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Molecular identification of Keratinase producing Bacteria isolated from chicken feather dumping site

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Abstract

In this article, we aimed to identify and characterize bacteria isolated from chicken feather dumping site soil of Aurangabad, Maharashtra, India. In the previous report, a single keratinase producing bacterial strain was identified based on the morphological, biochemical and cultural characteristics. In this report, we have identified bacterial strain using 16S rRNA gene sequence and performed bioinformatics analysis such as BLAST and phylogenetic tree. The isolated bacterial strain showed > 95 % similarity with *Lysinibacillus sp.* MG3-4 strain.

Keywords: Keratin, Feather, Bacteria, 16s rRNA

Introduction

Feather is composed of approximately 90 % pure keratin; predominantly in the form of β-keratin[1]. The increasing number of enzymes isolated from certain environmental microbial strains is known to degrade feather keratin [2-4]. Keratinolytic bacteria, particularly from the genus Bacillus, and actinomycetes were most often isolated from the plumage and bird feathers [5-6.] In bacteria, feather keratin-degrading abilities are observed mostly in strains of *Bacillus licheniformis* [5,7] less frequently in populations of *Bacillus pumilis* , *B. cereus* and *B. subtilis* [8] and non-spore forming bacteria belonging to, for instance, *Stenotrophomonas sp*.[9] *Fervidobacterium pannavorans* [10] and *F. islandicum* [11]. The use of microbial enzymes overcomes the limitation and hence improves the nutritional value of feather waste of which, keratinases are a group of mostly extracellular serine proteases and are known for their potent activity to degrade keratin [12]. The use of microbial keratinases represents a potentially improved alternative technology for recycling keratinous byproducts, leading to nutritional upgrading, as well as cost-effective and globally environment-consistent prospects [13] Bacterial keratinases are of particular interest because of their action on insoluble keratin substrates ,for dehairing processes in the leather industry , discarded feathers are currently used to produce feather meal through thermal processing , resulting in a low nutritional value product . Feather hydrolysates produced by bacterial keratinases have been used as additives for animal feed. In addition keratin hydrolysates have potential use as

organic fertilizers, production of edible films and rare amino acids [14]. In the present, we have identified a bacterial pure culture colony based on molecular method. Here, genomic DNA was extracted and performed PCR amplification using universal 16s rRNA pair of primers. Further, we sequenced and analyzed based on bioinformatics tools such as NCBI BLAST and CLUSTAL 2 and neighbour-joining tree. After compilation of this data, we could assign the correct taxon to isolated sequence as a *Lysinibacillus sp.*

Materials and methods

Collection of soil samples, Isolation and Screening

Soil samples were collected from the various feather dumping sites of Aurangabad, Maharashtra, India. Isolation of bacteria was performed by serial dilution and plating method on Nutrient agar medium (Himedia M-001) . Screening of keratinase producing bacteria was done by using Skim Milk Agar (Himedia M-763) and minimal feather meal media [15].

Characterization and Identification of Keratinase producing Bacteria

Initial identification of the bacteria was carried out based on their morphological, physiological and biochemical characteristics. This consisted of colony morphology , Gram's staining , endospore stain, motility Test , Oxidase Test , Nitrate reduction Test, Sugar Tests, Casein & Starch hydrolysis Test, Indole production Test, Methyl red (MR) Test, Vogus-Proskauer (VP) Test, Citrate utilization Test etc. by comparing with Bergey's Manual of Determinative Bacteriology, 8th edition (Buchanan and Gibbons, 1974)[15].

DNA Extraction, PCR amplification and 16s rDNA Sequencing

Genomic DNA was isolated from pure culture colony of bacteria by alkaline lysis method.

The precipitated DNA was washed with 70% ethanol and finally dissolved in TE buffer. Quantity and quality were confirmed by agarose gel electrophoresis and Nanodrop (Thermo Scientific, USA). Genomic DNA was amplified by Polymerase Chain Reaction (PCR) using universal bacterial primers 16s forward primer:

5'–AGAGTTTGATCMTGGCTCAG–3'

16s reverse primer:

5'–TACGGYTACCTTGTTACGACTT–3'.

PCR amplification of 16s rRNA was carried out in the 50-µl reaction mixture containing 10-20ng of template DNA, 200 µM of dNTPs, 0.1 µM of each primer and 1 unit of Taq DNA polymerase (Thermo Scientific, USA). The reaction mixture was amplified in Applied Bio systems thermal cycler with temperature profile 95°C for 5 min; 35 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 1.30 min; final extension 72°C for 7 min, The amplified products were separated by agarose gel (1.2%) electrophoresis and stained with ethidium bromide (Sambrook et al. 1989). PCR products were purified according to manufacturer's instruction (Chromous Biotech) and further sequencing reactions were carried out using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and analyzed on ABI 3500xL Genetic Analyzer (Applied Biosystems).

Data Analysis

The sequences obtained using 16s rRNA gene sequencing was submitted to the NCBI GenBank (Accession numbers MF361145), and publicly accessible through the NCBI dataset. The NCBI BLAST was performed to confirm identity of specimens (Altschul et al.1990). The 'BLASTn' tool was used for sequence assignment against NCBI database

and highest-scoring hit from each query is taken as the bacterial identification.

Phylogenetic Analysis

Phylogenetic Tree Builder uses sequences aligned with System Software aligner. A distance matrix is generated using the Jukes-Cantor corrected distance model. When generating the distance matrix, only alignment model positions are used, alignment inserts are ignored and the minimum comparable position is 200. The tree is created using Weighbor with alphabet size 4 and length size 1000.

Weighbor Tree

Weighbor is a weighted version of Neighbor Joining that gives significantly less weight to the longer distances in the distance matrix. The weights are based on variances and covariances expected in a simple Jukes-Cantor model.

Jukes-Cantor Correction

The Jukes-Cantor distance correction is a model which considers that as two sequences diverge, the probability of a second substitution at any nucleotide site increases. For distance-based trees such as a Weighbor, the difference in nucleotides is considered for the distance, therefore, second substitutions will not be counted and the distance will be underestimated. Jukes and Cantor created a formula that calculates the distance taking into account more than just the individual differences (1969; *Evol. of Protein Molecules*, Academic Press).

Bootstrap

Bootstrapping is a statistical method for estimating the sampling distribution by resampling with replacement from the original sample. In making phylogenetic trees, the approach is to create a pseudo alignment by taking random positions of the original alignment. Some columns of the alignment could be selected more than once or not selected at all. The pseudo alignment will be as long as the original alignment and will be used to create a distance matrix and a tree. The process is repeated 100 times and a majority consensus tree is displayed showing the number (or percentage) of times a particular group was on each side of a branch without concerning the subgrouping [20].

Results and discussion

Since the first report on the isolation of extracellular alkaline protease from *Bacillus* sp. strain 221 [16], the search for industrially relevant protease from different environmental sources such as farmyard wastes [17] and sewage sludge [18] has been continuous. We have already isolated, screened, and identified the potential keratinase producing bacteria on the basis of morphological, physiological and biochemical characteristics as *Bacillus* sp. [15]. For further identification by 16S rDNA sequencing, the genomic DNA (gDNA) was extracted according to the standard bacterial gDNA extraction protocol (See fig. 1). The amplification of the DNA by using PCR was done (fig. 2). In this report we have supplemented the molecular identification of bacteria on the basis of 16S rDNA analysis.

Sequence Analysis

A high quality bidirectional 16s ribosomal gene sequences were received and formed single aligned full length sequence. Further, this sequence was analyzed using various tools such as NCBI blast and phylogenetic analysis[19]. The Blast result predicted based on percent match given bacterial sequence belongs to *Lysenibacillus* genus (Table 1). and alignment of gene sequence with 10 closely related gene sequence was performed and phylogenetic tree was constructed (See fig. 3). The sequence was submitted in the database Gene Bank and got accession number as MF361145. The bacterial strain was identified as *sphaericus sp.*

Figure 1: Genomic DNA from bacterial sample using the bacterial genomic DNA isolation kit loaded in 1 % agarose gel.

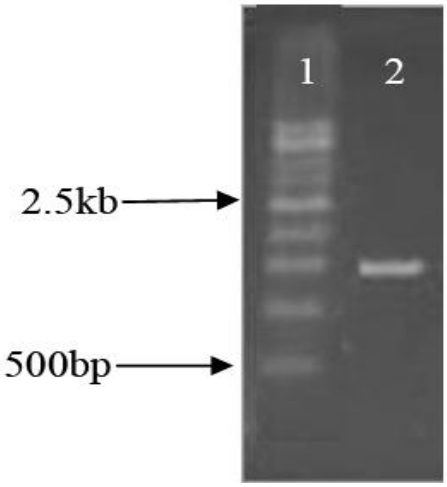

Figure 2: PCR amplification of 16s rDNA fragment from bacterial sample. The size of PCR amplified product is ~ 1.5 kb.

Table 1: Blast results based on percent match.

Sl. No.	Organism Name	Accession No.	Percentage Match
1	Lysinibacillus sp. MG3-4 16S ribosomal RNA gene	KP992161.1	100%
2	Bacillus sp. VTGP.D30808 16S ribosomal RNA gene	HQ848387.1	100%
3	Lysinibacillus fusiformis strain GF15 16S ribosomal RNA gene	KY312812.1	100%
4	Lysinibacillus sphaericus strain LSR1 16S ribosomal RNA gene	MF000302.1	100%
5	Lysinibacillus sphaericus strain S5-24 16S ribosomal RNA gene	MF112004.1	100%
6	Lysinibacillus fusiformis strain BH45 16S ribosomal RNA gene	KY910256.1	100%
7	Lysinibacillus sp. strain M2c 16S ribosomal RNA gene	KY705015.1	100%
8	Lysinibacillus sphaericus strain Endo Ya6 16S ribosomal RNA gene	KY565423.1	100%
9	Lysinibacillus sphaericus strain SADAAB_31 16S ribosomal RNA gene	KX908032.1	100%
10	Lysinibacillus fusiformis strain CKT-190 16S ribosomal RNA gene	KX432210.1	100%

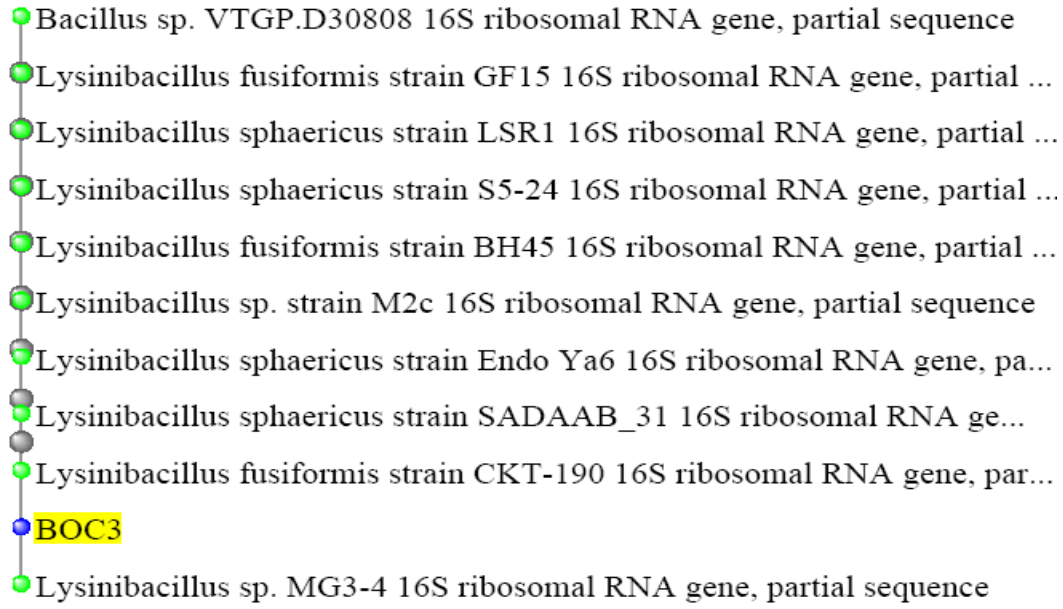

Figure 3: Phylogenetic position of *Lysinibacillus* sp. BOC 3 based on 16s rDNA sequence within the genus bacillus.

Conclusion

The results suggest that the keratinase producing bacteria is the *Lysinibacillus sphaericus* . strain.

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Conflict of interest

None

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