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A novel strain of *Bacillus thuringiensis* which efficiently degrades feather

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Abstract:

Bacillus thuringiensis is a bacterium known for the production of proteinaceous crystalline inclusions with insecticidal activity against the *Lepidopteran* larvae. We report a new strain of *Bacillus thuringiensis* designated, S3KUBOT which adds to the classification of *B.thuringiensis* based on crystal protein morphology, since it produces two morphologically different proteinaceous crystal inclusions on sporulation. The candidate *B. thuringiensis* isolate was recovered from a site in Thiruvananthapuram, where poultry wastes had been deposited from a long time. Characterization of the strain was based on the molecular (16S rRNA sequence analysis) and microscopic techniques. Characterization of the strain based on 16S rRNA gene was difficult because it showed 99% similarity to the *Bacillus cereus* group of organisms. Thus the microscopic examinations of the strain need to be depended. It is due to the difficulty in differentiating the isolates belonging to the *B. cereus* group, that several isolates are still identified as *Bacillus* species within the GenBank. Disposal of waste is a major concern of poultry industry especially when developing countries are concerned. Keratin containing waste materials are even very slowly degraded in nature and thus considered as hazardous waste. Chemical treatment of feather results in the loss of nutritionally important amino acids, the problem being solved when keratin degrading microorganisms are employed. Thus there is a need for exploration of efficient strains in the current scenario of biodegradation of poultry waste. The feather degradation by microorganisms is attributed to the production of an inducible enzyme keratinase. Keratinases are a group of proteolytic enzymes that are able to hydrolyze insoluble keratins. The present work reports a new strain of *Bacillus thuringiensis* which produces keratinase in the presence of feather waste. The study also recommends the need for a strong molecular tool which could efficiently distinguish between the species of *Bacillus cereus* group of organisms.

Keywords: Feather degradation; characterization; cloning; scanning electron microscopy, parasporal crystals.

Introduction

Feathers; making up about 5% of the body weight of poultry, is a considerable waste product of the poultry industry being produced about 8.5 billion tons per year world-wide (20). Disposal of waste feathers is a major concern for poultry industry. Accumulation of this huge volume of waste feathers results in protein wastage and environmental pollution of land and underground water resources. Discarded feather also causes various human ailments including chlorosis, mycoplasmosis and fowl cholera (28(a)). There is always a requirement of isolation of enzymes from new sources to meet the industrial and environmental demand. *Bacillus thuringiensis* is a Gram positive, spore forming and rod shaped bacterium which has an ability to produce parasporal crystal inclusions. We could isolate six feather degrading bacteria from various poultry-waste dumping sites of Thiruvananthapuram, Kerala, India. The feather degradation efficiency of a novel strain of *Bacillus thuringiensis* was found to be appealing from among the isolates. The achievement of the investigation lies in that our isolate is a novel strain of *Bacillus thuringiensis* which can be considered as another class on the basis of crystal protein morphology analysis. We report the occurrence of a new strain of *B.thuringiensis* which produces cuboidal and spherical crystals on sporulation.

Materials and Methods

Feathers were obtained from a local poultry farm of Thiruvananthapuram and washed thoroughly so as to remove dirt and blood. The well cleaned feathers were dried under sunlight and stored as such for further use. For solid medium, the feathers were cut into small pieces of a few millimeters. For enzyme activity determination, whole feathers were used. The basic medium used for isolation and biodegradation experiment contained 0.5 g/L NaCl, 0.7 g/L KH_2PO_4, 1.4 g/L K_2HPO_4, 0.1 g/L $MgSO_4$ and 10 g/L feather (pH 7.2). Cultivation was done using 250 ml Erlenmeyer flasks containing 100 ml medium. Feather agar medium containing the basic medium and 15 g/L of agar was used for screening the microorganisms in plates. 1ml of 24hr grown culture (4.25×10^5 CFU/ml) in LB broth was used as inoculum.

Keratinolytic activity determination

0.5 % (w/v) soluble keratin was used as substrate for keratinolytic activity determination (27). Crude enzyme was obtained from inoculated medium after 96 hrs of cultivation and done enzyme assay according to 3 (a). The control sample was prepared similarly, with uninoculated medium. One unit of enzyme activity (U/ml) was defined as an increase of corrected absorbance at 280 nm with that of the control for 0.01/minute under the conditions described and was calculated by the equation, $U = 4 \times n \times A_{280} / (0.01\times10)$, where, n is the dilution rate; 4 is the final reaction volume (ml); 10 is the incubation time (min.). The protein content of the crude enzyme preparation was done by (13).

Keratin Degradation *in situ*

The prepared soluble keratin was used to determine the keratinolytic activity of the crude enzyme preparation in native polyacrylamide gel. 12% resolving gel was prepared according to (10) containing 10% soluble keratin powder. 100µl of the crude culture supernatant obtained by centrifugation of the sample at 96^{th} hour of incubation was loaded along with the experimental control and run at constant current (40mA). After electrophoresis, the gel was washed twice with 2.5% Triton x-100 for 30 minutes at room temperature. Then it was washed with 0.05M Tris (pH 8) till the lather was almost removed. Then the gel was incubated in 0.05M Tris (pH 8) with 1mM DTT for 48hrs. After 48hrs, the gel was stained with Coomassie Brilliant Blue R-250 solution and destained.

Taxonomic studies

The cultural, morphological and biochemical characteristics of the selected bacterial strain were compared with the data from Bergey's Manual of Determinative Bacteriology (18). Molecular characterization of 16S rRNA gene was also carried out. Bacterial 16S rDNA was amplified by using the following universal primers, 8F and 1492R. Polymerase chain reaction was performed in Thermal Cycler (Techni, Germany) with a 25 µl reaction mixture. The amplified band was eluted using QIA quick extraction kit protocol (QIAGEN) and cloned in *E.coli* DH5α cells using pGEMT Easy vector system (Promega, Madison, WI, USA). The transformants were selected and screened using Luria Agar plates containing x-gal, IPTG and ampicillin. The transformed colony was inoculated into LB broth and incubated at 37ºC in a rotary shaker at 180 rpm, overnight. Plasmid DNA was isolated in which the 16S rDNA insert was integrated. The insert was sequenced by automated sequencing. Sanger's dideoxy chain termination method based on PCR (thermal cycle sequencing) was adopted for DNA sequencing. Automated sequencing was carried out using Big Dye Terminator Cycle Sequencing Ready Reaction Kit Version 3.1 from Applied Biosystems. The sample was denatured at 95ºC for 5 minutes and loaded into the Applied Biosystems 3730 Genetic Analyzer. The sample was resolved through POP 7 polymer and the sequencing data was analyzed using the sequence analysis software (5.2). The electrophorogram was analyzed for the sequence. The sequence obtained was aligned using BioEdit sequence alignment software and verified for correctness using the BLAST option available at: http://www.ncbi.nlm.nih.gov/. The16S rRNA sequence of the strain S3KUBOT was then submitted to GenBank and obtained the accession number. A phylogenetic tree was constructed using TREECONW 3.1 software package.

Scanning Electron Microscopy

Spore-crystal mixture was prepared in sterile distilled water after heat treatment for 1hr and a thin smear of the suspension was prepared on glass mounts , coated with gold in JEOL-JFC 1200 sputtering unit and photographed in JEOL-JSM 5600LV scanning electron microscope operated at a voltage of 20 kV.

Results

Six morphologically different bacteria were isolated from the collected soil samples. All the strains were maintained in Nutrient Agar for elucidating their keratinolytic activity as well as feather degrading capacity. S3KUBOT showed maximum activity as per the keratinolytic activity determining assay.

Keratinolytic activity determination

All the six strains isolated were able to grow and produce extracellular keratinase in the medium in which whole chicken feather served as the sole carbon and nitrogen sources. The maximum enzyme activity was observed on the 4th day of inoculation for all the isolates. The strain 3 designated as S3KUBOT was selected which was found to exhibit maximum enzyme activity (31.92 U/ml) on the basis of the assay (Fig 1).

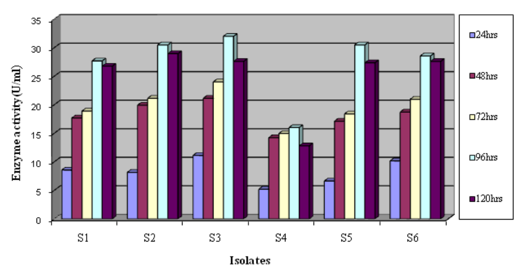

Fig. (1) Keratinolytic activity exhibited by the isolates at different incubation period.

Keratin Degradation *in situ*

Keratin degradation *in vitro* was found to be associated with the release of a large amount of extracellular keratinase into the medium by the strain. The presence of extracellular keratinase in the culture supernatant of the isolate was detected by keratin zymography. Figure 2 shows the degradation of the gel-incorporated soluble keratin by the culture supernatant obtained by centrifugation at 10000 rpm for 10 minutes. The arrow indicates a clear area where the keratin has been degraded by the crude enzyme preparation.

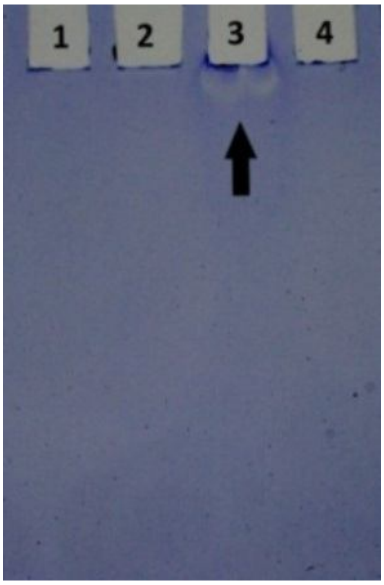

Fig. (2) *In situ* keratin degradation. Lane 1 Uninoculated control Lane 3 Culture supernatant of S3KUBOT

Taxonomic studies

The cultural, morphological and biochemical features agreed with the description of *Bacillus cereus* group in the Bergey's Manual of Determinative Bacteriology. The PCR product which corresponds to the 16S rRNA gene was eluted and cloned in pGEMT Easy vector system and was transformed in plasmid deficient *E.coli* DH5α cells. The plasmids of the transformed white colonies, where the 16S rDNA had integrated were used as such, for sequencing. The 16S rRNA sequences devoid of the vector sequences were used for determining homologous sequences in the GenBank (http://www.ncbi.nlm.nih.gov/blast). The BLAST result showed 99% sequence similarity to the species within the *Bacillus cereus* group. Multiple alignments of the sequences were done using Clustal W programme. Phylogenetic tree was inferred from (25) distances using the neighbour-joining method and constructed using the software TREECONW 3.1. The branching pattern was checked by 100 bootstrap replicates (Fig.3). The 1514 bp sequence obtained was submitted to GenBank and obtained the accession number (HQ832565.)

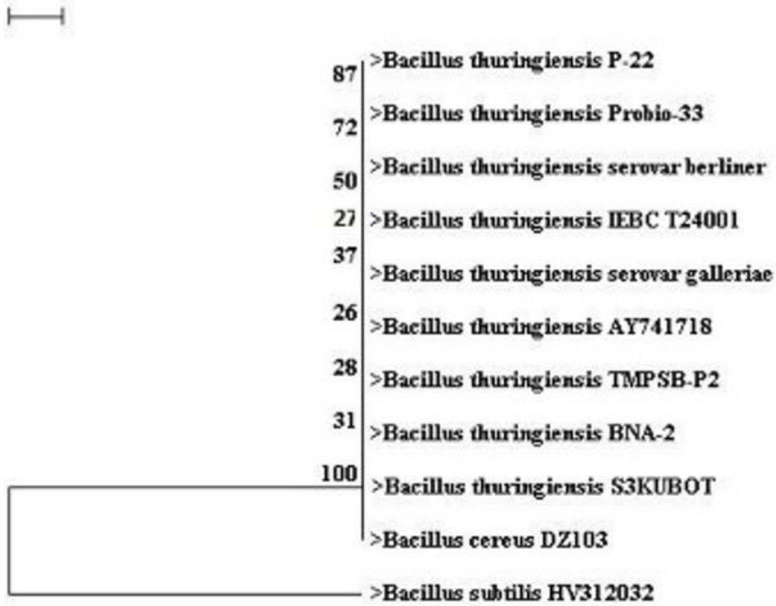

Fig. (3) Phylogenetic tree based on TREECONW 3.1

Scanning Electron Microscopy

The scanning electron micrograph of S3KUBOT before autolysis phase (Fig.4) clearly evidenced the presence of two morphologically different (spherical and cuboidal) parasporal crystals. The presence of parasporal crystals evidenced the identity of S3KUBOT as *Bacillus thuringiensis.*

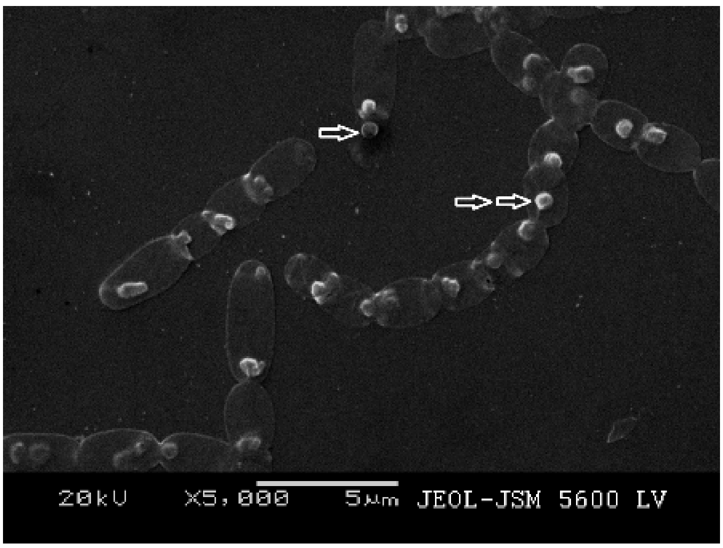

Fig. (4) Scanning Electron Micrograph of S3KUBOT before autolysis. Spherical crystal is indicated by a single arrow. Cuboidal crystal is indicated by double arrows

Discussion

Keratinases from microorganisms have attracted a great deal of attention in the last decade particularly due to their multitude of industrial applications. Biological treatment improves the nutritional value of feather waste. Keratinolytic enzymes find use in environment-friendly biotechnological processes that utilize keratin containing wastes from poultry industry. Also these enzymes could be interesting for various industrial purposes. (19, 22, 8, 12, 17, 7 and 28(b)). Developing countries including India should recycle the highly proteinaceous wastes generated from poultry industry because of the increasing need for improvement of their socio-economic status. Thus the isolation of potential strains of keratinolytic organisms has become important. Keeping all these in mind we started working in the current issue of biodegradation of poultry. We could isolate a new strain of *Bacillus thuringiensis* with efficient keratinolytic activity from a local poultry-waste dumping soil of Thiruvananthapuram. The enzyme activity of S3KUBOT was found to be appealing when previous reports were compared. The enzyme activity of the wild-type strain of *Bacillus subtilis* isolated by 3(b) was 24.3±1.31 U/ml. (24) have reported a *Bacillus* species with a maximum keratinase production of 0.9U/ml. Our strain could grow on the medium where feather served as the sole carbon and nitrogen sources and completely degrade whole feathers within 96 hrs of incubation. The ability of the strain to degrade whole feathers can offer a great potential when both industrial and environmental aspects are concerned.

B.cereus group strains share many phenotypic characters and a high level of genetic similarity. So it is important to develop methods for the rapid detection of the *B. cereus* strains. Analysis of 16S rRNA sequences is a simple, commonly used method for the identification of microorganisms (23, 29). However, early studies of the isolates from the *Bacillus cereus* group revealed that the 16S rRNA sequences of species in this group had as high as 99 to 100% similarity and, thus, suggested that rRNA sequences might not be useful for discrimination of members of that group (1). The *B. cereus* group contains seven closely related species: *Bacillus anthracis, B. cereus, Bacillus thuringiensis, Bacillus mycoides* (26, 21, 6), *Bacillus pseudomycoides* (15), *Bacillus weihenstephanensis* (11), and *Bacillus medusa* (4).

One of the most striking aspects of *Bacillus thuringiensis* sporogenesis is the synthesis of parasporal crystal. An ovoid inclusion was observed by (5) in *Bacillus thuringiensis* subsp. *kurstaki* and they had reported it as the first case in the species. The PCR results and sequencing evidence as observed by (2) makes clear that except for cry gene, it was difficult to develop a reliable molecular method for the differentiation of *B.cereus* and *B.thuringiensis* strains. Therefore to distinguish *B.thuringiensis* from *B.cereus*, a single feature such as the presence of a parasporal crystal protein may be reliable. The crystal and spore of *B.thuringiensis* isolates are released separately during sporulation.

The isolate S3KUBOT was confirmed to possess parasporal crystals by scanning electron microscopy. The morphology of the crystals was noted as spherical and cuboidal ones. (9) have reported the presence of bipyramidal and cuboidal crystals of the strain Bn-1. The occurrence of more than one type of crystals has been reported by (16). They could isolate the *B.thuringiensis* strains producing parasporal crystal inclusions with a variety of shapes and sizes. They grouped the isolates into three on the basis of distribution of crystals, as class 1 which consisted of spherical, class 2 which consisted of bipyramidal and class 3 which consisted of both bipyramidal and cuboidal crystals. Our results add to their classification; since we observed only spherical and cuboidal crystals in our isolate, making chance for a fourth class within the species. According to 14, the differences in the parasporal crystal shape distribution might be related to the genetic variation caused by the difference in environmental conditions. The present study highlights S3KUBOT as a novel strain of *Bacillus thuringiensis* since it produces a new combination of two morphologically distinct proteinaceous crystal inclusions, on sporulation.

Conclusion
The strain S3KUBOT has been reported as the first strain of *Bacillus thuringiensis* to produce cuboidal and spherical crystals during sporulation as well as produces keratinase during vegetative phase. Reports on the keratinolytic activity of *B. thuringiensis* are very few. Identification of the strain was laborious since the molecular analysis of 16S rRNA gene was found inefficient in identifying the species. This may be the prime reason that a lot much of isolates belonging to the *Bacillus cereus* group are still been identified as *Bacillus* species within the GenBank. Thus we strongly recommend the need to explore new molecular probes for the rapid detection of the environmental isolates of *Bacillus thuringiensis* as well as report a new strain from India.

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