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(RESEARCH ARTICLE)

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Screening and molecular identification of l-asparaginase producing bacteria with ansB gene from sewage water

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Abstract

Enzymes are vital biocatalysts facilitating biochemical reactions in organisms, playing pivotal roles in both biological systems and numerous industrial applications. This study focuses on the isolation and screening of asparaginase-producing bacteria from sewage water samples in Bhopal City. A modified M9 media, supplemented with asparagine and phenol red, was used to isolate asparaginase-producing bacteria. Out of the ten samples, seven exhibited asparaginase activity. Two bacterial strains, identified as *Bacillus tropicus* (S-1) and *Priestiaveravalensis* (S-2), were found to harbor the ansB gene. Sequence analysis revealed a 96.52% and 95.09% resemblance to *Bacillus tropicus* MCCC 1A01406 and *Priestiaveravalensis* strain SGD-V-76, respectively. These findings highlight the potential of these bacterial strains in producing L-asparaginase, emphasizing their significance in pharmaceutical applications, particularly in cancer treatment.

Keywords: Anticancer enzymes; L-Asparaginase; Sewage water; Microbial metabolites

1. Introduction

Enzymes act as crucial biocatalysts that enable biochemical reactions within living organisms under the gentle conditions found in cellular settings (Robinson, 2015; Blanco and Blanco, 2017). These catalysts are essential for the various chemical transformations that maintain life, dramatically speeding up metabolic processes by as much as 10 billion times compared to standard chemical reactions (Gurung *et al*., 2013). Beyond their role in biological systems, enzymes play a vital role in a wide range of industrial applications. They are instrumental in the creation of sweetening agents, the modification of antibiotics, and are used in cleaning products and analytical tools with applications in clinical, forensic, and environmental sectors (Robinson, 2015).

The biotechnology sector prioritizes enzyme production, utilizing traditional and advanced methods like genetic engineering to scale up production of naturally scarce proteins (Headon and Walsh, 1994). Asparaginase is a key enzyme with roles in pharmaceuticals, biosensors, and food industries. It shows promise in cancer treatment by degrading L-asparagine, leading to nutrient deprivation and cancer cell death (Karpel-Massler *et al*., 2016; Martinez-Outschoorn*et al*., 2017).

Microorganisms, especially bacteria and fungi, are key enzyme sources due to their rapid large-scale cultivation and the potential for genetic enhancements to boost enzyme yield (Laatsch, 2006; Lebaret*et al.,* 2007). While *Erwinia chrysanthemi* and *E. coli* are known asparaginase producers for commercial use (Alrumman*et al.,* 2019), there's a need

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for new asparaginase variants with better stability, reduced glutaminase activity, and increased substrate affinity (Mohamed *et al.,* 2015). This study explores native sewage water microbes containing the ansB gene, from slum areas of Bhopal, M.P. India, aiming to identify strains with improved enzyme production potential for future applications.

2. Materials and Methods

2.1. Sample Collection

The sampling of the sewage water was done between the period of October 2022 to December 2022 from different locations in and around Bhopal city on a random basis.100 ml of water samples were collected from each location following all the necessary sampling procedures and brought to the laboratory and provided with suitable codes.

2.2. Screening and Isolation of Asparaginase +ve Bacteria

Modified M9 media supplemented with amino acid asparagine and phenol red indicator was used for isolation and enumeration of asparaginase-producing bacteria, and nutrient agar media was used for their subsequent pure culture preparation. The composition of modified M9 agar media is mentioned in table 1.

Table 1 Composition of modified M9 agar media supplemented with amino acid and phenol red indicator

S.N.	Ingredients	Quantity in Grams/Litre
1.	KH_2PO_4	3 gm
2.	Na_2HPO_4	6 gm
3.	NaCl	0.5 gm
4.	$MgSO_4.7H_2O$	0.12 gm
5.	$CaCl_2.2H_2O$	0.001 gm
6.	L-asparagine	10 gm
7.	phenol red	0.05 gm
8.	Agar	15 gm
9.	Final pH	7.2±0.2

*All ingredients homogenized in per liter distilled water then sterilized and poured into sterile plates

Microbial Enumeration: In the present investigation, a digital colony counter (EI-362) was used for microbial enumeration. The total number of cells or bacteria per ml of water sample or substrate may be counted using the following *formulae*;

$$\text{CFU / ml} = \frac{\text{No. of Colonies} \times \text{Total dilution factor}}{\text{Total Vol. Plated in ml}}$$

[Where: CFU= colony forming unit**]**

2.3. Detection of ansB Gene and Molecular Identification

The selected unknown bacterial isolates that were reported positive for *in vitro* asparaginase enzyme production potential in the present study were subjected to detection of ansB gene and the identification by partial sequence analysis of 16S rRNA gene.

- **DNA Extraction:** The genomic DNA from unidentified bacterial isolates was extracted using the conventional boiling method by selecting representative colonies from nutrient agar plates (Wilson, 2001). Each selected colony from the water samples was inoculated into a 1.5 ml microfuge tube containing 1 ml of nutrient broth. The tubes were then transferred and incubated for 24 hours at 37°C. After incubation, the tubes were centrifuged at 10,000 rpm for 7 minutes, the supernatant was discarded, and the pellet was washed with 500 µl of sterile distilled water. Subsequently, the pellet was centrifuged at 12,000 rpm for 5 minutes. After

discarding the supernatant, the pellet was homogenized with 200 µL of sterile distilled water and heated in a digital boiling water bath at 100°C (Navyug, India) for 10 minutes. The sample was cooled for 7 minutes in an ice bath and then centrifuged at 10,000 rpm for 5 minutes. The resulting supernatants were transferred to fresh sterile 1.5 ml microfuge tubes and stored at −20°C for future use.

- **Detection of ansB gene:** the PCR reaction was carried out to amplify the ansB gene fragments using the set of amplification primers ansB-F/ansB-Robtained from *Eurofin Genomic* India Pvt. Ltd, Bangalore, Karnataka, India. The sequence of forward primers ansB-F is "5-GGTGGATCCTTACCCAATATCACCATTTTAG-3" and the sequence of reverse primers ansB-R is "5-GGGAAGCTTTTAGTACTGATTGAAGATCTG-3". The PCR reaction mixture was prepared by mixing genomic DNA, universal primers, dNTP mix, assay 10X buffer and $MgCl_2$ and molecular grad distilled water all procured from HiMedia, India Pvt Ltd, Mumbai, Maharashtra, India. 2µl of Genomic DNA (25-50 ng/µl) of the bacterial isolates was used as a template and PCR was performed in a 25µl reaction volume. The PCR reaction mixture was prepared by mixing genomic DNA, forward and reverse primers, dNTP mix, assay 10X buffer, and $MgCl_2$ and molecular grad distilled water. The PCR was performed on Thermal Cycler Machine "Prima-96" from HiMedia India Pvt Ltd, Mumbai, with an initial denaturation step at 94°C for 4 minutes, followed by 35 cycles of denaturation at 94°C for 40 seconds and then annealing at 52°C for 40 seconds followed by extension at 72°C for 1 minutes and final extension at 72°C for 10 minutes with final holding at 4°C, as described by Mohamed, *et al*., (2015). The confirmation of ansB gene was confirmed by visual detection of bands of single sharp band at 981 BP region on 1% agarose gel after electrophoresis.
- **Amplification of 16S rDNA:** bacterial genomic DNA of selected samples were subjected to amplify the 16S rRNA gene fragments using universal 16S rDNA or rRNA amplification primers 27F/1492R obtained from *BioServe* Biotechnologies India Pvt. Ltd, Hyderabad, A.P. India. The sequence of universal forward primers 27F is "5-AGAGTTTGATCMTGGCTCAG-3" and sequence of universal reverse primers 1492R is "5-TACGGYTACCTTGTTACGACTT-3". The PCR reaction mixture was prepared by mixing genomic DNA, universal primers, dNTP mix, assay 10X buffer and $MgCl_2$ and molecular grad distilled water all procured from HiMedia, India Pvt Ltd, Mumbai, Maharashtra, India. The PCR reaction was performed using an initial denaturation stage at 94°C for 5 minutes was followed by 30 cycles of 30 seconds at 94°C, 1 minute at 45°C, and 30 seconds at 72°C, and a final step of 10 minutes at 72°C. Agarose gel electrophoresis was conducted according to Sambrook *et al*., (2001) on 1% agarose gel to confirm the amplification of 16S rDNA fragment in PCR reaction. The amplified 16S rDNA fragment was visualized in E-gel Imager gel documentation systemfrom ABI Invitrogen (Thermo).
- **Partial Sequencing of 16S rRNA and Analysis:** The partial sequencing of amplified 16S rRNA gene using universal primers 27F/1492R was get done from *HiMedia Laboratories* Pvt. Ltd, Mumbai, Maharashtra, India by utilizing the outsource facility provided by Lenience Biotech Lab, Bhopal. The provided sequences in FASTA format were used for analysis on website of NCBI using nucleotide BLAST search in GenBank.

3. Results and Discussion

3.1. Isolation & Screening of Asparaginase Producing Bacteria

Modified M9 media was supplemented with amino acid asparagine and phenol red indicator was used for isolation and enumeration of asparaginase producing bacteria, which worked very well in this study. There were total of 10 sewage water samples collected from different areas of Bhopal City which were evaluated for their total CFU count and incidence of Asparaginase producing unknown bacterial species. The outcomes of the Asparaginase producing unknown bacterial speciesas CFUs within each samples source are depicted in table 2.

Table 2 The CFU count of Sewage water samples from slum areas at different dilution

S.N.	Samples Resource	Samples Codes	Total CFU count at 10^{-7}dilution
1	Anna Nagar	S-1	45
2	Abbas Nagar	S-2	26
3	Pratap Ward	S-3	34
4	Barkherha Pathani	S-4	46
5	Jaatkhedi	S-5	52
6	Ambedkar Nagar	S-6	53
7	Slums, Arera Hill	S-7	52

8	Arjun Nagar	S-8	63
9	Durga Basti	S-9	47
10	Habibjang Area	S-10	39

Out of the 10 sewage water samples used for isolation of desired bacterial species, there were total 7 samples observed to be responded for the incidence of asparaginase producing bacterial in the form of CFUs with pink zone around them due to the hydrolysis of asparagine amino acid present in the media used for the study.

3.2. Detection of ansB Gene and Molecular Identification

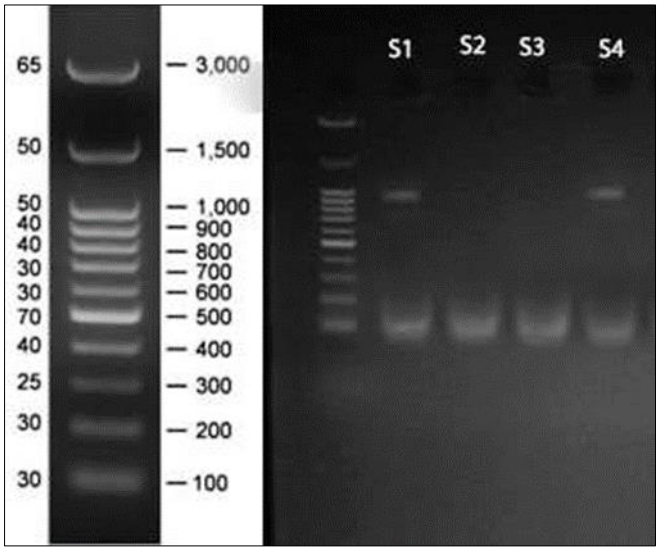

Figure 1 Amplified fragment of ansB gene by PCR in 13 asparaginase enzyme positivebacterial isolates

Referring to the image as depicted in figure 1 in present investigation it is clear that the amplicons of samples S1, and S4 were reported to show clear sharp band of 981 bp approx. visible near 1000 bp marker in this study that coincides with the studies by Mohamed, *et al*., (2015) who used this fragment in cloning. This indicates that these bacterial isolates bear ansB gene, hence these bacterial isolates confirms that they could probably be the potential producer of L-asparaginase like oncotherapeutic enzymes and hence could be commercially exploited upon further extensive investigations.

In present study, the two selected bacterial isolates coded as S-1 and S-2when subjected to partial gene sequencing and analysis of their 16S rRNA fragment were found to be resembling with *Bacillus tropicus MCCC 1A01406*and *Priestiaveravalensis strain SGD-V-76*upon 16S rRNA sequence alignment as per the BLAST analysis performed on NCBI's webserver with maximum percentage resemblance of 96.52% and 95.09%, respectively as depicted in table 4.The partial sequence of 16S rRNA regionof indigenous bacterial isolated S-1 and S-2 in FASTA format and other details are mentioned table 3.

Table 3 The partial sequence of 16S rRNA regionof indigenous bacterial isolated S-1 and S-2 in FASTA formatand query details obtained from NCBI's BLAST tool

S.N.	Bacterial Isolate Code	16S rRNA Sequence in FASTA format	Query Details
1.	Isolate S-1	>MBR01-27F_D04.ab1 TGGGTACAGCATGATGGGTGGGTACGTTAGAA CGGAAGTGGGATTAACAGCTTGCATCTTATGAG GTTAGCGGCGGACGGGTGAAGTAACACCTGGGT AACCTGCCCATAAGACTGGGATAACTCCGGGAA	Request Identifier (RID): UFF3X5XF013 Query ID: lcl|Query_3090385 Length: 527 bp

		ACCGGGGCTAATACCGGATAACATTTTGAACCG CATGGTTCGAAATTGAAAGGCGGCTTCGGCTGT CACTTATGGATGGACCCGCGTCGCATTAACTAG TTGGTGAGGTAACAGCTCACCAAGGCAACGATG CGTAGCCGACCTGAGAGGGTGATCGGCCACACT GGGACTGAGACACGGCCCAGACTCCTACGGGAG GCAGCAGTAGGGAATCTTCCGCAATGGACGAAA GTCTGACGGAGCAACGCCGCGTGAGTGATGAAG GCTATCGGGTCGTAAAACTCTGTTGTTAGGGAAG AACATGTGCTACTTGAATAAGCTGGCACCTTGAC GGTACCTAACCAGAAAGCCGCTGCTAACTACGTG CCACAGCCGCGGAATACCATGTGGCAAGTG	>*Bacillus tropicus strain MCCC 1A01406* small subunit ribosomal RNA gene, partial sequence Sequence ID: NR_157736.1 Length: 1509 Range 1: 65 to 550 Score: 802 bits (434), Expect:0.0 Identities: 471/488(97%), Gaps:5/488(1%), Strand: Plus/Plus
2.	Isolate S-2	>MBR02-27F_F04.ab1 AAGGGTCCAGAAGGGGGTGAAGAGTTCAGGAAGCG AACGTGTATTAGGAAGCTTGCTTCTAGTGACGTTAG CGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCT GTAAGACTGGGATAACTCCGGGAAACCGGAGCTAAT ACCGGATAACATTTTTTCTTGCATAAGAGAAAATTG A AAGATGGTTTCGGCTATCACTTACAGATGGGCCCGCG GCGCATTAGCTAGTTGGTGAGGTAACCGCTCACCAA G GCAACGATGCATAGCCGACCTGAGAGGGTGATCGGC C ACACTGGGACTGAGACACGGCCCAGACTCCTACGGGA GGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGT CTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTAT C GGGTCGGAAAACTCTGTTGTTAGGGAAGAACCTAAA C ATTAGTAACTGCTTGTACCTTGACGGTACCTAAACA GT CAGGAACGGGTAACTGCTTGCCAACTGCCATAGTAA G ATGTCAGAGGTAAGGGGT	Request Identifier (RID): UFF4R9UY016 Query ID: lcl|Query_2551277 Length: 531 bp >*Priestiaveravalensis strain SGD-V-76* small subunit ribosomal RNA gene, partial sequence Sequence ID: NR_178610.1 Length: 1480 Range 1: 37 to 500 Score: 734 bits(397), Expect:0.0 Identities:445/468(95%),Gaps:4/468(0%), Strand: Plus/Plus

Table 4 Closest resemblance of indigenous bacterial isolates with reference to 16S rRNA region sequence analysis using NCBI BLAST tool

S.N.	Bacterial Isolate Code	Closest Neighbour upon Alignment	Accession	Percentage Resemblance
1.	Isolate S-1	*Bacillus tropicus MCCC 1A01406*	NR_157736.1	96.52%
2.	Isolate S-2	*Priestiaveravalensis strain SGD-V-76*	NR_178610.1	95.09%

Microorganisms with the ansB gene are increasingly recognized for their ability to produce the enzyme L-asparaginase, which shows potential in cancer treatment. The ansB gene codes for L-asparaginase, an enzyme that breaks down asparagine into aspartic acid and ammonia, starving cancer cells of vital nutrients and causing cytotoxicity. Various microbial species, including *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, and certain fungi, carry the ansB gene and serve as valuable sources of L-asparaginase with diverse biochemical properties. Research by Kumar et al. (2020) detailed the molecular characteristics and expression patterns of the ansB gene in bacteria, shedding light on its

genetic role in L-asparaginase production. Additionally, studies by Prakasham et al. (2016) and Gomathi et al. (2018) highlighted the enzymatic features and anticancer potential of L-asparaginase derived from ansB gene-containing microbes.

Several microbial species, including bacteria like *Erwinia chrysanthemi*, *Erwinia carotovora*, and *Aeromonas hydrophila*, along with certain fungal varieties, carry the ansB gene and can produce L-asparaginase. Khushoo et al. (2004) conducted research on the biochemical characteristics and kinetic properties of L-asparaginase from *Erwinia carotovora*. Additionally, studies by Vignardet al. (2020) and Alkhalaf &Ganguly, (2018) explored the anticancer properties and potential modes of action of L-asparaginase from microbes with the ansB gene, emphasizing its therapeutic potential in cancer treatment.

The pharmaceutical sector extensively utilizes microorganisms to produce various compounds, from antibiotics to enzymes (Demain & Sanchez, 2009). *Bacillus tropicus* and *Priestiaveravalensis* have emerged as notable microbial candidates. *Bacillus tropicus* is recognized for producing diverse bioactive compounds, including antimicrobials and enzymes, with potential in antibiotic production (Kumar et al., 2013). Meanwhile, *Priestiaveravalensis*, though less researched, has shown promising antimicrobial properties, making it a potential source for new antibiotics in pharmaceutical research (Rajivgandhi et al., 2016).

4. Conclusion

In this research, sewage samples from different slum areas in Bhopal City were examined for bacteria that produce asparaginase. Among the ten samples tested, seven showed asparaginase activity. Two bacterial strains, S-1 and S-2, were identified as *Bacillus tropicus*and *Priestiaveravalensis*, respectively. These strains were found to contain the ansB gene and closely matched known bacterial strains, suggesting their potential for producing L-asparaginase. This underscores their importance in pharmaceuticals, especially for cancer treatment. Overall, these microorganisms represent valuable resources for the pharmaceutical industry, offering potential for the development of novel antibiotics, enzymes, and other therapeutic agents upon extensive scientific investigations.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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