


Short Communication

PCR detection of *ansA* from marine bacteria and its sequence characteristics from *Bacillus tequilensis* NIOS4

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As many as 71 marine bacterial DNA extracts were PCR screened for L-asparaginase (*ansA*), a key gene in anti-cancer molecular-searches. Over 62% (44) of them were positive for *ansA* gene. The positive cultures were from genera *Bacillus* and *Staphylococcus*. The *ansA* gene cloned from isolate NIOS4 belonging to recently described *Bacillus tequilensis* is 1099 bp in length with a 990 bp ORF coding for 329 amino acids. BLASTx analysis revealed this sequence to be 98% similar to earlier reported *ansA* sequence from *B. subtilis* (Accession no. NP390239.1). By comparing its deduced amino acid sequence with other bacterial asparaginase sequences six substitutions at positions 305(Thr), 313(Lys), 314(Leu), 315(Asp), 318(Arg), and 320(Gln) are observed. Key residues like Thr (12), Thr(85), Asp(86), Lys(156), and Phe(165) taking part in active-site formation and imparting catalytic properties are conserved. The phylogenetic tree based of the *ansA* amino acid sequences revealed close relatedness of the NIOS4 *ansA* sequence with *B. subtilis* (Accession no. NP 390239.1). It's very close genetic resemblance to *B. subtilis* and conservation of certain key amino acid residues suggest it as a prospective candidate for evaluation and, production of L-asparaginases.

 Additional supporting information may be found in the online version of this article at the publisher's web-site.

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Introduction

Biologically active secondary metabolites and commercially important bioactive molecules of marine microbes make them the best subjects of many bioprospecting efforts for developing novel and economically important products. For instance, owing to their well-established roles in food and therapeutics industry [1], bacterial L-asparaginase enzymes have remained the prime focus of research. Asparaginase (L-asparagine amido hydrolase, E. C.3.5.1.1) catalyzes the conversion of L-asparagine to L-aspartic acid and ammonia. This basic catalytic property makes it an indispensable therapeutic agent to decrease the blood concentration of L-asparagines significantly and thereby starving and affecting the neoplastic cells [2].

Further, L-asparaginase is very effective in mitigation of acrylamide, a potent carcinogen generated during baking of starchy food products [3]. Type II asparaginases are well known as effective oncolytics in acute lymphatic leukemia (ALL) [4]. Tumor- or neoplastic-cells are generally devoid of L-asparagine due to the absence of L-asparagine synthetase.

Asparaginases produced from *Escherichia coli* and *Erwinia chrysanthemi* are currently in use in the treatment of ALL. However, their long-term use has shown some immunological side-effects [5]. Hence, the search for new asparaginase producing bacteria from different/exotic environmental conditions with better and novel antigenic properties continues. Marine bacteria have also been reported to produce asparaginases [6] and, many marine *Bacillus* species produce asparaginases [7]. In this perspective, asparaginase produced from *Bacillus* species that are free of health-concerns as well as safe and efficient enough for use in food industry might prove to be effective alternative anticancer agents with a fewer/no side effects.

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We report here the 16S rRNA gene-sequence based identification, diversity of 71 marine bacterial isolates and, their screening for presence of L-asparaginase gene. Further, cloning of *ansA* gene coding for L-asparaginase from an isolate *Bacillus tequilensis* strain NIOS4 and comparison of its amino acid sequence with earlier reported *ansA* from different bacteria were carried out for elucidating the possible functional and compositional features.

Materials and methods

Sample collection, isolation of bacterial cultures, and extraction of genomic DNA

For screening of marine bacteria for *ansA* gene, sediment samples collected from the coastal region of Goa were serially diluted and spread plated on 50% seawater nutrient agar. A total of 71 isolates were chosen randomly from plates with well isolated colonies for examining their 16S rRNA gene sequence based phylogeny and to screen for *ansA* gene. Following culture-purification steps, genomic DNA was extracted from all 71 isolates using standard CTAB (Cetyl trimethyl ammonium bromide) method.

PCR, sequencing of 16S rRNA gene, and phylogenetic analysis

The 16S rRNA gene from all 71 DNA samples was amplified using 27F 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R 5'-GGTTACCTTGTTACGACTT-3' universal 16S rRNA primers. Sequencing of the purified PCR products was carried out in both directions. All sequences were checked for chimera formation using the PINTAIL program version 1.1 [8]. The 16S rRNA gene sequences were searched in public databases using BLAST (NCBI) and EzTaxon analysis. Sequence alignment was carried out using the multiple sequence alignment program Clustal X. Neighbor joining method was followed to construct the phylogenetic tree using 16S rRNA gene sequences using Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0 [9]. Tree topology was tested by bootstrap analysis of 1000 samplings. The 16S rRNA gene sequences were deposited in GenBank with accession numbers from JQ818351 to JQ818421 (Supporting Information Table S1).

PCR screening for *ansA* gene

Screening for presence of L-asparaginase gene was carried out on all 71 DNA samples using primers *ansA* F 5'-TACGGAATTCATACCTTCTCGCAACCCATC-3' and *ansA* R 5'-TTACAGGACAACGTCATCAGCTATCGG-3' [10]. The PCR

reaction was performed in final volume of 25 μ l containing 2.5 μ l of 10X PCR buffer, 0.5 μ l of dNTP mix, 1.0 μ l each of forward and reverse primers, 0.5 μ l of Taq DNA polymerase and 1 μ l each of template genomic DNA. Thermal cycling was carried out with an initial denaturation of 94°C for 25 s followed by 35 cycles at 94°C for 10 s, 55°C at 30 s and 72°C for 1.5 min followed by a final extension of 72°C for 10 min.

Cloning of the *ansA* gene from isolate, *Bacillus tequilensis* NIOS4

A gene fragment coding for the *ansA* gene was amplified using primers previously described and cloned from genomic DNA of isolate NIOS4 (Accession No. Q818355). The amplified product was cloned into pGEM-T Easy Vector. Ligation product was transformed into *E. coli* Top 10 cells by electroporation. Recombinant clones were picked and plasmids isolated using plasmid mini prep kit. Plasmids were sequenced in both directions using M13 forward and reverse primers. The *ansA* gene sequence was deposited in GenBank with accession number gb|JQ911764|. Deduced amino acid sequences of *ansA* gene were used as query in the BLASTp algorithm to search for related proteins in the non-redundant protein database at NCBI. Amino acid sequences of asparaginase gene retrieved from GenBank (Supporting Information Table S2) and a phylogenetic tree was constructed based on the using Clustal X and Mega 3 software. The 3D structure of the protein was generated using (PS) ² Protein structure prediction server [11]. Final 3D structure was generated using the modeling package MODELLER. The obtained 3D structure was then validated by Ramachandran plot using the online tool RAMPAGE [12].

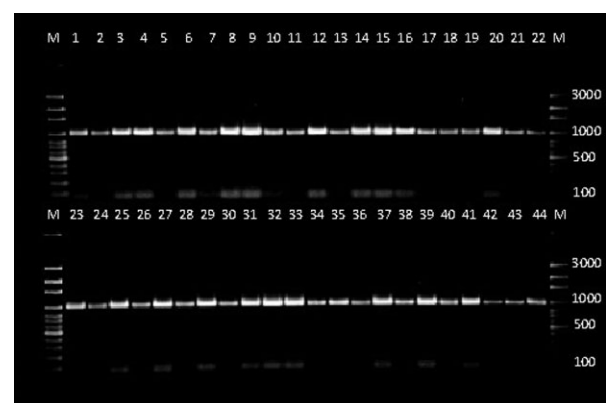


Figure 1. The *ansA* amplicons (989 bp long) obtained by PCR from 44 different isolates. Lanes 1 to 44 – Bacterial isolates positive for *ansA* gene, M-100 bp DNA Ladder.

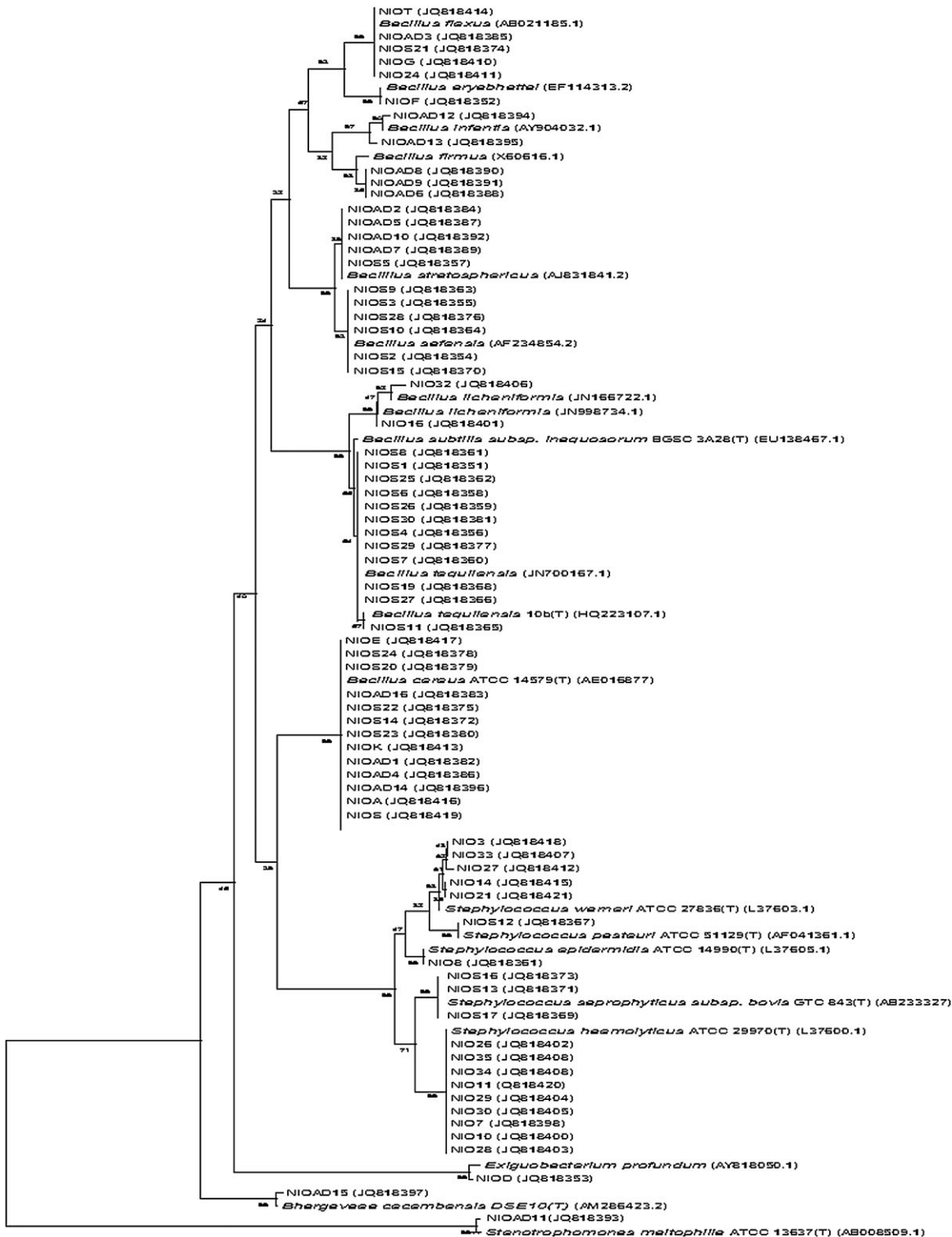


Figure 2. Phylogenetic relatedness of 71 bacterial isolates from marine sediments. Sequence alignment was carried out using the multiple sequence alignment program Clustal X. Neighbor joining method was followed to construct the phylogenetic tree using 16S rRNA gene sequences using Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0 [9]. Figures at the nodes depict bootstrap values generated from 1000 replications. All reference sequences with their corresponding accession numbers used for construction of this tree were retrieved from GenBank.

Results and discussion

Identification of bacterial isolates and bacterial diversity

Similarity matching using BLAST and EzTaxon server for our 16S rRNA gene sequences revealed 99–100% similarity with previously deposited sequences in the GenBank. *Bacillus* was the largest represented genus with 49 sequences showing 99–100% similarity to it (Supporting Information Table S1). Among those aligning within *Bacillus* belonged to *B. safensis*, *B. tequilensis*, *B. subtilis*, *B. cereus*, *B. flexus*, *B. stratosphericus*, *B. firmus*, *B. infantis*, *B. licheniformis*, *B. aryabhattai*, and *B. anthracis*. As many as 19 sequences belonged to *Staphylococcus* species. This genus was represented by *Staphylococcus pasteurii*, *S. saprophyticus*, *S. epidermidis*, *S. haemolyticus*, and *S. warneri*. The other bacteria identified were *Bhargavaea cecembensis*, *Exiguobacterium profundum*, and *Stenotrophomonas maltophilia* (Supporting Information Table S1).

PCR screening of isolates for *ansA* gene

In *Bacillus subtilis*, the *ansA* gene codes for L-asparaginase I and, is located in an operon with another gene *ansB*, which encodes L-asparatase [10]. Our PCR screening revealed amplification of *ansA* gene fragment in 44 of the 71 DNA samples (Fig. 1). As identified through 16S rRNA gene sequencing 29 of 44 belonged to *Bacillus* sp. This observation is useful to infer the preponderance of L-asparaginase in about 60% of marine sediment bacteria. In this regard, molecular screening of bacterial isolates using PCR is apparently a swift and reliable approach to identify L-asparaginase producing strains within a species/genus. As such, *Bacillus* sp. are widespread, common inhabitants of soil and aquatic ecosystems. Larger numbers of L-asparaginase positive *Bacillus* strains detected in this study is useful to suggest that one can narrow down the screening efforts for this enzyme to the strains of this genus, from which promising strain(s) can be chosen for further work. Some of the strains of *Bacillus* sp. are well known for their antimicrobial and anticancer activities [13, 14]. Earlier studies by Prakasham *et al.* [15] have also reported many species of *Bacillus* as effective producers of L-asparaginase. Especially *B. subtilis* has been reported to have both the *ansA* and *ansZ* genes that code for L-asparaginase type I and II enzyme, respectively [16]. Only a few earlier studies have examined L-asparaginase from *Staphylococcus* sp. (see Prakasham *et al.* [17]). Since *ansA* gene was detected in 15 *Staphylococcus* isolates, we propose that they may also form an important source of L-asparaginases. Notably, isolates of *B. cecembensis*, *E. profundum*, and *S. maltophilia* were negative for asparaginase gene.

Molecular characterization and phylogenetic analysis of *ansA* gene from *Bacillus tequilensis* NIOS4

As noted recently by Pieters *et al.* [1], bacterial L-asparaginases are used in various food and pharmaceutical industries owing to its ability to remove asparagine from starchy foods. L-asparaginases are used in the effective removal of acrylamide a potent carcinogen formed during baking and frying of starch containing food products [3]. Since a large number of isolates identified as *B. tequilensis* were positive for Type I asparaginase gene, a representative strain NIOS4 identified as *B. tequilensis* (16S rRNA gene Accession no. Q818355) was taken up for further analyses. Upon cloning and sequencing the 1099 bp long *ansA* gene fragment (Supporting Information Fig. S1) had an amino acid coding region from position 88 to 1074, with an ORF of 990 bases encoding 329 amino acids (Supporting Information Fig. S2). Its theoretical molecular weight was calculated to be 36585.88 Da and the isoelectric point (pI) of the peptide was 4.1 (Compute pI/Mw tool at ExPASy). Putative ribosome binding sites were identified at positions from 9 to 22 (Supporting Information Fig. S2). The amino acid composition showed high percentages of serine, glutamine, isoleucine, and threonine residues (Supporting Information Table S3). Protein 3D structure was generated using (PS)² Protein structure prediction server (Supporting Information Fig. S3). The obtained 3D structure was then validated by Ramachandran plot (Supporting Information Fig. S4).

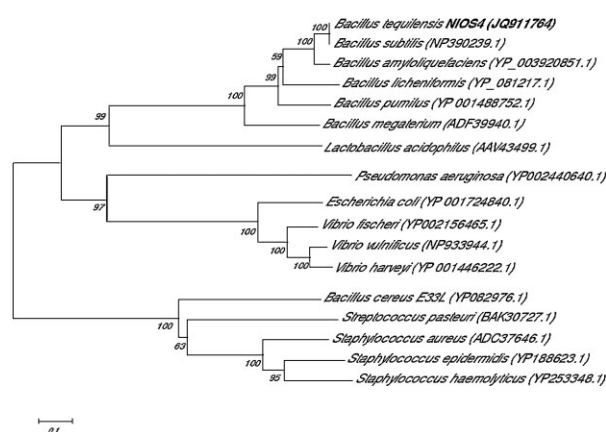


Figure 3. Evolutionary relatedness of different bacterial *ansA* amino acid sequences. Sequence alignment was carried out using the multiple sequence alignment program Clustal X. The tree was constructed using Neighbour joining method using Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0 [9]. The topology of the tree was tested with 1000 bootstrap replications. All the reference sequences used for construction of the tree were retrieved from GenBank. Shown in bold letters is the bacterial isolate from which we obtained the *ansA* amino acid sequence.

The BLASTx analysis of NIOS4 *ansA* sequence revealed 98% similarity to the one from *B. subtilis* (Accession no. NP390239.1). This might, in all likelihood, be due to the close phylogenetic proximity and resemblance of *B. tequilensis* to *B. subtilis*. Further, full length 16S rRNA gene sequence of NIOS4 has 99.57% similarity to *B. tequilensis* strain 10B (T) (Accession No-HQ223107; Fig. 2) reported recently as a new species by Gatson et al. [18] who also implied that *B. tequilensis* must be very closely related to *B. subtilis*. From the significantly high homology observed between the cloned L-asparaginase ORF of NIOS4 and *B. subtilis* 168 (Fig. 3), and that there are no previous reports of this enzyme from this species, it is suggested that *ansA* gene codes for a functional protein with characteristics

similar to L-asparaginase produced by *B. subtilis*, many strains of which are recognized as industrially important. Multiple-alignment of the *ansA* protein sequences indicate conservation of some significant residues like Thr (12), Thr (85), Asp (86), Lys (156), and Phe (165) that are universally conserved in asparaginase family and are part of the active site of the functional enzyme (Fig. 4). These residues are conserved throughout the asparaginase family and are also present in type II L-asparaginase which is a potential antineoplastic agent used in the treatment of ALL [19]. These key amino acid residues are important members leading to formation of active sites and are also responsible for the catalytic properties of this enzyme [20]. Alignment of the deduced NIOS4 *ansA*

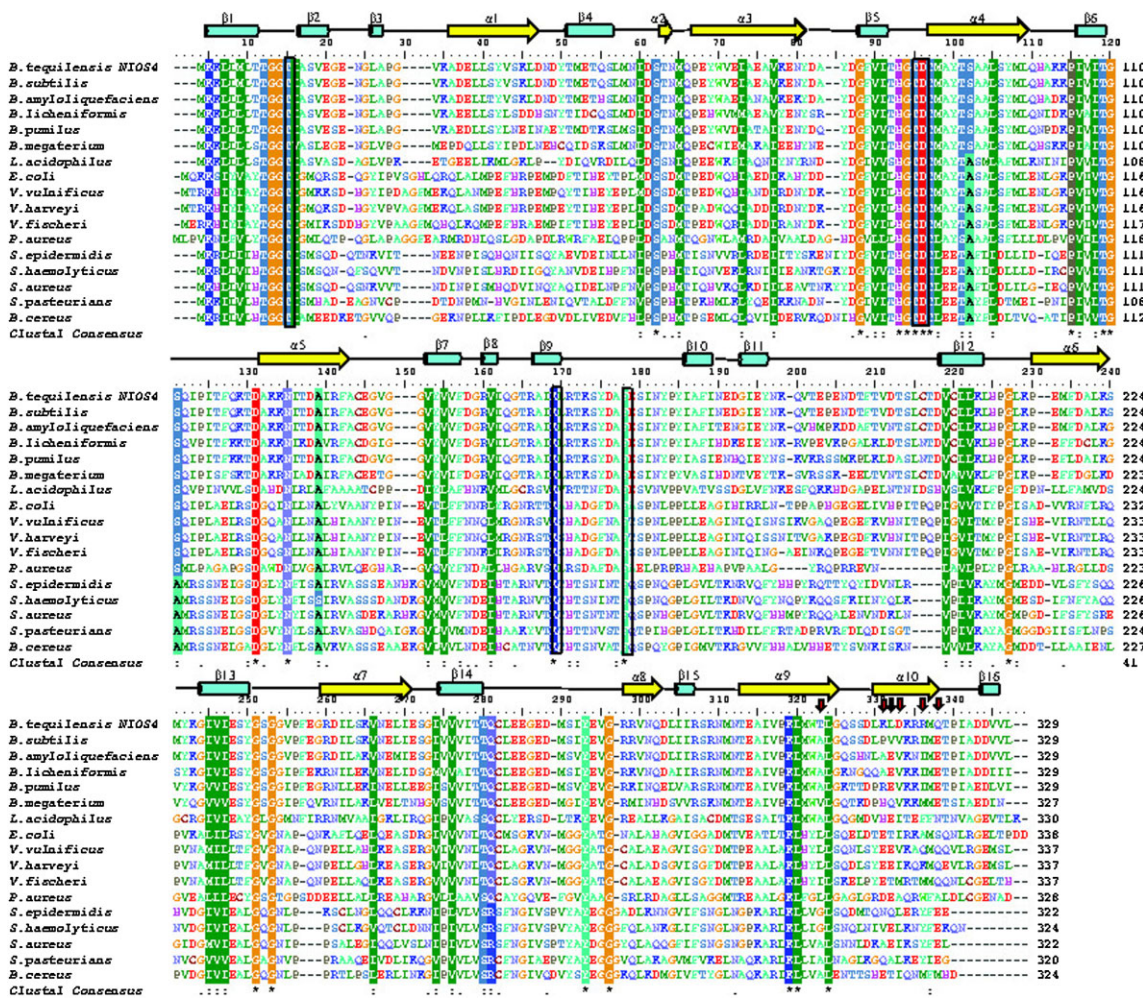


Figure 4. Multiple sequence alignment comparing the *Bacillus tequilensis* NIOS4 *ansA* amino acid sequence (Accession No gb|JQ911764) with other representative bacterial asparaginase amino acid sequences. Alignment was generated using BioEdit Sequence Alignment Editor. Conserved amino acid residues are shaded in color. (*) indicates identity; colon (:) and period (.) denote amino acid conserved substitutions. The secondary structure elements (α – alpha helix, β – Beta strand and coils) are shown as yellow arrows, blue cylinders, and black lines, respectively. Catalytic amino acid residues which are conserved in all family of L-asparaginase are shown in black outlined boxes.

amino acid sequence along with other bacterial asparaginase sequences show six amino acid substitutions at positions 305(Thr), 313(Lys), 314(Leu), 315(Asp), 318Arg, and 320(Gln), respectively (Fig. 4). Further substitutions at positions 305, 313, 314, 315, and 318 are specific for the species *B. tequilensis*. These substitutions might confer different novel properties to the enzyme produced by this isolate. Its efficiency and specific properties are yet to be evaluated. As there is no earlier work or, data on its activity, our observations would be useful in future studies. Additionally, its phylogenetic similarity with that of *B. subtilis* might make it an ideal candidate for exploring the possibilities of its use for commercial purposes.

Concluding remarks

PCR screening is seemingly reliable for circumventing long drawn protocols for searching for genes of specific enzyme(s). In this regard, detection of *ansA* in ~60% of the examined isolates and its molecular characterization from the isolate NIOS4, which belongs to the newly described species *B. tequilensis*, brings out subtle differences in its amino acid make-up. Comparison of its amino acid sequences with other known asparaginases has revealed some important substitutions and also conservation of some key amino acid residue(s) which might impart some enhanced properties to the enzyme from this isolate.

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Conflict of interest statement

All authors declare that there are no financial/commercial conflicts of interest.

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