IJCRT.ORG ISSN : 2320-2882

INTERNATIONAL JOURNAL OF CREATIVE RESEARCH THOUGHTS (IJCRT)

An International Open Access, Peer-reviewed, Refereed Journal

ISOLATION OF L-ASPARAGINASE PRODUCING BACTERIA FROM SOIL

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Abstract: The current study deals with the isolation of L-Asparaginase enzyme from bacterial strains, through serial diluting the soil samples from Coimbatore, India. A total of 15 bacterial strains were isolated from the soil samples. The bacterial strains were subjected to primary screening where the positive strains are known to produce pink color zone around the colonies after incubation for 48 hrs on M9 media. Only 5 isolates were positive for producing L-Asparaginase. The positive strains were subjected to morphological studies, cultural characteristics and biochemical tests. The strains were subjected to secondary screening by submerged fermentation and the crude enzyme activity was determined by Nesselerization method, the highest enzyme activity was found to be 1.902 U/ml (B–I–13) and estimation of protein was determined by using Lowry's method. The organism which produced highest enzyme activity was identified by 16S rRNA sequencing method and phylogenetic tree was constructed. The isolated organism was found to be *Bacillus subtilis* which can be used to produce L-Asparaginase.

Keywords: L-Asparaginase, Bacillus subtilis, acute lymphoblastic leukemia, acrylamide.

I. INTRODUCTION:

Asparaginase is an anti-carcinogenic enzyme used to treat acute lymphoblastic leukemia (Rachel *et al*., 2016) which is a type of cancer of the blood and bone marrow that affects white blood cells (Jha *et al*., 2012). Asparaginase was first used to treat Acute lymphoblastic leukemia (ALL) patients. L-asparagine is an essential amino acid used for nutritional requirement of both normal and cancer cells. Lymphatic cancer cells require huge amount of L-asparagine for their growth. Leukemic cells are deprived of the asparagine required for DNA, RNA, and protein synthesis when asparaginase catalyses the conversion of asparagine to aspartic acid and ammonia, which eventually results in inhibiting cell development, and ultimately activates apoptotic cell-death processes. So the tumor cells die due to starvation of L-asparagine (Joao *et al*., 2020). The asparaginase is added to some foods in order to reduce the quantity of the amino acid asparagine (Shivaji & Priyanka., 2021). Clinically available asparaginase is generated from two sources, namely *Escherichia coli* and *Erwinia chrysanthemi*, according to investigations conducted utilizing bacteria to find alternative sources of asparaginase in the 1960s (Schwartz *et al*., 1966). L-asparaginase from *Erwinia* showed less allergic responses compared to the *E. coli* L-asparaginase. Also, *Erwinia* asparaginase had a shorter half-life than *E. coli* asparaginase. Therefore, L-asparaginase from new sources may have great financial impacts in treatment of ALL (Asselin *et al*., 1993). Accordingly, this study is aimed at isolating L-Asparaginase producing bacteria from soil. The identified bacteria can be optimized and used in the treatment of acute lymphoblastic leukemia.

II. METHODOLOGY:

2.1 Isolation of soil bacteria:

For the isolation of L-Asparaginase enzyme producing microorganism, 5 soil samples were collected from Coimbatore in sterile plastic covers. 1g of soil is dispensed in 100 ml of sterile distilled water and it was serially diluted to get different dilutions, and 0.1 ml of each dilution was plated onto a nutrient media by spread plate method (Murugesan & Palaniswamy, 2018). Different colonies on the media is isolated and plated on media, pure culture are isolated and stored for further studies. The cultural characteristics of the colonies developed on the nutrient media were observed (Murugesan & Palaniswamy, 2018).

2.2 Primary screening on M9 media:

Using the M9 media, bacterial strains were tested for early L-Asparaginase activity. The pH of the medium was change to 6.2 and 0.005% phenol red dye was added. The bacterial strains were inoculated and incubated for 48 hours at 37^0C. The intensity of the pink color developed on the streaked plates after incubation allowed for the identification of L-Asparaginase activity (Mahmoud *et al*., 2019).

2.3 Screening of L-Asparaginase by submerged fermentation:

The bacterial strains that showed pink zone around the colonies on modified-M9 solid media were then used for the submerged fermentation process that produces L-Asparaginase. Bacterial suspension was added to 50 ml of M9 broth before being incubated on a rotary shaker at 37 °C for 18 to 20 hours. The 72-hour-old cultures were taken and centrifuged for 10 minutes at 10,000 rpm at 4°C. The supernatant was collected and used as a source of the crude enzyme (Amany *et al*., 2020).

2.4 Cell separation:

The 50 ml of culture was taken in the sterile centrifuge tubes and were centrifuged at 10000 rpm for 1 minute in a cooling centrifuge. The supernatant was then transferred carefully into another sterile storing vial were stored in refrigeration conditions (Robert *et al*., 1997).

2.5 L-Asparaginase assay:

The hydrolysis of L-Asparagine to release ammonia was measured by Nessler's reaction, was used to estimate the L-Asparaginase activity (Yim & Kim 2019). A 0.1 ml of enzyme extract, 0.2 ml of 0.05M tris-HCl buffer solution (pH 8.6), and 1.7 ml of 0.01M L-asparagine waas incubated for 10 min at 37°C. The reaction was stopped by adding 0.5 ml of 1.5 M Trichloroacetic acid (TCA) and centrifuged at 10000 rpm for 10 mins. 0.5 ml of supernatant was then diluted by adding 7ml of distilled water and then treated with 1 ml of Nessler's reagent. Absorbance at 480 nm was measured after the color reaction had been allowed to develop for 10 minutes (Kishor *et al*., 2015). One international unit (IU) of L-asparaginase was defined as amount of enzyme that liberates 1micro mole of ammonia per minute (Prakasham *et al*., 2007).

2.6 Estimation of protein by Lowry's method:

Proteins are estimated by quantifying the amino acids present in the sample. The intensity of the color development depends on the amount of theses aromatic amino acids present in the protein sample and absorbance at 600nm was measured in spectrophotometer (Lowry *et.al*., 1951).

2.7 Biochemical tests:

The morphological & biochemical characteristics of the bacteria were studied by using Gram staining (Bartholomew & Mittwer., 1952), Indole test (Maria & Willams., 2009), methyl red test (Devitt., 2009), Voges-Proskauer test (Eddy., 1961) and citrate utilization (Maria & Willams., 2009) to find the bacteria in the genus level

2.8 Molecular identification of bacterial strain:

The 16S rRNA gene sequencing technique was used to identify the bacteria that passed the screening. By using the Marmur technique, DNA was extracted from the bacterial species and the purity of the extracted DNA was assessed using a spectrophotometer set to 260/280 values. The extracted DNA was examined by gel documentation unit and polymerase chain reaction (PCR) was used to amplify the recovered DNA. The forward primer and reverse primer were the Universal Primer sequences. Using an Eppendrof Master Cycler Personal, the PCR amplification cycle was completed (Alrumman *et al*., 2019). To visualize the PCR product that had been amplified, 16 µl of the amplified product was subjected to

electrophoresis and visualized under a Gel documentation unit (Pradhan *et al*., 2013). The Forward primer (5'-GAGTTTGATCCTGGCTCAG-3') and Reverse primer (5'-ACGGCTACCTTGTTACGACTT-3') were used for PCR.

2.9 Phylogenetic tree:

Using the NCBI BLAST, the 16S rRNA gene sequences were compared to the reference species of bacteria found in the genomic database library. The homology was less than 100% in all cases probably indicating new bacterial strains. The sequencing results were eventually blasted on the NCBI website, and the sequences were compared with those available in the gene bank to determine the samples in the phylogenetic tree (Pourmolaei and Tobi., 2020) . Using the Clustal W software, multiple alignments of the sequences were carried out. Phylogenetic tree was constructed using Mega 5 software (Thandeeswaran *et al*., 2016). The similar organisms related to our species are also studied and reported.

III. RESULTS AND DISCUSSION:

3.1 Isolation of soil bacteria

Totally 15 bacterial isolates were recovered from the soil samples and their cultural characteristics like shape color, size and opacity based on the colonies developed on the nutrient media are studied. The colonies developed on the nutrient media are sub cultured on the media and incubated and then they are stored at 4^0C for further analysis.

3.2 Primary screening on M9 media

L-Asparaginase hydrolyzes asparagine into aspartic acid and ammonia so it produces pink color zone around the colonies which are positive for L asparaginase producing bacteria. When ammonia is released, phenol red indicator, which has a pale yellow color in an acidic environment, changes color to pink as the pH rises (Dalfard, 2016). In this study 5 bacteria are known to produce L - Asparaginase enzyme when they are cultured on the M9 media producing pink color zone around the colonies which contain L asparaginase. These bacterial strains were used further for the analysis of L asparaginase as shown in **(Fig 1).**

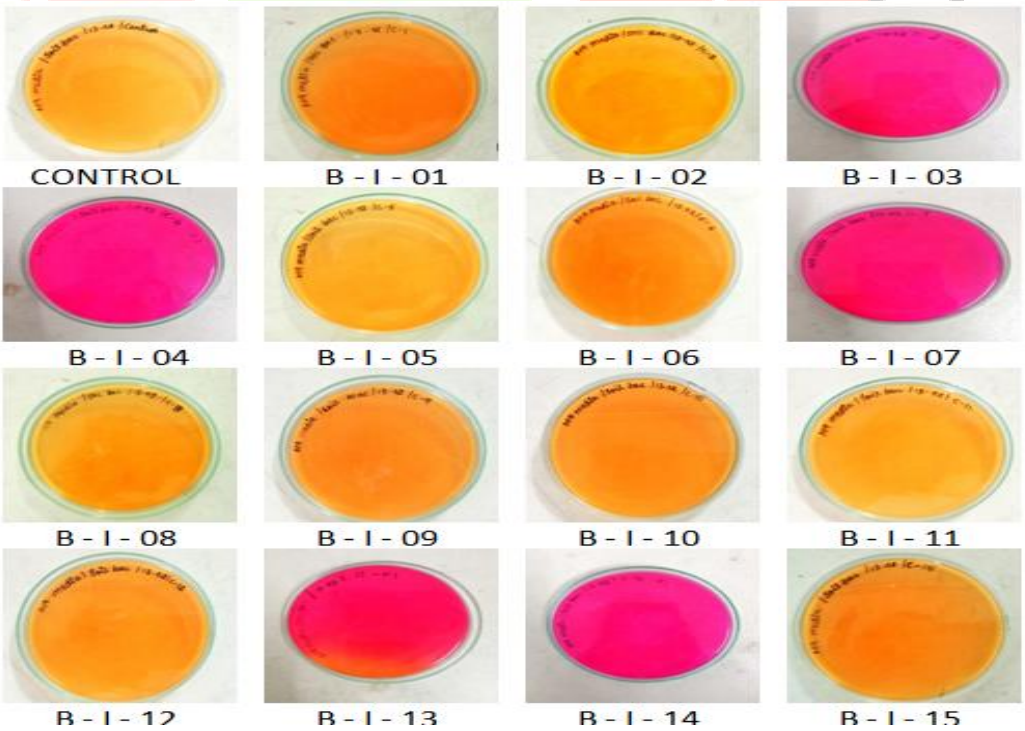

Fig 1: Primary screening on M9 media

This is consistent with our findings of Gulati *et al*. (1997) who demonstrated that the synthesis of L-asparaginase was the cause of color change. Good L-asparaginase producers are strains with zones that are at least 0.9 cm in diameter; moderate and weak L-asparaginase producers are those with zones that are between 0.6 and 0.9 cm in diameter respectively.

3.3 Estimation of enzyme activity through nesselrization method:

The chosen isolates were then tested for L-Asparaginase activity in submerged fermentation using M-9 medium and incubated at 120rpm in orbital shaker at 37^0C for 24 hrs. The bacterial cell mass was centrifuged, the supernatant was used as the crude enzyme source (Tallur *et al*., 2013). According to Mashburn and Wriston's (1964) description, asparaginase activities were regularly measured by direct nesseleriztion, where the rate of asparagine hydrolysis is determined by measuring released ammonia.

Table 1: Enzyme activity of L-Asparaginase produced by the bacterial strains

S .No	Bacterial Isolates	Specific activity (U/ml)	Protein concentrations (U/ml)
1	B – I – 03	1.240	0.38
2	B – I – 04	0.671	0.34
3	B – I – 07	1.586	0.42
4	B – I – 13	1.902	0.46
5	B – I – 14	1.019	0.28

Kothari and Deshmukh (2014) found 1.79 U/ml with 1.33 U/ml of a total protein concentration. In another study the higher specific activity would be *E.coli* CTLS20 (1.787 U/ml). (Thandeeswaran *et al*., 2016). Similarly Robert *et al*. (1968) showed 0.950 U/ml of specific activity from *E.coli* HAP strain.
In this study the maximum specific activity from the estimation of enzyme through nesselerization of the bacterial strain (B –I – 13) was known to be (1.902 U/ml) followed by the isolate (B – I – 7) to be (1.586 U/ml) as shown in Table 1**.** Further the organism which has higher specific activity would be identified by morphological, biochemical characteristics and molecular identification of bacterial strain, that would be used as a source for the L Asparaginase enzyme production used in the cancer treatment.

3.4 Identification of bacterial strain

Morphological and biochemical tests indicated the bacterial strains found to be *Bacillus spp*. and further the selected strain was subjected to 16S rRNA sequencing. The genomic DNA was isolated from the efficient strains and PCR amplification was performed. FASTA format of the bacterial strains was obtained. The result obtained by 16S rRNA gene sequencing and by using BLAST tool showed the bacterial strain B-I-13 was *Bacillus subtilis*.

GGATGGTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACTTACAGAT
GGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGC
CGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGG
CAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGAT
GAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGCG
GTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAAT
ACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAA
GTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGT
GCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACA
CCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGC
GAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGG
TTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAA
GACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTC
GAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAATCCTAGAGATAGGACGT
CCCCTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTT
GGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTC
TAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCC
TTATGACCTGGGCTACACACGTGCTACAATGGACAGAACAAAGGGCAGCGAAACCGCGAGG
TTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAA
GCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTAC
ACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTTAGGA
GCCAGCC

FASTA format of *Bacillus subtilis*

Bacillus subtilis was found to be an ideal producer of extracellular L-Asparaginase. (Pradhan *et al*, 2013). According to Alrumman *et al*. (2019), the study reported that the isolated organism was found to be *Bacillus licheniformis* by 16S rRNA gene sequencing method. In another study the Bacillus subtilis was isolated from the fermented soybeans (Lim *et al*., 2021).

3.5 Phylogenetic tree construction:

The phylogenetic tree was constructed using MEGA5 or Clustal W software. The similar organisms related to our species are also reported. From the phylogentic tree, the organisms similar to the Bacillus subtilis were obtained as shown in (**Fig 2).**

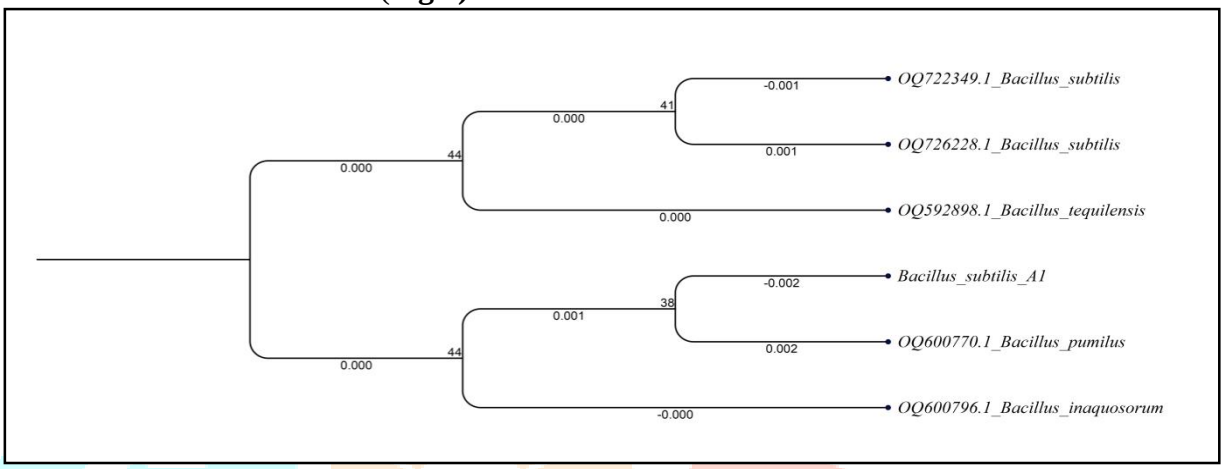

Fig 2: Phylogenetic tree

Mahboobi *et al*., (2017) focused on the application of bioinformatics tools to study about E.coli model because it is a good source of L-Asparaginase enzyme which has an antitumor ability.

IV. SUMMARY AND CONCLUSION:

In this study, B-I-13 was the best L-asparaginase producer, showed higher enzyme activity through nesslerization method. From the morphologically, biochemically & genetically identification of bacterial strain, the isolated organisms was known to be *Bacillus subtilis.* Further studies are aimed at optimization and purification studies of L-Asparaginase and evaluation of anticancer activity

V. ACKNOWLEDGEMENT:

The authors are grateful to the management of Dr. N.G.P Arts and Science college, Principal and Department of Biotechnology for their constant support. The communication number is DRNGPASC 2022-23 BS021.

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