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## 16S rRNA gene sequencing for bacterial identification of pullulanase synthesizing thermophilic bacteria contributing to big data

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### Abstract

16S rRNA gene sequences has been by far the most common housekeeping genetic marker used to study bacterial phylogeny and taxonomy because 16S rRNA gene is present in almost all bacteria and its functioning has not changed over time. Present experiment aimed isolation and characterization of thermostable pullulanase producing bacteria. Characterization was done by morphological and biochemical test and was confirmed by molecular approach using 16S rRNA gene sequencing technology. 16S rRNA gene sequence was analyzed with various bacterial genomes using BLASTn tool in NCBI (<https://www.ncbi.nlm.nih.gov/>) sequence database. Phylogenetic relationships and DNA sequencing showed that the bacterial isolate has 96% similarity with *Bacillus licheniformis* strain BCRC 11702. Phylogenetic analysis and pairwise alignment revealed its close synteny with related species. A total of 100 bootstrap replications were performed to represent the evolutionary history of the taxa analysed. 16S rRNA gene sequencing has been used to characterize numerous bacterial genera and species. Discovery, phylogenetic relationships and the classification of novel bacterial species has been facilitated by using 16S rRNA gene sequencing. Further, in last decade sequencing of various bacterial genomes and comparison between genomes has confirmed the representativeness of the 16S rRNA gene in bacterial phylogeny. Characterization of thermostable pullulanase synthesizing bacteria would be helpful in defining wide variety of applications in near future as thermostable pullulanase enzyme has many applications in textiles, detergents, food, pharmaceuticals, leather, bioremediation of waste material, starch processing industry and it has a great potential to convert agro-waste into biofuel as it ferments starch to ethanol etc.

**Keywords:** 16s rRNA, gene sequencing, BLASTn, thermophilic bacteria, pullulanase

### Introduction

Isolation of bacteria from new sources is being done since ages and different types of bacteria have been reported from various types of habitats such as extreme environments, ocean beds, amorphous solids, effluents etc (Francois *et al.* 2012) [5]. Traditional method of classification depends on similarities and differences in the phenotypic characteristics, dividing them into prokaryotes and eukaryotes and these were in turn further classified into various phyla, classes, orders, families, genera and species. However, taxonomic classification by these methods can be difficult because of the variations in phenotypic characteristics. Carl Woese and George E Fox started to analyse and sequence the 16S rRNA genes of various bacteria, using DNA/RNA sequencing and used the sequences for phylogenesis (Wose and Fox, 1977) [13]. Molecular characterization technology is now widely used for broad range of bacterial species (Petti *et al.* 2007) [8]. The analysis of DNA has been used in a large number of studies on bacterial taxonomy and bacterial typing and as well to further understand the basic mechanisms of evolution. The availability of DNA sequences for the analysis of a number of bacteria has paved the way for the identification of orthologous families of genes within genomes based on DNA sequence and gene function (Eisen, 1998) [4]. Invention of PCR and automated DNA sequencing three decades ago and subsequent work on 16S rRNA gene sequencing of bacteria, as well as 18S rRNA gene sequencing of eukaryotes, has led to the accumulation of a vast amount of sequence data on the rRNA/rDNA genes of the smaller subunit of the ribosomes in a large number of living organisms. Comparison of these sequences has shown that the rRNA gene sequences are highly conserved within living organisms of the same genus and species, but that these differ between organisms of other genera and species (Woo *et al.* 2008) [15]. 16S

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rRNA gene sequencing has played a pivotal role in bacterial classification and discovery of novel bacteria (Woo *et al.* 2003) [14] and it depends on significant inter-species differences and small intra-species differences in 16S rRNA gene sequences (Stackebrandt *et al.* 1994) [11].

A detailed analysis of 16S rRNA oligonucleotide catalogues or complete sequences revealed that there are both variable and highly conserved regions of the molecule called signature sequences which leads to determination of both close and distant relationships. Using 16S rRNA gene sequences, numerous bacterial genera and species have been reclassified and renamed, classification of uncultivable bacteria has been made possible, phylogenetic relationships have been determined, and discovery and classification of novel bacterial species has been facilitated (Mignard *et al.* 2006) [7]. In the last decade, sequencing of various bacterial genomes and comparison between genome and 16S rRNA gene phylogeny has confirmed the representativeness of the 16S rRNA gene in bacterial phylogeny (Spiegelman *et al.* 2005) [10].

Advances in computational biology and bioinformatics has been remarkable in the last few years, that established large scale sequencing, structure and function determination, gene prediction and specific landmarks on the genome as well as proteome analysis on strong foundations. Several services such as NCBI and EMBL are used when homologous sequences are to be compared and are essential precursors to numerous further analyses. Use of these tools in multiple sequence alignment, computational phylogenetic studies and proteomics has been carried out.

Keeping in view above considerations, the present study was framed for isolation, and molecular identification of thermostable pullulanase synthesizing bacteria by use of 16S rRNA technology to determine evolutionary rates of different bacterial isolates and close synteny between them by using various bioinformatics tools.

## Materials and methods

### Isolation of pullulanase synthesizing bacteria

Survey was conducted for the selection of hot water springs of Himachal Pradesh. Various hot water springs located in Kullu and Mandi districts of Himachal Pradesh were surveyed and selected. Samples in the form of water, soil, pebbles and rock matting from different sites of Manikaran, Vashist, Khirganga, Kasol, Kalath and Tattapani were collected in sterilized screw capped vials and jars.

Isolation and screening of pullulanase synthesizing bacteria was carried out using selective medium i. e. nutrient medium containing pullulan as carbon source at pH 7.5. Pullulanase synthesizing bacterial isolates were characterized morphologically and biochemically and confirmed using PCR-16S rDNA technology.

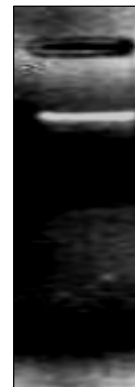
### Bacterial DNA isolation and PCR amplification of rRNA gene

Total genomic DNA of selected bacterial isolates was extracted using Genomic DNA extraction Mini kit (Real Genomics) used as template for amplification of the 16S rRNA gene (Fig. 1) using universal primers for 16S rRNA gene of bacteria. Amplifications were performed using thermal cycler (Biorad) and with a temperature profile standardized for 16S rRNA gene amplification. The PCR amplification was carried out in 0.2 ml PCR tubes with 20 µl reaction volume containing Taq DNA polymerase (5U/reaction), PCR buffer (10x) with MgCl<sub>2</sub> (1.5 mM), primers (10 nmol/reaction), deoxynucleotide triphosphate

(dNTPs) (0.5 mM) of Thermo Fisher Scientific and template DNA. Initial cycle of 5 min at 94 °C followed by 30 cycles of 1 min at 94 °C, annealing temperature of 48 °C for 1 min, elongation step of 1.5 min at 72 °C and a final extension step of 8 min at 72 °C followed by a 4 °C soak until recovery.

## Appendices

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**Fig 1:** Gel image showing genomic DNA extracted from selected thermotable pullulanase producing bacterial isolate (KS2W).

### Agarose Gel Electrophoresis

PCR products were analysed by electrophoresis on 1% agarose (GeNei, Bangalore, India) in 1X TAE buffer containing ethidium bromide (10 µg/ml) and images were taken through Gel Documentation Unit (Syngene, UK). Size of the amplified products were determined by electrophoresis of 100bp standard molecular weight markers (GeNei, Bangalore, India). The selected bacterial isolates were further characterized using 16S rRNA gene technology and genomic DNA extracted from these isolates were selectively amplified using PCR technology. Universal primers B27F and U1492R for 16S rRNA gene were used.

### DNA sequencing of 16S rRNA gene fragment

The 16S rRNA purified PCR products (100ng concentration) were subjected to sequencing using the chain termination method developed by Sanger and his coworkers in 1977 (Applied Biosystem Inc). Sequencing of 16S rRNA gene fragments of selected bacterial isolates was done from both forward and reverse directions. The sequence obtained was subjected to BLASTn search leading to identification of bacterial species. The percentages of sequence matching were also analyzed and the sequence was submitted to NCBI-Gen Bank and accession number was obtained for the same.

### Computational analysis (BLAST) and identification of bacterial species

Basic Local Alignment Search Tool (BLAST) uses an algorithm of Altshul *et al.* (1997) [1] for searching similarities above certain threshold between a query sequence and all other sequences present in a database. 16S rRNA gene sequence of the selected bacterial isolate was analyzed using BLASTn to align it with corresponding sequences of 16S rRNA from the database (Sacchi *et al.* 2002) [9]. Multiple sequence alignment was performed using CLUSTAL W program (Thompson *et al.* 1994) [12]. From such alignment studies, inference of sequence homology and phylogenetic analysis has been drawn.

## Results and Discussion

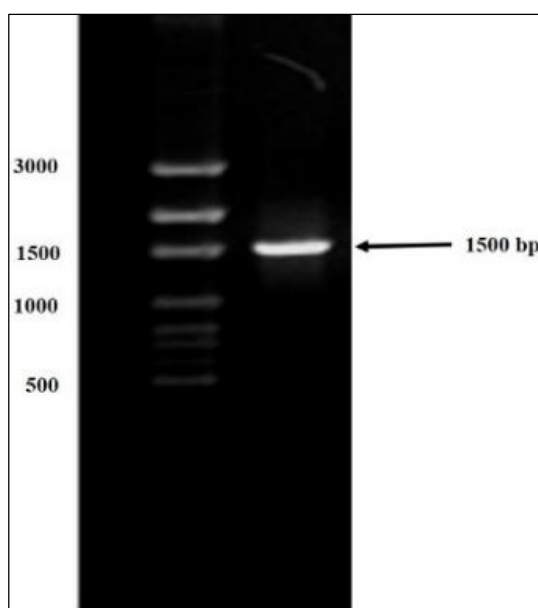
Genotypic methods of bacterial identification are more accurate as compared to the traditional techniques including phenotypic and metabolic characteristics. In recent times comparison of the bacterial 16S rRNA gene sequence which are conserved in nature has emerged as a preferred genetic technique. A total of 58 bacterial isolates were obtained initially out of which only 6 were quantified positive for synthesis of thermostable pullulanase. Only one maximum thermostable pullulanase synthesizing bacteria KS2W with 3.96 U/ml activity was selected for further studies. Total genomic DNA of this selected bacterial isolates was extracted successfully using Genomic DNA extraction Mini kit (Real Genomics). Genomic DNA was extracted from the isolate, was selectively amplified using PCR technology and characterized using 16S rRNA gene technology. After 30 cycles of PCR amplification, universal primers for 16S rRNA gene were able to successfully amplify 16S rRNA gene and produced an amplicon of expected size i.e. 1500 bp (Fig. 2). On the basis of results obtained from 16S rRNA gene analysis

and in addition to G+C content analysis (Table 1, 2, 3), the selected bacterial isolate was found to belong to genera *Bacillus*. Further *in silico* analysis pertaining to the sequence, so obtained, was carried out using various bioinformatics tools available online. Analysis of 16S rRNA gene of the selected bacterial isolates revealed homology with various other 16S rRNA gene sequences.

## List of tables

**Table 1:** Nucleotide base composition in the query sequence (KS2W isolate)

| Nitrogenous Base | Nucleotide Count |                |
|------------------|------------------|----------------|
|                  | Total            | Percentage (%) |
| Adenine (A)      | 123              | 22.12          |
| Thymine (T)      | 132              | 23.74          |
| Cytosine (C)     | 161              | 28.95          |
| Guanine (G)      | 140              | 25.17          |
| G+C              | 255              | 45.86          |
| A+T              | 301              | 54.13          |



**Fig 2:** Amplicon of 16S *rna* gene of selected thermostable pullulanase producing bacterial isolate KS2W

**Table 2:** Percent homology of nucleotide query sequence of KS2W thermos table pullulanase producing bacterial isolate from hot water springs of H.P. with other nucleotide sequences present in the database using BLASTn analysis

| Accession No. | Description                                                          | Percent Homology |
|---------------|----------------------------------------------------------------------|------------------|
| NR-116023.1   | <i>Bacillus licheniformis</i> strain BCRC 11702                      | 96%              |
| NR-13588.1    | <i>Bacillus licheniformis</i> strain NBRC 12200                      | 96%              |
| NR-042338.1   | <i>Bacillus aerius</i> strain 24K                                    | 95%              |
| NR-113993.1   | <i>Bacillus sonorensis</i> strain NBRC 101234                        | 94%              |
| NR-025130.1   | <i>Bacillus sonorensis</i> strain NRRL B-23154                       | 94%              |
| NR-118290.1   | <i>Bacillus mojavensis</i> strain ifo 15718                          | 92%              |
| NR-113994.1   | <i>Bacillus vallismortis</i> strain NBRC 101236                      | 91%              |
| NR-104873.1   | <i>Bacillus subtilis</i> subsp. <i>inaquosorum</i> strain BGSC 3A28  | 91%              |
| NR-117946.1   | <i>Bacillus amyloliquefaciens</i> strain MPA 1034                    | 91%              |
| NR-112725.1   | <i>Bacillus mojavensis</i> strain NBRC 15718                         | 91%              |
| NR-112723.1   | <i>Bacillus atrophaeus</i> strain NBRC 15539                         | 91%              |
| NR-112686.1   | <i>Bacillus subtilis</i> subsp. <i>spizizenii</i> strain NBRC 101239 | 91%              |
| NR-112685.1   | <i>Bacillus amyloliquefaciens</i> strain NBRC 15535                  | 91%              |
| NR-116022.1   | <i>Bacillus amyloliquefaciens</i> strain BCRC 11601                  | 91%              |
| NR-115930.1   | <i>Bacillus malacitensis</i> strain CECT 5687                        | 91%              |

## BLASTn

BLASTn search of selected bacterial sequences with the most similar 16S rRNA gene sequences of the Gen Bank database (<http://www.ncbi.nlm.nih.gov/blast>) revealed the closest

sequence identities from the sequence database. Sequence analysis revealed that KS2W is identical to *Bacillus licheniformis* as it showed maximum homology (96%) with *Bacillus licheniformis* strain BCRC 11702 (Table 2). Thus the

bacterial isolate KS2W was submitted to NCBI as *Bacillus licheniformis* strain ASM3 (Gen Bank accession no. MG016496.1).

### Multiple sequence alignment

Multiple sequence alignment of test nucleotide sequences of *Bacillus licheniformis* strain ASM3 with that of the selected nucleotide sequences was performed using Clustal W program (Higgins *et al.* 1992) [6] available online at European Bioinformatics Institute (EBI) (<http://www.ebi.ac.uk/>) and

the Clustal W output was then used in (MEGA 5.0 software) bioinformatics tool for constructing phylogenetic tree.

Pairwise percentage similarity score of 15 nucleotide sequences with test isolate *Bacillus licheniformis* strain ASM3 from Kasol, Himachal Pradesh (India) were arranged in tabular form. The pairwise similarity score of 15 nucleotide sequences with test sequence *Bacillus licheniformis* strain ASM3 elucidates that sequence-1 (*Bacillus licheniformis* strain BCRC 11702) showed maximum similarity score of 74.4% (Table 3).

**Table 3:** Pairwise percentage similarity score table of *Bacillus licheniformis* ASM3

| Seq A | Name            | Length (bp) | Seq B | Name                         | Length (bp) | Score |
|-------|-----------------|-------------|-------|------------------------------|-------------|-------|
| 1     | <i>Bacillus</i> | 556         | 2     | gi 636559963 ref NR_116023.1 | 1468 bp     | 74.4  |
| 1     | <i>Bacillus</i> | 556         | 3     | gi 126653866 gb EF433411.1   | 1468 bp     | 73.6  |
| 1     | <i>Bacillus</i> | 556         | 4     | gi 115333962 gb DQ993679.1   | 1549 bp     | 73.6  |
| 1     | <i>Bacillus</i> | 556         | 5     | gi 256265123 gb GQ375247.1   | 1448 bp     | 73.0  |
| 1     | <i>Bacillus</i> | 556         | 6     | gi 291088018 emb FN652005.1  | 845 bp      | 72.8  |
| 1     | <i>Bacillus</i> | 556         | 7     | gi 636559957 ref NR_116017.1 | 1468 bp     | 72.0  |
| 1     | <i>Bacillus</i> | 556         | 8     | gi 126567887 gb EF423603.1   | 1538 bp     | 72.0  |
| 1     | <i>Bacillus</i> | 556         | 9     | gi 291088017 emb FN652004.1  | 929 bp      | 70.4  |
| 1     | <i>Bacillus</i> | 556         | 10    | gi 636559466 ref NR_115526.1 | 1486 bp     | 68.0  |
| 1     | <i>Bacillus</i> | 556         | 11    | gi 343202909 ref NR_043403.1 | 1486 bp     | 67.2  |
| 1     | <i>Bacillus</i> | 556         | 12    | gi 295027210 emb FN667913.1  | 1355 bp     | 67.2  |
| 1     | <i>Bacillus</i> | 556         | 13    | gi 7321091 emb AJ288157.1    | 1340 bp     | 67.2  |
| 1     | <i>Bacillus</i> | 556         | 14    | gi 343202907 ref NR_043401.1 | 1486 bp     | 61.6  |
| 1     | <i>Bacillus</i> | 556         | 15    | gi 457650 dbj D16273.1       | 1486 bp     | 61.6  |

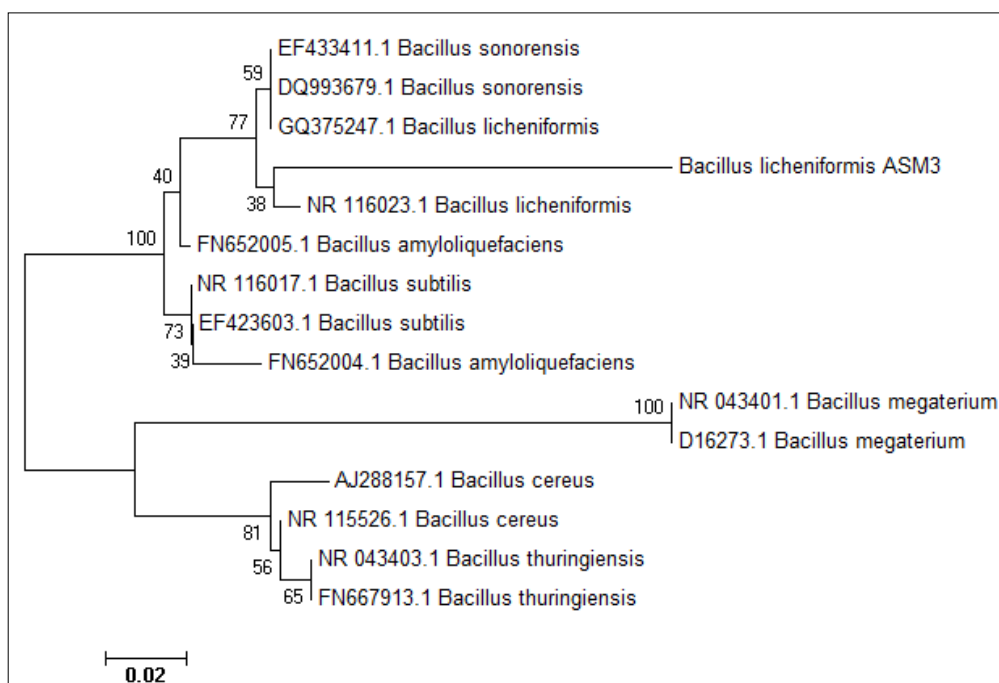
### Phylogenetic analysis

To gain insight of the evolutionary pattern, phylogenetic tree was constructed using MEGA 5.0. Neighbour-Joining (NJ) technique of mathematical averages (UPGMA) was used. The results have been presented in the form of phylogenetic tree (Fig. 3).

To trace out the evolutionary pattern of the selected KS2W bacterial isolate (Gen Bank accession no. MG016496.1), and assess its relationship with other selected sequences at NCBI, phylogenetic tree was constructed using Neighbour-Joining (NJ) method of mathematical averages (UPGMA) among 16S rRNA gene sequence and corresponding gene sequences. The

bacterial isolates selected were united with quite high statistical support by the bootstrap method estimates for 100 replications and values inferred greater than 38 percent, only were presented (Fig. 3).

By Neighbour-Joining algorithm, the phylogenetic tree (Fig. 3) has been divided and subdivided into clusters and sub clusters with bootstrap values ranging from 38 to 100. These clusters were sub clustered further and verified the bacterial isolate KS2W as *Bacillus licheniformis* (Gen Bank accession no. MG016496.1), as the isolate KS2W clustered closely with *Bacillus licheniformis* strain BCRC 11702 (NR-116023.1) with boot strap value of 38.



**Fig 3:** Phylogenetic tree of genus *Bacillus* 16S rDNA. The Gen Bank accession numbers of the analyzed sequences are shown. A total of 100 bootstrap replicates were performed, and the bootstrap values are indicated at the branching points.

## Conclusion

In conclusion, the present study reported use of 16S rRNA gene sequencing for definitive identification of thermophilic pullulanase synthesizing bacteria for harmonious interpretation of sequence data. This bacteria has the ability to synthesize thermophilic pullulanase extracellularly at a much higher temperature and pH, and thus are used in wide variety of applications such as bioremediation, detergents, starch hydrolysis, dental plaque control agent, enzymatic anti-staling treatment, bioethanol production etc. The appropriate use of such technology requires the adoption of standards similar to those previously defined for DNA-DNA hybridization (Darland *et al.* 1971; Deinhard *et al.* 1987) <sup>[2, 3]</sup> as the adaptation of 16S rRNA gene sequencing as a tool in species identification is still a relatively new phenomenon and will continue to evolve over time. Furthermore, use of microarray based technologies with 16S or other housekeeping gene targets may provide a much more sensitive and definitive platform for molecular species identification in the near future.

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