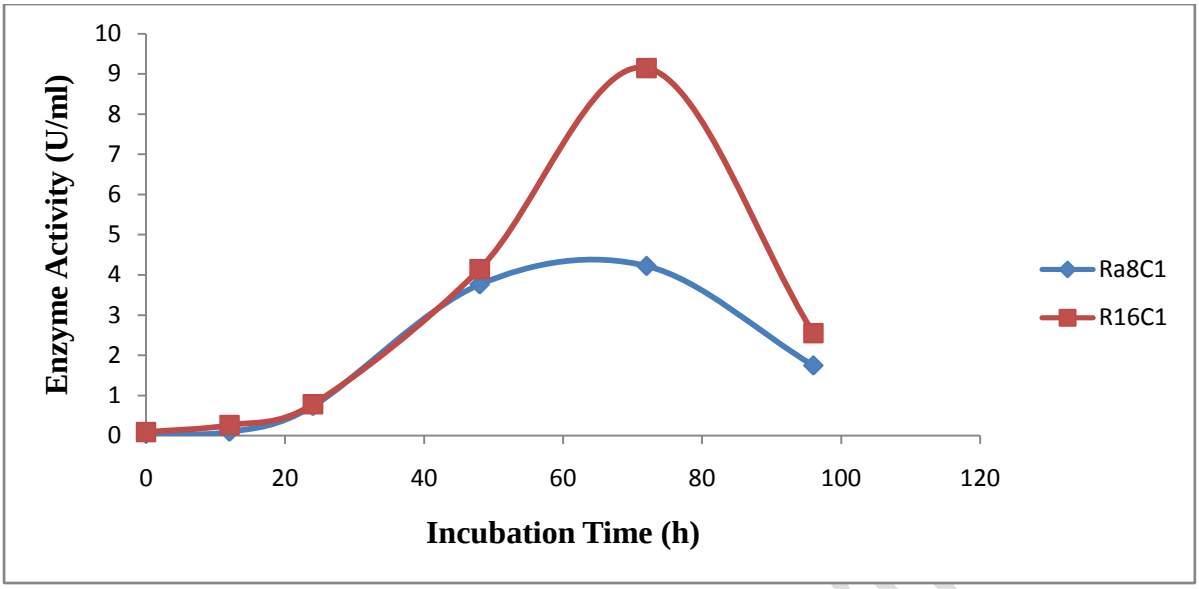

Figure # 2. Comparative enzyme activity study of isolated bacterial cultures -Ra8C1 and R16C1

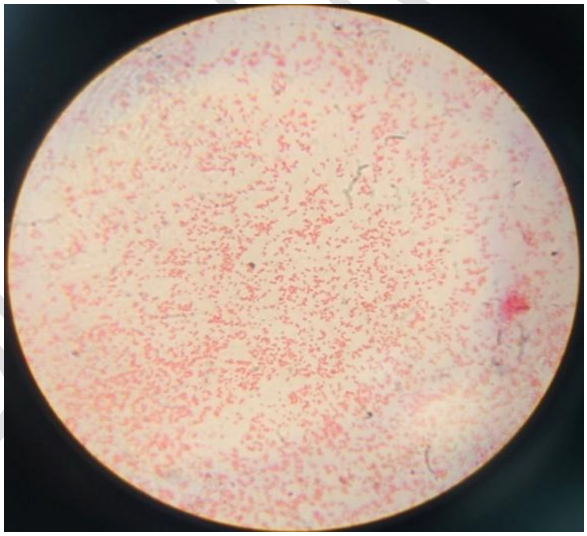

Figure # 3. 100X view of gram stained culture R16C1

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	

Table 1. Biochemical characterization of bacterial isolate R16C1

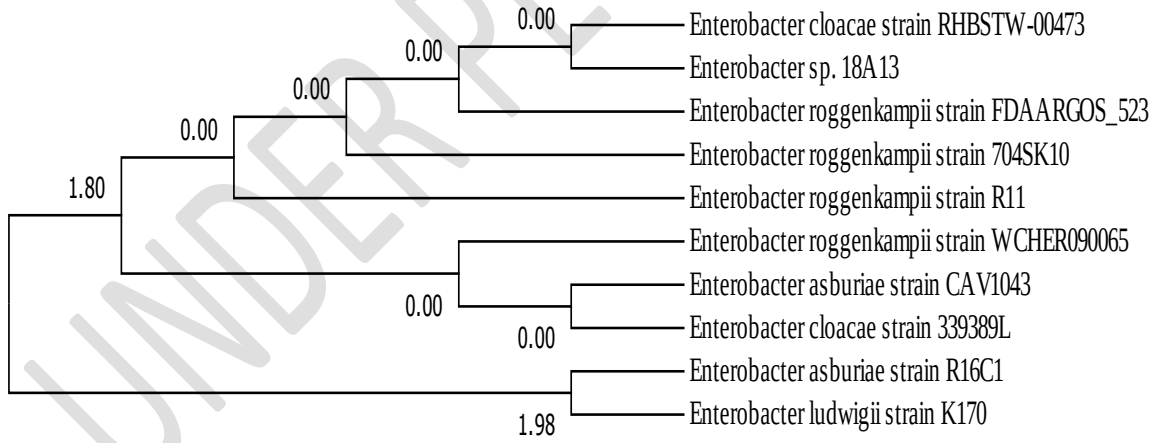

Figure # 4 Phylogenetic tree of the results obtained

GenBank

Enterobacter asburiae strain R16C1 16S ribosomal RNA gene, partial s

GenBank: MT936543.1

FASTA Graphics

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            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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https://www.ncbi.nlm.nih.gov/nuccore/MT936543


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     1561 gcgctt
//
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Figure # 5.*Enetrobaxter aesburiae*16rRNA sequence derived from isolated cultureR16C1