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Therapeutic L-Asparaginase Activity of Bacteria Isolated from Marine Sediments

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ABSTRACT

L-Asparaginase, a therapeutic enzyme used in lymphoblastic leukemia and lymphosarcoma chemotherapy which is derived mostly from the bacterial sources *Escherichia coli* and *Erwinia* sp. The long term administration of the drug leads to the development of resistant tumours and anaphylactic shock in certain individuals. Hence serologically different L-Asparaginase from novel microbial sources with enhanced therapeutic potential and immunological characteristics is an essential requirement. The marine bacteria having diverse range of potential enzymes might be a source for L-Asparaginase with novel properties, which are still unexplored. In this study, we have screened marine bacteria isolated from the coastal regions of Kerala which showed both intra and extra cellular L-Asparaginase activity. *Bacillus* sp. (Accession no KF142395) was found to have the highest extracellular enzyme activity (2.31 IU/ml) while *Shewanella* sp. (Accession no KF142390) showed maximum intracellular Asparaginase activity (2.16 IU/ml).The crude extracellular enzyme preparation from *Bacillus* sp. had cytotoxic effect on HL60 cell line with an IC_{50} value of 12.5µg/ml.

Keywords: L-Asparaginase, 16S rRNA, Antineoplastic, Therapeutic-enzyme, MTT assay.

INTRODUCTION

Bacterial L-Asparaginase (L-Asparagine amido hydrolase, E.C.3.5.1.1) is a chemotherapeutic agent used in the treatment of Acute Lymphoblastic Leukemia (ALL) and Lymphosarcoma in humans for the last 25 years. [1-2] The potential chemotherapeutic utility of the enzyme in treating ALL was first noticed when the guinea pig serum had an inhibitory effect on the lymphoma cell proliferation in mice. [3] Further studies on the guinea pig serum proved that the L-Asparaginase activity was responsible for the anti tumour activity of the serum. [4] Asparaginase is still preferred for the ALL chemotherapy since it is nontoxic biodegradable, can be administered at local sites without the fear of tissue irritation and is cost effective also. Due to the absence of L-Asparagine synthetase certain tumour cells cannot synthesise L-asparagine from aspartic acid. The L-Asparaginase reduces the activity of L-Asparagine in the circulatory system by catalysing the hydrolysis of L-Asparagine to aspartic acid and ammonia which leads to selective starvation of tumour cells and which causes protein synthesis inhibition and cytotoxicity of lymphoblastic cells. The enzyme has immense applications in food and medical industries. Recently it is found that L- Asparaginase reduces the formation of carcinogenic acrylamide in deep fried potato recipes. [5] Asparaginase based diagnostic biosensor for monitoring asparagine levels in patients suffering from acute lymphoblastic leukemia is also available.

L-Asparaginase is relatively wide spread in nature and was reported to be present in all the three domains of

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life. [6] The microbial sources are very common for L-Asparaginase, because they can be easily cultured; extraction and purification are also convenient, facilitating the Industrial scale production. [7] A variety of microorganisms like *Escherichia coli* [8], *Erwinia* sp. [9], *Proteus vulgaris* [10], *Serratia marcescens* [11], *Enterobacter aerogenes* [12], *Staphylococcus* sp. [13], *Pseudomonas aeruginosa* [14], *Thermus thermophiles* [15], *Bacillus* sp. [16] and *Streptomyces* sp. [17-18] were found to produce L-Asparaginase. Although L-Asparaginase can be obtained from a variety of sources, the enzyme purified from *Escherichia coli* and *Erwinia* sp. approved by FDA are being used exclusively as drug. The clinical utilization of Asparaginase was limited because of its severe side effects- pancreatitis, diabetes and anaphylactic shock. [19]

Microorganisms inhabiting the saline environments are known to produce proteins with novel properties. Hence the halophilic bacteria may be a source of L-Asparaginase with novel immunological and therapeutic properties. L-Asparaginase activity from halophiles has not been fully explored yet. There are several reports suggesting variations in the biochemical and kinetic property of L-Asparaginase with the changes in the genetic nature of the microbial strains analyzed. [20-21] Considering all these facts the study has been initiated for the isolation and characterization of potential L-Asparaginase producing bacteria from the marine sediments along the coastal regions of Kerala.

MATERIALS AND METHODS

Isolation of Marine Bacteria

Sediment samples were collected from the various coastal regions in Thiruvananthapuram district of Kerala. Suspensions were prepared by mixing each of the samples (1 g) with 100 ml sterile distilled water. The suspension was kept on rotary shaker for 30 min. After sedimentation, the supernatants from the samples were serially diluted and 100µl each of 10^{-3} and 10^{-4} dilution were spread on Zobell marine agar (Himedia) plates and incubated at 30°C for 24 h. The isolated strains were transferred onto marine agar slants and stored at 4°C.

Screening of L-Asparaginase Producing Bacterial Strains

L- Asparaginase production was screened with modified M9 minimal media supplemented with 7% NaCl (w/v) and L-asparagine as the sole N_2 source and phenol red as indicator. [7] Asparaginase producing colonies changed to pink color after incubation at 30°C for 48 hours due to pH change by the formation of NH_3 as byproduct of the enzyme catalysis were selected for further analysis.

Phylogenetic Analysis

The Asparaginase positive bacterial isolates were purified and broth cultures were prepared in Zobell marine broth (Himedia). The cultures were concentrated by centrifugation at 4500×g, 10 min, at 4°C and the pellets were selected for DNA isolation (Wizard genomic DNA purification kit, Promega) and PCR amplification. Amplification of approximately 1500bp 16S rRNA genes was performed in a thermal cycler (Bio-Rad, USA) with eubacterial primers 27F and 1492R. [22] PCR was performed in a final volume of 50µl containing PCR amplification buffer (1X), Hot-Start *Taq* polymerase (2.5U), dNTPs (4mM), $MgCl_2$ (2.5mM) primers (0.4µM) and template DNA (4ng). Amplification conditions were, initial denaturation at 94°C for 3 min followed by 30 cycles at 94°C for 1 min, 54°C for 1 min, extension at 72°C for 2 min and with a final extension of 72°C for 10 min. The resulting PCR product (approximately 1500bp) was purified with gel extraction kit (Qiagen). Sequencing of the 16S rRNA gene was carried out using ABI PRISM Big Dye Terminator V3.1 cycle sequencing kit, universal primers and AB 3730 automated DNA sequencer. The sequences obtained was viewed with ABI Sequence Scanner V.1, compiled and edited using software BioEdit V 5.0.6 [23] and compared with the public database (NCBI BLAST). The phylogenetic analyses of potential L-Asparaginase producing strains were done and a phylogenetic tree was constructed using Mega V.4 software. [24]

Crude L-Asparaginase Preparation and Enzyme Assay

The Asparaginase positive strains were transferred to 500 mL Erlen-meyer flasks with 100 ml broth medium containing in 1 L; KH_2PO_4 (0.75g) NaCl (0.5g), L-Asparagine (10g), maltose (1g), yeast extract (10g) and phenol red (0.05g) and incubated in a shaker incubator (150 rpm, 30°C) for 48 h. [25] After incubation, the cells were harvested by centrifugation at 4500×g for 5 min. The supernatant was used to assay the extracellular L-Asparaginase activity. For the determination of intracellular L-Asparaginase, bacterial pellets were washed with sterile water and freeze dried. 0.1g of dry cell weight was resuspended in 80µl of sonication buffer (50mM Tris and 10mM EDTA, pH7.5). Cells were disrupted using ultra sonication probe with 30s pulses at a 30s interval for 10 min. Cell disruptate thus obtained was centrifuged at 12,000×g for 15 min at 4°C. The cell free supernatant was used for intracellular L-Asparaginase assay. [26]

L-Asparaginase enzyme assay was performed by estimating the ammonia produced during L-Asparaginase catalysis using direct Nessler's method. The reaction mixture consisting of 0.5ml of 0.08M L-asparagine, 1.0 ml of 0.05M borate buffer (pH 7.5), and 0.5ml of enzyme solution was incubated for 10 min at 37°C. The reaction was stopped by the addition of 0.5ml of 15% trichloro acetic acid solution. [21] One unit of the L-Asparaginase (IU) is defined as the amount of enzyme capable of producing 1 µmol of ammonia per minute at 37°C.

Sequencing and Homology Analysis of L-Asparaginase Gene (*ansB*) from *Bacillus* sp. TVS 55

Periplasmic L-Asparaginase gene sequences were retrieved from NCBI database and primers were designed to amplify the gene in full length.

Amplification was done in a final volume of 50µl. The PCR master mix consist of 40ng of genomic DNA isolated from the isolate TVS 55 as template, Taq buffer (1X), High fidelity Taq polymerase (1.5U), dNTP mixture (25 mM each), $MgCl_2$ (2.5 mM), primers (0.4µM each). The PCR conditions as follows 94°C for 3 minutes initial denaturation, followed by 30 cycles of 94°C for 1 minute, 52°C for 1 minute, 72°C for 2 min and final extension of 72°C for 5 minutes. The amplicons were visualized in 1% agarose gel. Sequencing of the *ansB* gene was carried out using ABI PRISM Big Dye Terminator V3.1 cycle sequencing kit, designed primers and AB 3730 automated DNA sequencer. The sequences obtained was viewed with ABI Sequence Scanner V.1, compiled and edited using software BioEdit V 5.0.6 and homology of the gene was studied by taking similar sequences from NCBI database (BLASTx). The gene sequence was submitted in the NCBI public database.

Cell Viability Assay

The anti-proliferative effect of L-Asparaginase was measured by MTT assay that measures the reduction of yellow 3-(4, 5-dimethythiazol-2-yl) - 2, 5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase to an insoluble, colored (dark purple) formazan product which is solubilized in organic solvent and measured spectrophotometrically. [27] Briefly, HL-60 (ATCC CCL240) cells were plated at a cell density of 1×10^4 cells/well on flat bottomed 96-Well standard microplates. The partially purified L-Asparaginase was serially diluted in incomplete medium and added to cell cultures at a final concentration from 6.25-200µg/ml. After incubation for 24 h, 20µl of MTT at a concentration of 5 mg/ml in phosphate-buffer saline (PBS, pH 7.4) were added to each well. After 4 h of incubation the plates were centrifuged at 2000 rpm for 10 min, after which plates were rapidly inverted with a firm flick to remove the culture medium. The formazan precipitate was solubilised by the addition of 150µl of DMSO and the plates were further incubated for 20 min at room temperature. The absorbance was then determined with a micro plate reader, at wavelength of 620 nm.

Nucleotide sequence accession numbers

16S rRNA gene sequences of marine isolates in this study were submitted to NCBI GenBank under the accession numbers from KF142387-KF142396. The L-Asparaginase gene from the *Bacillus* isolate TVS 55 was submitted to NCBI GenBank under the accession number KP720593.

RESULTS

Among the major habitats of biosphere, marine ecosystem which covers 70% of the earth's surface provides the largest inhabitable space for microorganisms. Marine microorganisms are the central catalysts of global element cycling. Exploring the exotic marine environment has opened up a new horizon for finding new microbial resources for therapeutic enzymes. A total of 27 bacterial colonies were isolated from the marine sediments using standard isolation techniques. The L-Asparaginase producing capacities of all the isolates were evaluated using M9 minimal media supplemented with L-Asparagine as sole energy source and phenol red as pH indicator. The 10 isolates showing L-Asparaginase production potential in plate assay were selected for further studies.

Table 1: L-Asparaginase producing marine bacterial isolates

Strain Name	Bacteria	Accession number	Length (bp)
TVS 6	*Vibrio alginolyticus*	KF142387	1419
TVS 8	*Vibrio parahemolyticus*	KF142388	1453
TVS 37	*Marinomonas* sp.	KF142389	1430
TVS 38	*Shewanella* sp.	KF142390	1405
TVS 40	*Gracilibacillus* sp.	KF142391	1451
TVS 41	*Micrococcus* sp.	KF142392	1464
TVS 49	*Erythrobacter sp.*	KF142393	1357
TVS 54	*Photobacterium* sp.	KF142394	1409
TVS 55	*Bacillus* sp.	KF142395	1441
TVS 71	*Cyclobacterium* sp.	KF142396	1446

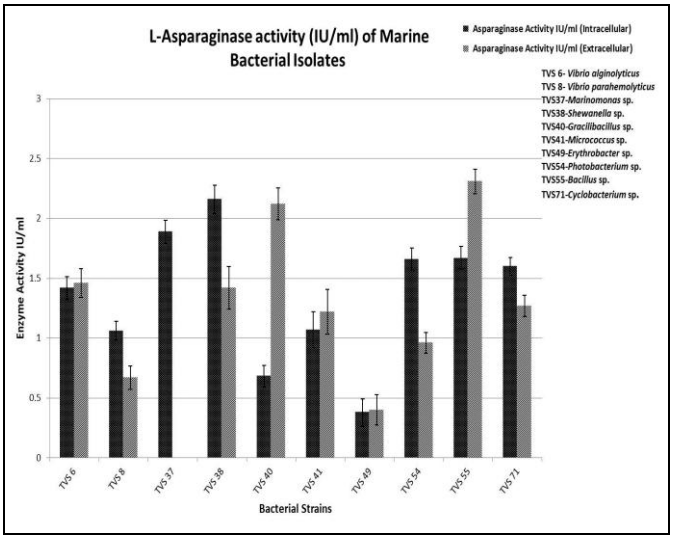

Fig. 1: Intra and extra cellular L-Asparaginase activity of Marine bacterial isolates

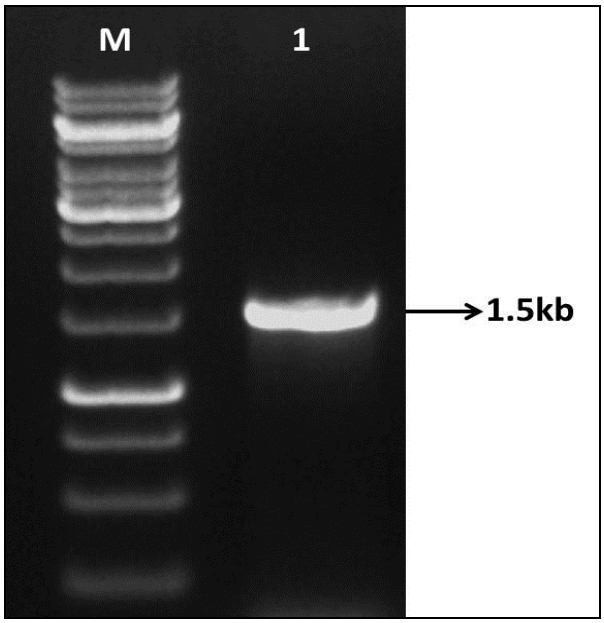

Fig. 2: 16 S rRNA gene amplified from marine bacterial isolates

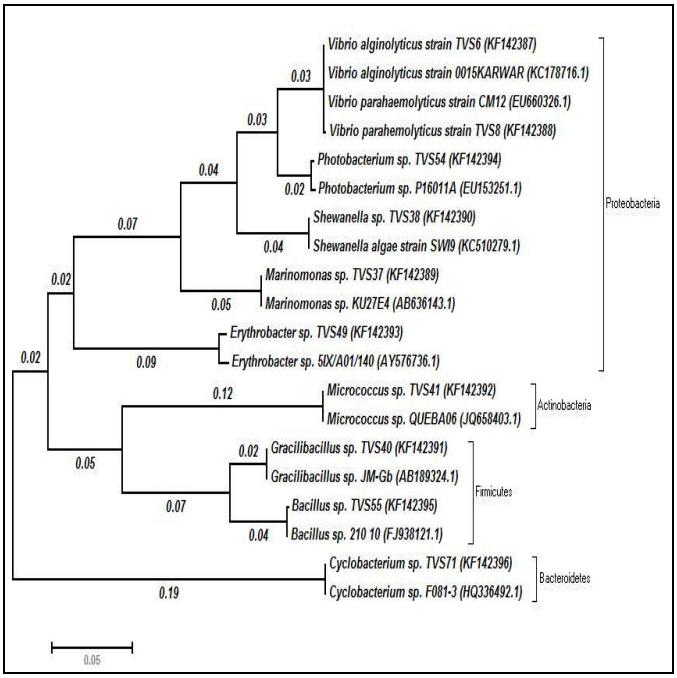

Fig. 3: Phylogenetic tree of L-Asparaginase producing marine isolates by neighbour joining method

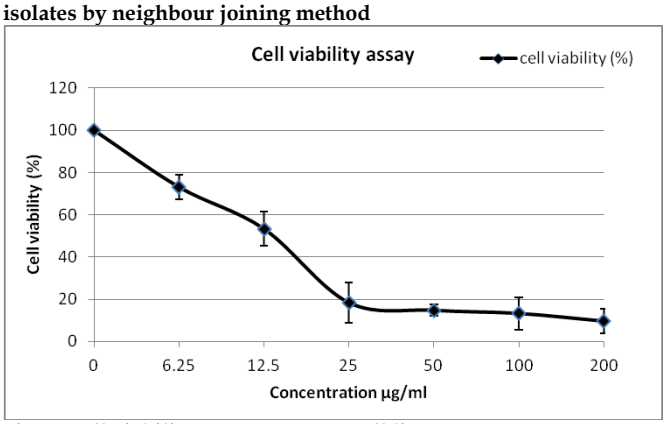

Fig. 4: Cell viability assay on HL-60 cell line

Out of the 27 bacterial isolates from the marine sediments, only 10 isolates showed L-Asparaginase activity (37.03%). Among them 90% of the strains had showed significant levels of intra and extra cellular Asparaginase activity and 10% showed only the intracellular Asparaginase activity (Fig. 1). *Bacillus* sp. (Accession no: KF142395) was found to be the most potent producer of extracellular L- Asparaginase, (2.31 IU/ml) and *Shewanella* sp. (Accession no: KF142390) exhibited maximum intracellular Asparaginase production (2.16IU/ml). More or less similar observations were reported earlier from marine bacteria. [28]

The PCR amplification of 16S rRNA gene produced ~1500 bp amplicon (Fig. 2)and the DNA sequences were submitted to the GenBank database under accession numbers KF142387 to KF142396 (Table 1.). The sequences were used as queries in NCBI BLASTn searches to determine the nearest identifiable match present in the database. The phylogeny tree (Fig. 3) revealed that, 60% of the L-Asparaginase producing isolates were belonged to the group *Proteobacteria*, which is the largest and most diverse bacterial domain. *Shewanella* sp. (Accession no: KF142390), showing the highest intracellular L-Asparaginase activity belonged to *Proteobacteria* and the maximum producer of extracellular L-Asparaginase, *Bacillus* sp. (Accession no: KF142395), representing the 20% of the isolated Asparaginase producers belonged to the group *Firmicutes*.

The *Bacillus* sp. TVS 55 was producing the L-Asparaginase enzyme extracellularly. It will give the isolate an industrial advantage since the extracellular enzymes are easy to purify. The *ansB* gene was successfully amplified and sequenced in full length from TVS 55. The sequence was submitted in NCBI public database under the accession number KP720593. The sequence showed maximum similarity of 98% with the bacterial periplasmic L-Asparaginase gene of *Bacillus* sp. Aph1 Accession no WP_034267445 in the NCBI GenBank database.

The cell free extracellular extract of *Bacillus* was used for the preparation of drug dilutions for cytotoxicity studies. The *in-vitro* cytotoxic effect of extracellular L-Asparaginase from the most potent enzyme producer *Bacillus* sp TVS55 on the growth of HL-60 (Human leukemia cell line) was analysed by MTT assay and found that the crude extracellular enzyme extracts have significant anti-proliferative effects on HL-60 cells. At 200µg/ml enzyme concentration it showed 90.4% cell death when compared with the growth of untreated control cells (Fig. 4). The IC_{50} value of the crude enzyme was found to be 12.5µg/ml which is much better when compared with the observations of Rani *et al.* [29] and Selvam and Vishnupriya. [30] In the present study we demonstrated that the extracellular L-Asparaginase inhibited cell proliferation and induces mortality in HL-60 cells. Therefore further purification of the enzyme will improve its antitumor activity.

DISCUSSION

Marine microorganisms are salt tolerant living in saline habitats which resembles blood plasma of humans biochemically. So the enzymes, especially therapeutic enzymes produced by them can be used as drug in human systems safely with less toxicity and side effects. [31] Marine bacteria are a promising source of therapeutic enzymes which are more functional at human physiological conditions than the enzyme produced by bacteria that from other non-marine sources.

The L-Asparaginase production among the isolated bacterial strains was screened by the pH change occurred in the medium due to enzymatic reaction using phenol red as pH indicator. The colour change in the screening media was due to L-Asparaginase activity alone and was confirmed by control experiments. The isolated strains did not produce any pink colour in both the control experiments - M-9 minimal media without either L-asparagine or phenol red. This indicates that the formation of pink colour is only due to L-Asparaginase production by these strains.

Many reports are available on the occurrence of L-Asparaginase in marine bacteria but the correlation between their distribution and microbial phylogeny has not yet been discussed. Such a study may be valuable in understanding how the present classification based on genetic level is correlated with the distribution of therapeutic enzymes in nature. The amidase activity in microorganisms is already reported to be concentrated in certain taxonomic groups of bacteria. Most of the members of *Enterobacteriaceae* family were reported to be having L-Asparaginase activity when compared to other amidases. Gram positive bacteria were usually showing less amidase activity as compared to Gram negative bacteria. [32] A complete cataloging of Marine bacteria which has potential to produce the therapeutic enzyme L-Asparaginase is not possible but a representative survey is worthwhile to understand the phylogenetic distribution of the L-Asparaginase gene among various taxonomic groups.

In the present observation when compared to Gram negative isolates, Gram positive isolates depicted low L-Asparaginase activity. Among the L-Asparaginase producing isolates 70% were Gram negative. Even then Gram positive organisms are potential source of L-Asparaginase since most of them secrete the enzymes to the production medium that eliminates the cost of enzyme extraction and simplifies the large scale industrial production. Many *Bacillus* sp. were reported for their extracellular L-Asparaginase production from soil [33] and marine sediments. [34]

L-Asparaginase is widely used in the chemotherapy of acute lymphoblastic leukemia, lymphosarcoma and many other tumours in humans. Prolonged administration of L-Asparaginase will produce corresponding antibodies in human sera which causes neutralization of the drug or anaphylactic shock. Therefore, it is worthwhile to screen new microbial sources for novel serologically different L-Asparaginase with improved therapeutic effects. Marine sediments are the favourable habitats of a wide range of unique microorganisms. [35-36] A little is known about the bacterial diversity of marine sediments, which is an inexhaustible resource of many potential therapeutic enzymes that has not been properly exploited.

The present study reveals the fact that the exotic marine environments of Kerala are potential source of therapeutic enzymes like L-Asparaginase. Bioactive compounds like antimicrobials and antifungal have been extensively studied and characterized from marine bacteria, but a similar attention has not been paid towards therapeutic enzymes till date. The diversity of marine bacteria and the unique environmental properties of Kerala coastal region potentially contribute to the novelty and uniqueness of the L-Asparaginase strains isolated. Furthermore, the isolated strains appear to be a promising source for L-Asparaginase which requires further investigation to elucidate their potential antileukemic activity. Our results demonstrate that marine bacteria are a powerful resource of novel therapeutic enzymes and potential industrially important enzymes. Enlargement of the pool of L-Asparaginase producing strains will provide an opportunity to develop new improved drug preparations for the treatment of acute lymphoblastic leukaemia.

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