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Research Article

DISCOVERY OF POLYGALACTURONASE PRODUCING *BACILLUS TEQUILENSIS* STRAIN ARMATI USING 16S rRNA GENE SEQUENCING

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ABSTRACT

Objective: To identify a novel strain of polygalacturonase producing *Bacillus tequilensis* from poultry farm.

Methods: Poultry feces sample was serially diluted, and the pure isolate was subjected to morphological tests, biochemical tests, genomic DNA isolation, polymerase chain reaction amplification, amplicon purification and 16S rRNA gene sequencing. The strain was screened for polygalacturonase production using plate assay method. The nucleotide sequences obtained from the isolate were subjected to BLAST tool for the pairwise alignment. RNA secondary structure was predicted through RNAStructure Web Server. Multiple antibiotic resistance (MAR) index was determined against six antibiotics through disc diffusion method. Phylogenetic trees were inferred using the neighbor-joining algorithm in Molecular Evolution Genetic Analysis software version 4.0.

Results: *B. tequilensis* strain ARMATI showed the production of polygalacturonase. MAR index of this novel strain was found to be zero. RNA secondary structure with a minimum free energy of −281.40 kcal/mol was obtained. Phylogenetic tree of *Bacillus* 16S rRNA genes separate each *Bacillus* according to their taxonomic positions and were supported statistically.

Conclusion: The present investigation clearly indicates the isolation, molecular characterization and sequence analysis of *B. tequilensis* strain ARMATI from poultry farm, and industrial application of this novel strain for the production of the enzyme.

Keywords: *Bacillus tequilensis*, Multiple antibiotic resistance index, Polygalacturonase, Phylogenetic tree, RNA secondary structure, RNAStructure Web Server.

INTRODUCTION

Pectin is a polysaccharide present in the primary cell wall and middle lamella of higher plants which contribute to the firmness and structural integrity of plant tissues [1]. Pectinases are a heterogeneous group of enzymes that catalyse pectic substance through depolymerization and de-esterification reactions. Based on the mode of action, pectinases are classified into pectin lyase, pectinesterase and polygalacturonase. Polygalacturonase is a prime importance for plants since they assist in cell wall extension and softening of some plant tissues during maturation and storage [2,3]. Polygalacturonases are subdivided into endopolygalacturonase and exo-polygalacturonase which hydrolyse the internal and external α-(1,4) glycosidic linkages of pectin, respectively [4]. Polygalacturonase are widely used in food industries and facilitate maceration, liquefaction and extraction as well as filtration process of fruits and vegetables juices [5]. Apart from this the pectinolytic enzymes have numerous applications in the various types of industries such as production of papers and fibers treatment in textile industries, paper and pulp industry [6,7]. Polygalacturonases are also used in the industrial processing of wine, coffee and tea fermentation [8]. *Aspergillus niger* is usually used for the commercial production of polygalacturonase on industrial scale [9]. However, very few researches have been done for the production of polygalacturonase from bacterial sources. Little is known about the bacteria present in the poultry environment such as in poultry litter and air of poultry house [10]. Bacteria present in poultry environment may enter into the flock to produce disease. There are also reports indicating that poultry feed and water may act as a source for various infectious diseases [11]. The genus *Bacillus* species consists of Gram-positive, rod-shaped, aerobic or facultatively anaerobic, spore forming bacteria of diverse phenotypic characteristics, including differences with respect to nutritional requirements, growth conditions and DNA base composition [12]. *Bacillus tequilensis* is a Gram-positive, single cell and motile rod shaped bacteria. Biochemically *B. tequilensis* is quite similar to *B. subtilis* which can be differentiated by positive arginine hydrolases, lysine decarboxylase, ornithine decarboxylase and acid production from rhamnose [13]. This study was concerned to search novel bacterial strain from poultry farm for large scale commercial production of polygalacturonase. In this study, we determined the 16S rRNA gene sequences of *Bacillus* strains isolated from poultry farm. By using molecular composition we classified and separated the poultry farm isolate into *Bacillus* species and were identified as a novel strain of polygalacturonase producing *B. tequilensis*. The present study was also aimed to determine the *in vitro* antibiotic sensitivity test of the isolate.

METHODS

Collection, isolation and screening of sample

Feces sample was collected from poultry farm of Guduvanchery, Tamil Nadu, India. Feces soils were brought to the laboratory in aseptic condition. One gram of sample was suspended in 9 ml of saline and mixed vigorously to make uniform suspension. After that soil sample was serially diluted up to 10^{-5} and 0.1 ml of aliquots were spread over nutrient agar plates from 10^{-5} dilution. The plate was incubated at 37°C for 24 hrs. The pure strain was picked out and purified by repeated streaking on nutrient agar slants. The culture was streaked on slants and kept in an incubator at 37°C for 24 hrs and were preserved in slants at 4±2°C.

Organism identification

Purified isolate was characterized by biochemical analysis using indole test, methyl red test, Voges–Proskauer test, citrate utilization test, catalase test, urease test, oxidase test and amylase test (according to the Bergey's Manual of Systemic Bacteriology). Gram-staining and Motility test were performed under morphological test.

Genomic DNA isolation

2 ml of bacterial culture were centrifuged at 6000 rpm for 5 minutes. The supernatant was discarded. 1 ml of UniFlex™ Buffer 1 and 10 µl of RNase were added to the pellet obtained. Mixed well by pipetting and incubated for 30 minutes at 37°C in a water bath. To the lysed samples, 1 ml of 1:1 phenol:chloroform were added and mixed well. The samples were centrifuged at 10,000 rpm for 15 minutes at room temperature. The aqueous layers were separated in a fresh 1.5 ml vial. To the aqueous layer 1 ml of UniFlex™ Buffer 2 were added and mixed well by pipetting. The mixture was centrifuged at 12,000 rpm for 15 minutes at room temperature. The supernatant was discarded. To the pellet 500 µl of 70% ethanol were mixed. Again it was centrifuged at 10,000 rpm for 10 minutes at 4°C. The supernatant was discarded. The pellet was air-dried for about 10-15 minutes till the ethanol evaporate. The pellet was resuspended in 50-100 µl of UniFlex™ Elution Buffer. DNA was stored at −20°C.

Polymerase chain reaction (PCR) amplification and sequence of 16S rRNA

The 16S ribosomal RNA was amplified by using the PCR (ependorfep.Gradient) with *Taq* DNA polymerase and primers 27F (5`AGTTTGATCCTGGCTCAG 3`) and 1492R (5`ACGGCTACC TTGTTACGACTT 3`). The conditions for thermal cycling were as follows: Denaturation of the target DNA at 94°C for 4 minutes followed by 30 cycles at 94°C for 1 minute, primer annealing at 52°C for 1 minute and primer extension at 72°C for 1 minute. At the end of the cycling, the reaction mixture was held at 72°C for 10 minutes and then cooled to 4°C. PCR amplification was detected by agarose gel electrophoresis and visualized by alpha image gel doc after ethidium bromide staining.

Purification of amplified product

PCR sample was taken in fresh vial and 5 µl of 3 M sodium acetate solution (pH - 4.6) and 100 µl of absolute ethanol were added into it. The vial was mixed thoroughly. The vial was kept at −20°C for 30-40 minutes to precipitate the PCR product. Then it was centrifuged at 10,000 rpm for 5 minutes. 300 µl of 70% ethanol were added to the pellet, without mixing, and the centrifugation was repeated at same rpm. The pellet was air dried until the ethanol effervescence was removed. The pellet was suspended in 10 µl of sterile distilled water [14].

Sequencing of PCR product

The PCR product obtained was sequenced by an automated sequencer (Genetic Analyzer 3130, Applied Biosystems, and the USA). The same primers as above were used for sequencing. The sequence was compared for similarity with the reference species of bacteria contained in genomic database banks, using the NCBI BLAST available at http://www.ncbi-nlm-nih.gov/.

Qualitative screening for polygalacturonase production

Pectin agar medium (1%) was prepared and was autoclaved. The agar media were poured onto the sterilized petri plate and allowed to cool. The overnight grown novel bacterial strain was streaked onto the solidified agar plates. The plates were incubated overnight at 37°C. After overnight incubation the streaked plates were flooded with iodine solution containing 0.25% iodine, 0.5% potassium iodide and 31 ml of 20% ethanol. The plates were observed for the clear zone of inhibition around the streaked culture. Further confirmation for the extracellular production of polygalacturonase was done by agar well diffusion method. Wells of 6 mm were made on pectin agar plates using cork borer and 100 µl of overnight grown novel bacterial culture was poured into the well. The plates were kept in upright position overnight at 37°C in order to check zone of inhibition around the well. The plates were stained with iodine solution for the visualization of zone of inhibition.

RNA secondary structure prediction

RNA secondary structure prediction was performed to determine the stability of chemical or biological molecules or entities of the isolate. RNA structure prediction was determined using RNAStructure Web Server. Minimum free energy (MFE) and the mountain plot representation were also identified.

Neighbor-joining tree analyses of *Bacillus* 16S rRNA gene for sequence comparisons

Phylogenetic relationship of the isolate with other *Bacillus* species were inferred from phylogenetic comparison of the 16S rRNA sequences using Molecular Evolution Genetic Analysis software version 4.0 [15].

Multiple antibiotic resistances (MAR) index determination

The antibiotic susceptibility pattern of the test organism was performed as per standard procedure. A homogeneous bacterial lawn was prepared on Mueller Hinton Agar plates using sterile cotton swabs. The sterile discs of 6 mm diameter were soaked with 25 µl of antibiotics. Using an ethanol dipped and flamed forceps the standard antibiotic and soaked discs were aseptically placed over the agar plates sufficiently separated to avoid overlapping of zone of inhibition. Plates were incubated at 37°C for 24 hrs. After 24 hrs, diameter of zone of inhibition was measured in mm and results were recorded. MAR index was calculated by the ratio of number of antibiotics ineffective over the organisms to the number of antibiotics exposed [16]. The antibiotics used in this study were ampicillin - 10 µg, kanamycin - 30 µg, nalidixicacid - 30 µg, streptomycin - 10 µg, cefotaxime - 30 µg and penicillin G - 10 µg.

RESULTS

Morphological and biochemical test analysis

The morphological and biochemical characteristics of the isolate were studied (Table 1 and Fig. 1). The isolated bacterial strain was identified as *Bacillus* sp. based on the taxonomical characteristics.

PCR amplification and sequencing of 16SrRNA

Genomic DNA of the isolate was visualized under UV. The amplicon of 740 bp was observed using PCR amplification (Fig. 2). In the present study, 16S rRNA gene sequencing of the isolate was investigated. The isolate was identified as *B. tequilensis* strain ARMATI by comparing the similarity with the reference species of bacteria contained in genomic database banks, using the NCBI BLAST. The comparison showed that the similarities of 16S rRNA gene sequences were 100%. The identities of strain ARMATI were determined by comparing them with the available

Table 1: Morphological and biochemical test report

Serial number	Tests	Result
1	Morphology	Rod shaped
2	Gram-staining	Positive
3	Motility	Positive
4	Indole	Negative
5	Methyl red	Negative
6	Voges–Proskauer	Positive
7	Citrate utilization	Positive
8	Urease	Negative
9	Catalase	Positive
10	Amylase	Positive
11	Oxidase	Positive

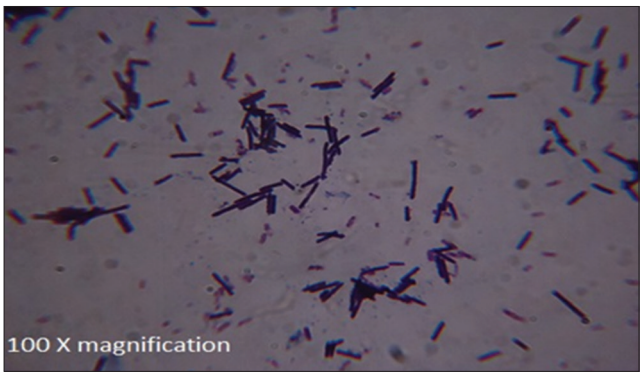

Fig. 1: Gram-staining of *Bacillus tequilensis* strain ARMATI

sequences of the strains and with high scored rRNA sequences in BLAST search. The novel isolated sequence was deposited in GenBank (Accession number-KC424491), maintained by NCBI, USA.

Qualitative screening for polygalacturonase production

Clear zone around the streaked novel bacterial culture and around the well onto the agar plate were observed indicating the production of polygalacturonase.

RNA secondary structure prediction

The optimal secondary structure with a MFE of −281.40 kcal/mol was represented in Fig. 3. Mountain plot representation and entropy for each position were also determined (Fig. 4). A mountain plot represents a secondary structure in a plot of height versus position, where the height is given by the number of base pairs enclosing the base at given position i.e. loops correspond to plateaus (hairpin loops are peaks), helices no slopes.

Phylogenetic tree of strain ARMATI 16S rRNA

A Neighbor- joining tree of *Bacillus* 16S rRNA sequences, including different strains of *Bacillus* species, clustered all the isolates belonging to the previously identified species to the corresponding species (Fig. 5).

MAR index determination

The MAR index value of the test organisms was reported in Table 2. The MAR value is a ratio of the number of ineffective antibiotics to the number of antibiotics exposed. The MAR value of the test organisms was found to be zero.

DISCUSSION

The current classification and identification of the species within the genus *Bacillus* and related genera is well established and is based on

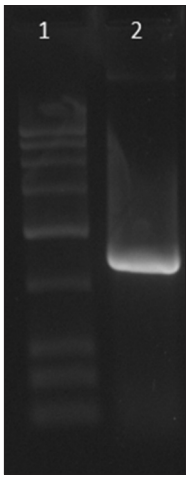

Fig. 2: 16S rRNA polymerase chain reaction (PCR) Product. Lane 1: Marker; Lane 2: PCR Product

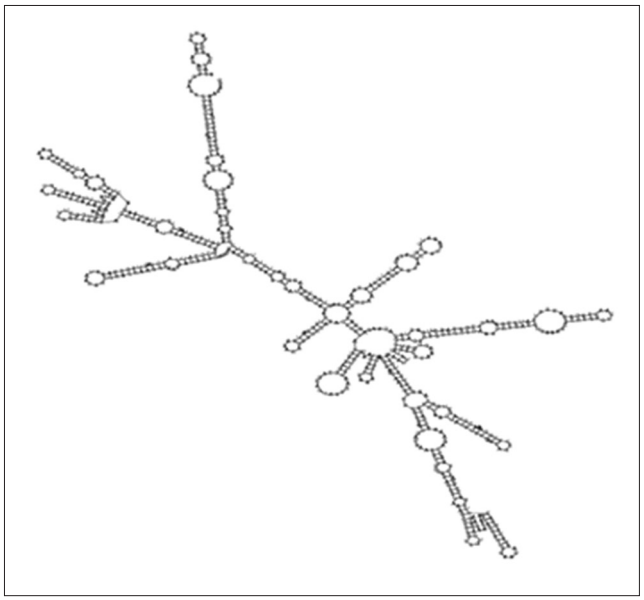

Fig. 3: RNA secondary structure of *Bacillus tequilensis* strain ARMATI with minimum free energy of −281.40 kcal/mol

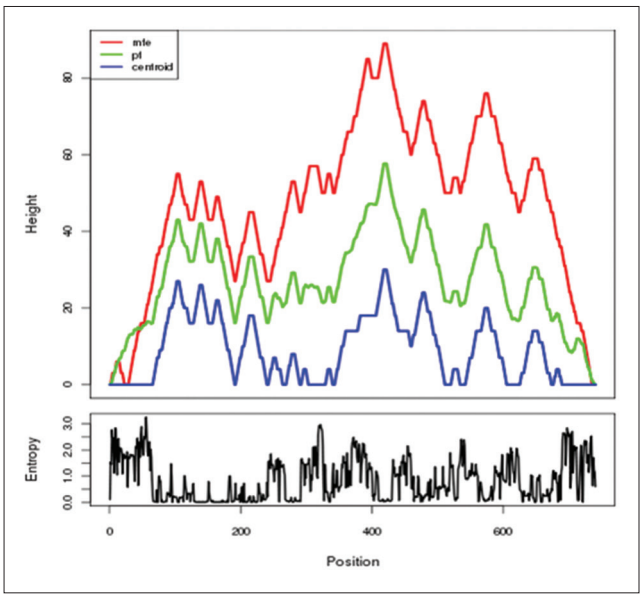

Fig. 4: Mountain plot and entropy for each position

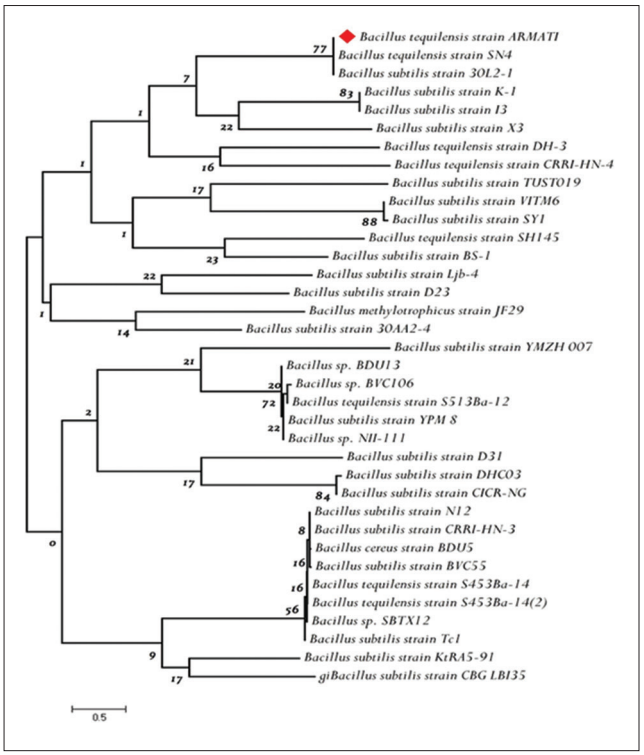

Fig. 5: Dendrogram depicting the phylogenetic relationship of strain

Table 2: MAR index determination of the bacterial isolate

Bacteria	MAR value	Number of ineffective antibiotics	Name of ineffective antibiotics
B. tequilensis strain ARMATI	Zero	Zero	None

MAR: Multiple antibiotic resistance, *B. tequilensis: Bacillus tequilensis*

a combination of numerous experimental approaches [17]. *Bacillus* strains are ubiquitous microorganisms, which can grow on natural media without any special requirements. Catabolic repression of biosynthesis of extracellular enzymes by fiber and other readily metabolized carbon sources has long been documented in *Bacillus* sp. [18]. In the present study, *Bacillus* species had been isolated and 16S rRNA determination was done. *B. tequilensis* strain ARMATI was further analyzed phylogenetically and also searched with BLAST-N algorithmic in NCBI database. Miranda *et al.* [19] reported *Bacillus* species from marine sediments by conventional biochemical tests and sequencing analysis of 16S rRNA genes. Two new strains of *Bacillus licheniformis* were identified and characterized from poultry farm using 16S rRNA gene sequencing [20]. The susceptibility and the resistant nature of *B. tequilensis* strain ARMATI in the presence of natural plant products have been reported in previous research [21]. Shah *et al.* [14] reported a novel strain of polygalacturonase producing *B. tequilensis* isolated from soil using 16S rRNA gene sequencing. This study was concerned with the searching of novel bacterial cultures from different habitats for the large scale commercial production of polygalacturonase. According to Bonala and Mangamoori [22], *B. tequilensis* NRRL B-41771 isolated from oil mill wastes on the basis of morphological and biochemical analysis, produced extracellular lipase. The previous reports clearly indicated that *B. tequilensis* is a potential bacterium to produce different types of enzymes which has commercially a lot of applications. Reports of several workers showed that *Bacillus* sp. was considered as prime producer of keratinase [23]. Jahan *et al.* [24] characterized keratinolytic bacteria isolated from poultry waste. According to the study, *Bacillus* Z4 is a potent producer of keratinase, which can be used for production of the enzymes at large scale. Mehta *et al.* [25] isolated new strain of *Bacillus* species with keratinolytic activity using microscopic, biochemical and 16S rRNA gene sequencing. The 16S rRNA gene is now used as a framework for the modern classification of bacteria including *Bacillus* sp. However, 16S rRNA gene sequences sometimes show limited variation for members of closely related taxa [26] due to the conserved nature of the gene. In such cases, DNA sequencing of certain housekeeping genes can provide more sensitive DNA sequencing subtyping than 16S rRNA sequencing for a number of bacterial species [27]. MFE for the prediction of optimal secondary structure is the method for searching the structure with stable energies. First a dot matrix analysis is carried out to highlight complementary regions (diagonal indicates succession of complementary nucleotides). The energy is then calculated for each predicted structure by summing negative base stacking energies. Using one sequence can determine structure of complementary regions that are energetically stable. MFE value determines it as a stable model. Many phylogenetic studies of the *Bacillus* [28] have been done, most of which are biased toward *B. subtilis*, particularly because of clinical concern about certain pathogens such as *B. cereus* and *B. anthracis* [29]. As a DNA sequence-based identification scheme for *Bacillus* [30], we consider 16S rRNA sequences appropriate for the identification of poultry farm *Bacillus* sp. The isolate was examined as *B. tequilensis* strain ARMATI depending on their taxonomic positions. The phylogenetic analysis and similarity analysis of the new strain undoubtedly reflects diversity within the targeted DNA regions.

CONCLUSION

Systematic studies of *Bacillus* group have been biased toward the isolation from different types of sources and environment and relatively studies have examined *Bacillus* species from poultry farm. In this study polygalacturonase producing newly isolated strain from poultry farm was studied and this strain was identified as *B. tequilensis* strain ARMATI after 16S rRNA gene analysis. This bacterium can be used for the commercial production of polygalacturonase at a large scale. Polygalacturonase enzyme from strain ARMATI will have the promising biotechnological approaches in industrial applications. As pathogenicity of *B. tequilensis* to human beings is undetermined so further research is necessary to find out the virulent and non-virulent gene of this novel bacterial strain. Another research should be continued to find out the improved methods of molecular characterization of this strain other than 16S rRNA gene sequencing.

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