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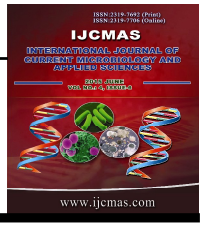

Original Research Article

Sequencing and characterization of L-asparaginase (ansB) gene of *Bacillus megaterium* isolated from Western Ghats, Kerala, India

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Keywords

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A B S T R A C T

L-asparaginase is a clinically approved bacterial therapeutic enzyme widely used in the treatment of Acute Lymphoblastic Leukaemia and Lymphosarcoma. In this study, periplasmic L-asparaginase producing bacteria were isolated from forest soil. The enzyme coding region (*ansB* gene) was amplified from the most productive isolate *Bacillus megaterium* PHB29, using group specific primers. The gene was cloned in pGEMT Easy vector and sequenced using automated DNA sequencer. The phylogeny of the gene was studied and compared with related sequences from NCBI public database. The secondary structure of the gene was analysed with SOPMA software. The antigenicity of the protein was predicted and compared with other commercially available enzymes using semi-empirical method. The present study indicates that soil bacteria are a promising source of commercially and therapeutically important drugs which are still unexplored.

Introduction

Asparaginase-II (L-asparagine amidohydrolase, E.C.3.5.1.1) catalyses the cleavage of L-asparagine to aspartic acid and ammonia. Normal cells can synthesize its own L-asparagine from aspartic acid by the action of L-asparagine synthetase enzyme which is defective or deficient in certain tumour cell lines. While injecting the drug intravenously it cleaves all the circulating L-asparagine which leads to selective starvation of L-asparagine in tumour cells, causing impaired protein synthesis and cell death (Deokar *et al*., 2010). The enzyme is relatively wide spread in the bacterial kingdom even though their actual physiological roles in bacterial cells are not fully elucidated yet. The enzyme probably has a special functional role in the anaerobic fumarate respiration. The FDA approved drugs available in the market are native asparaginase from *E. coli* and *Erwinia* and also a PEG conjugated form of *E. coli* asparaginase. The enzyme is in great demand since the ever increasing requirements in the therapeutic and food industries and its limited production. In most of the cases the treatment is interrupted by the severe side effects and immunological

reactions in patients (Avarmis and Tiwari, 2006). So it is worthwhile to explore the exotic soil environment of Kerala for L-asparaginase with novel properties and better therapeutic activities.

Forest soil is one among the most diverse natural environmentsw with respect to microbial assemblage. One gram of forest soil contains an estimated 4×10^7 prokaryotic cells (Richter and Markewitz, 1995). A little work has been done in the field of bacterial therapeutic enzymes from soil microorganisms so far which is not sufficient to explore the myriads of beneficial genes present in microorganisms. Keeping all these facts in mind we screened the Western Ghats forest soil for bacterial strains producing potential therapeutic enzymes. One of the isolates, PHB29 was found to be harbouring the periplasmic L-asparaginase gene (*ansB*). The full length gene was successfully amplified from the isolate and sequenced. The sequence was subjected to phylogeny analysis and further characterization using bioinformatics software. The study reveals that the forest soil of Western Ghats region in Kerala is an exotic resource of bacterial diversity which harbours hidden treasures in the form of genes coding for potential biological molecules.

Materials and Methods

Isolation of L-asparaginase producing bacteria

The soil samples were collected from the Western Ghats forest region of Thiruvananthapuram district Kerala, India. The samples were collected in sterile containers and transported to the laboratory and stored permanently at $^{-}20^{o}$C. The soil was serially diluted up to 10^{-6} and spread plated on nutrient agar plates supplemented with antifungal agent nystatin to avoid fungal growth. The plates were incubated at room temperature. Bacterial isolates were screened for L-asparaginase production using plate diffusion assay as described by Gulati *et al*., (1997) with M9 minimal agar supplemented with L-asparagine as sole energy source and phenol red as pH indicator.

Molecular characterisation and identification of the isolate

The bacterial genomic DNA was isolated using Wizard genomic DNA purification kit (Promega) and visualized by agarose gel electrophoresis. The 16S rRNA gene was amplified using high fidelity Taq polymerase and with universal primers 27F and 1492R and size checked by electrophoresis (Polz *et al*., 1999). PCR was performed in a final volume of 25µL containing PCR amplification buffer (1X), Hot-Start Taq polymerase (2.5U), dNTPs mixture (4mM), $MgCl_2$ (2.5mM) primers (0.4µM) and template DNA (4ng). Amplification conditions were, initial denaturation at 94^{o}C for 3 min followed by 30 cycles at 94^{o}C for 1 min, 54^{o}C for 1 min, extension at 72^{o}C for 2 min and with a final extension of 72^{o}C for 10 min. The resulting PCR product was purified with gel extraction kit (Qiagen). Sequencing reaction 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 were viewed with ABI Sequence Scanner V.1, compiled and edited using software BioEdit V 5.0.6 (Hall, 1999) and compared with the public database (NCBI-BLASTn).

Rapid flask assay for L-asparaginase production

The L-asparaginase producing capacity of the strain PHB29 was confirmed functionally by rapid flask assay method

(Jayaramu *et al*., 2010). The modified M9 medium supplemented with 0.1% phenol red indicator, 0.1% Glucose and 1% L-asparagine as sole energy source.

PCR amplification and characterisation of *ansB* gene

The *ansB* gene was amplified from genomic DNA of PHB29 using primers designed based on the L-asparaginase gene sequences from GenBank. PCR was performed in a final volume of 50 µL containing PCR amplification buffer (1X), Hot-Start Taq polymerase (2.5U), dNTPs mixture (4mM), $MgCl_2$ (2.5mM) primers (0.4µM) and template DNA (40ng). Amplification conditions were, initial denaturation at 95^{o}C for 3 min followed by 30 cycles at 95^{o}C for 1 min, 52^{o}C for 1 min , extension at 72^{o}C for 2 min and with a final extension of 72^{o}C for 10 min. The resulting PCR product was purified using gel extraction kit (Qiagen) and sequenced with the vector specific primers. Sequence homology study of the gene was carried out with *ansB* gene sequences from NCBI database (BLASTx).

Protein structure and antigenicity prediction

The protein sequences were multiple aligned and homology analysis was done using BioEdit software. Secondary structure of the protein was predicted through SOPMA program which determined the role of individual amino acids in the building of the secondary confirmation of protein (Geourjon and Deleage, 1995). In the second step the 3D protein structure was predicted by homology modelling using Swiss model workspace (Arnold *et al*., 2006). The antigenicity of the protein was analysed and compared by semi-empirical method (Kolaskar and Prasad, 1990). The molecular weight and isoelectric point (pI) was predicted using ExPasy tool (Bjellqvist *et al*., 1993).

Results and Discussion

Isolation of asparaginase producing bacteria

Eighty-three bacterial isolates were screened for L-asparaginase production using plate diffusion assay in M9 minimal agar medium supplemented with L-asparagine as sole energy source and phenol red as pH indicator. After incubation, 17 isolates showed pink colouration in agar medium and they were assumed as L-asparaginase positive. Among these isolates, PHB29 which showed comparatively higher enzyme activity was selected for further studies.

Molecular characterisation and identification of the isolate

The 16S rRNA gene of PHB29 was amplified using universal primers. The amplicon DNA was gel checked and a single band at 1500 bp size was visualized (Fig 1). The 16S rRNA gene was sequenced in triplicates and the sequences were aligned and corrected to minimize the errors. BLAST analysis of the sequence showed 99% similarity with *Bacillus megaterium* QM B1551 and the sequence was submitted to GenBank as *Bacillus megaterium* PHB29 under the accession number KF056893.

Rapid flask assay for L-asparaginase production

The strain was inoculated in M9 minimal media supplemented with L-asparagine as sole energy source and phenol red as pH indicator. After incubation at 37^{o}C for 24 hrs, colour of the flask was changed from yellow to pink (Fig 2).

PCR amplification and characterisation of *ansB* gene

The full length periplasmic L-asparaginase gene was successfully amplified from the strain PHB29. The agarose gel electrophoresis of the amplicon revealed an approximately 1200 bp amplicon which consist of the target gene along with some flanking regions (Fig 3). The gene was cloned into pGEM-T Easy vector (Promega) and transformed into JM109 cells. The positive clones were selected using blue white screening. The inserts were amplified using T7-SP6 promoter primers (Promega) and sequenced. The full length gene sequence was submitted in NCBI database under the Accession number KP768443. The complete gene sequence was 1113 bases long which encode the protein sequence of 370 amino acids. The protein sequence showed a maximum similarity of 94% with L-asparaginase of *Bacillus megaterium* (Accession no: WP_013058423). The protein sequence alignment is depicted in Fig 4.

Protein structure and antigenicity prediction

The ORF responsible for L-asparaginase production in *Bacillus megaterium* was identified from the clones. The ORF consist of 1113 bases which codes for the monomer of *ansB* gene which is 370 amino acids long. Fig 5 & Fig 6 describe the secondary and tertiary structures of the protein. The results showed that 31.89% of amino acids are present in the α-helical region, 25.41% in extended strands, 10.27% in β turn and 32.43% present in random coil respectively. The theoretical isoelectric point (pI) and molecular weight of the protein was determined through ExPasy tool and was found to be 9.23 and 39.5 kDa respectively. The antigenicity of the L-asparaginase from PHB29 was predicted using semi-empirical method and the obtained score was 1.032.

Fig.1 16S rRNA gene amplified from PHB29 M- 1kb DNA ladder (Fermentas); Lane 1, 2- 16S rRNA gene

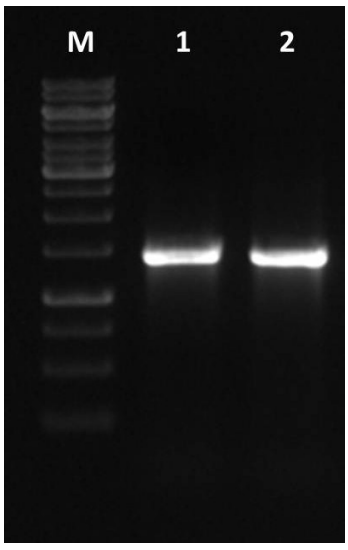


Fig.2 Rapid flask assay for L-Asparaginase production
A- Negative control, B- PHB29

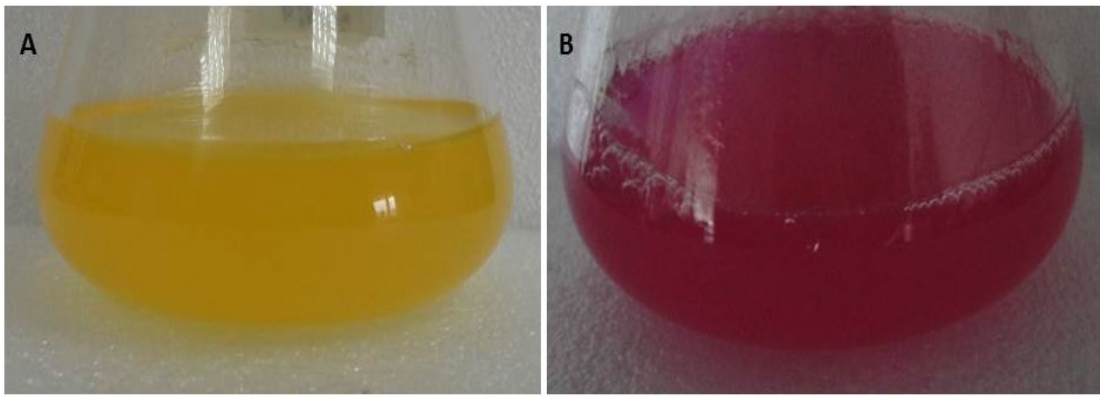


Fig.3 L-Asparaginase gene amplified from PHB29
M-1kb DNA ladder (Fermentas), 1- ansB gene (~ 1200kb)

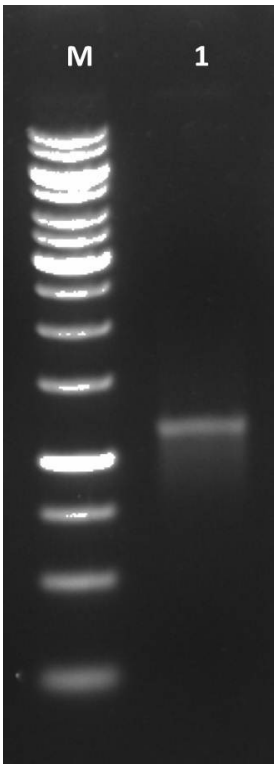


Fig.4 Multiple alignment of ansB gene from PHB29 with its homologues from GenBank

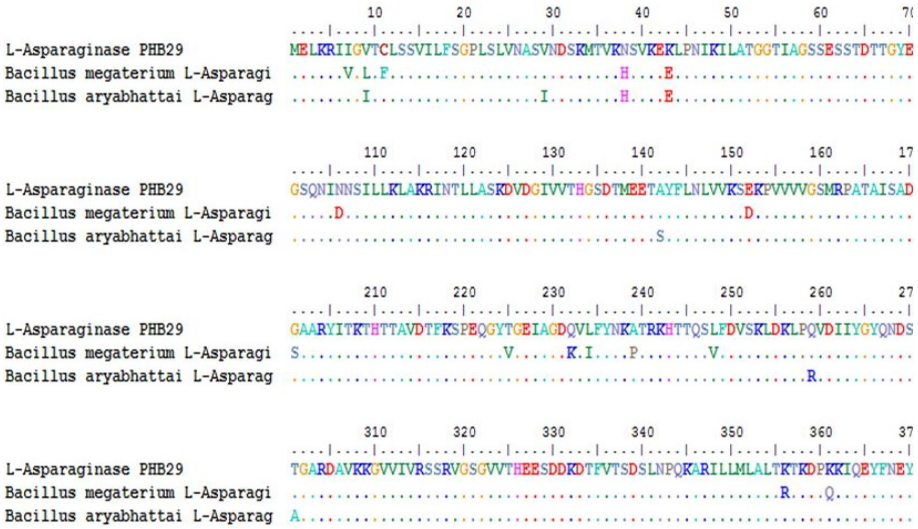


Fig.5 Secondary structure of ansB from PHB29 using SOPMA tool h-alpha helix, e-extended strand, t-beta turn, c-random coil

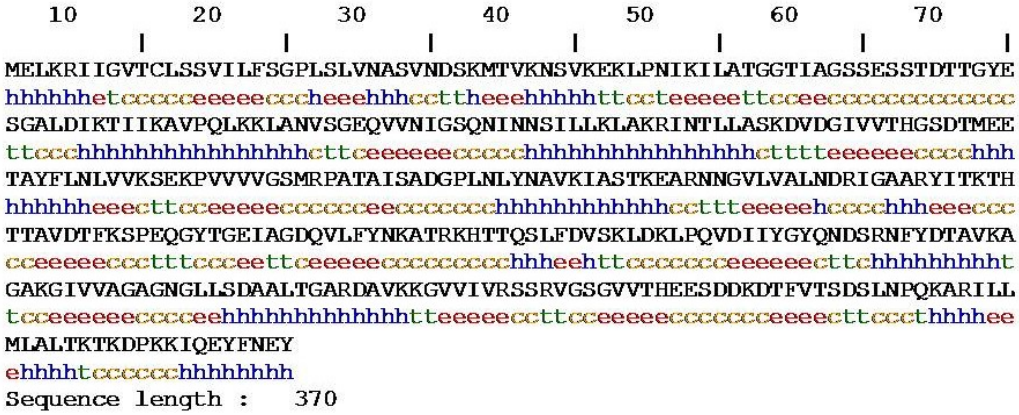
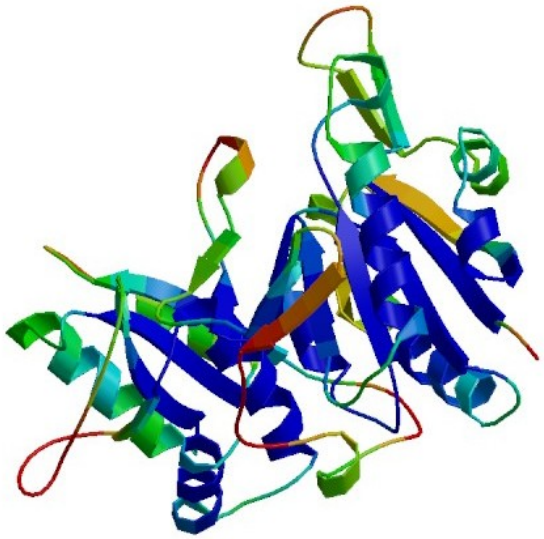


Fig.6 3-D structure of L-Asparaginase monomer designed using Swiss Model workspace



Forest soil is one of the most diverse microbial habitats on the earth. The soil bacterial community is a versatile resource of novel therapeutic enzymes. The isolates screened in this study were obtained from forest soil of Western Ghats region. Many reports are available so far on the occurrence of many soil bacteria with L-asparaginase activity. The forest soil of Kerala is rich in bacterial diversity, which has hidden treasures of many therapeutically and industrially important metabolites and their genes. It is worthwhile to explore the soil bacteria which are a powerful resource of therapeutic enzyme L-asparaginase with novel properties.

In this study we isolated a bacterial strain PHB29 which harbours a potential periplasmic L-asparaginase (*ansB*) gene. The plate diffusion assay used here, is a rapid screening method for screening asparaginase producing bacteria (Gulati *et al*., 1997). The bacterium was identified as *Bacillus megaterium* through 16S rDNA sequencing and its *ansB* gene was PCR amplified. Similarly many *Bacillus* spp. isolated from soil and marine sediments were reported for their L-asparaginase activity (Prakasham *et al*., 2010; Moorthy *et al*., 2010). The L-asparaginase producing capacity of the strain was confirmed by rapid flask assay in which the colour change occurred from yellow to pink, due to the presence of NH_4^+ formed as a by-product of L-asparaginase catalysis. The gene was sequenced and the secondary and tertiary structures were studied. The molecular weight of the protein was found to be 39.5 kDa, and this smaller size of the protein has an advantage to be used as a drug in human physiological conditions. The predicted antigenicity score of the enzyme was 1.032 which is comparable with the antigenicity of *E. coli* and *Erwinia* L-asparaginases.

The results confirmed the potential of the enzyme to develop into a good anticancer drug. Commercially L-asparaginase is produced by fermentation of wild strains. The production of large quantities of the enzyme is restricted due to the low efficiency of the technique. The clinical application of the drug is limited because of the extremely high price of the drug. So it is beneficial to search the soil micro biome to explore potential L-asparaginase genes and construct suitable recombinants to over

produce the enzyme to meet the ever increasing clinical demands.

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