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Screening, Isolation and Identification of Polygalacturonase Producing *Bacillus tequilensis* Strain EMBS083 Using 16S rRNA Gene Sequencing

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Abstract: Polygalacturonase is depolymerizing enzymes which are produced in plants and are foremost for industrial applications such as pretreatment of waste water from vegetable food processing industries. Mostly pectinaceous containing material are produced by bacteria and fungi. Therefore, in the present study, We aimed to identify a novel strain of Polygalacturonase producing Bacillus tequilensis. The sample was isolated from soil and rotten vegetables found in the geographical location of Indore, Madhyapradesh, India. The same has been serially diluted and were subjected to 16S rDNA sequencing. The sequence obtained from the isolate has been followed for a pair wise alignment using BLAST against other species of Bacillus tequilensis which constitute to be a novel species of the same, which was named further as *Bacillus tequilensis sp. EMBS083*. After the characterization the sequence of isolate has been deposited in GenBank with accession number KC171015.

Key words: *Bacillus tequilensis* · *Bacillus tequilensis Sp. EMBS083* · Polygalacturonase Producing Bacteria · 16S rRNA Gene Sequencing

INTRODUCTION

Pectinases are a heterogeneous group of enzymes that hydrolyze the pectic substances present in plants. Pectinase is an enzyme group that catalyzes pectic substance by degradation reactions through depolymerization (hydrolases and lyases) and de-esterification (esterases) reactions. These polysaccharide degrading enzymes are suitable tools to study the structure of pectin [1]. Polygalacturonase is of prime importance for plants since they assist in cell-wall extension and softening of some plant tissues during maturation and storage [2, 3]. Pectinases are most widely distributed enzymes in bacteria, fungi and plants [4]. The pectinolytic enzymes degrade the pectic substances found in plant tissues, thereby, having numerous applications in various types of industries such as production of papers and bast fibres, natural fibers treatment in textile industries, paper and pulp Industry [3, 5, 6]. *Bacillus tequilensis* is a Gram-positive, 0.9x4.0 µm, single cell, motile rod shape bacteria. Biochemically *Bacillus tequilensis* is quite similar to *Bacillus subtilis* which can be differentiated by positive arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and acid production from rhamnose [7].

This study was concerned with the searching of novel bacterial cultures from different habitats for the large scale commercial production of polygalacturonases. This effort can fulfill the national demand and strengthen the industry and economy because valuable foreign exchange is spent on import of industrial enzymes. Therefore in the present study, we describe the isolation of a novel strain of pectinase producing *Bacillus tequilensis*.

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MATERIALS AND METHODS

Isolation and Screening of Sample: The samples were collected from rotten vegetables from Indore area, India. Rotten vegetables were washed with distilled water and heated up to 10 minutes at 60°C. After drying for 10min, 30% vinegar and 70% salt water (1% NaCl) were applied over the mixed ingredients. The beaker was sealed and kept for fermentation for 3 months. Isolation of bacteria was performed after the completion of fermentation from the liquids and ingredients. Briefly, the ingredients were separated, homogenized and serially 10-fold diluted in phosphate-buffered saline solution (PBS; pH 6.6). 100 µl of each dilution was then plated on (NA) nutrient agar supplemented with 2% NaCl and incubated at 30°C for 24-48hrs. Single colonies were Replica-plated on to plates containing 10 µl Muller Hinton agar supplemented with 1% NaCl and V. harveyi, which was previously grown overnight in LB broth (Difco, Spark, NV, USA)[8]. There after these plates were then incubated for 24hrs at 30°C. Clear zones around replica-plated bacteria were interpreted as antagonistic activity against the pathogen and they were picked from the original plates.

Identification of Bacterial Strains: In order to identify the strain, extraction and amplification of genomic DNA, 16S rRNA sequence analysis were carried out as described previously [9, 10]. The sequences obtained were compared against the sequences available in the NCBI, EMBL and DDBJ databases obtained from the National Center for Biotechnology Information using the BLASTn [9, 10].

PCR Amplification and Sequencing of the 16S rRNA: The 16s rRNA coding gene from the DNA was amplified for 25 cycles. The first step here is Pre-Denaturation which involves the separation of entire DNA in single strands. It is done at 95°C for 5 minutes. It is followed by Denaturation which is done for 1 min at 94°C. In this step only the zone of interest is separated into single strands. Annealing, it involves the addition of primer to its complementary sequence on the strand which here is 16 s rRNA gene. It was carried at 52°C just for 1 minute in each cycle. Renaturation was done at 72°C for 1 min in each cycle. The final step of Extension is at 72°C for 7 minutes, with the help of DNA polymerase was carried out. The benefit of using 16sr RNA gene sequencing technique is that though we don't know the sequence of DNA still we can create the primers for PCR amplification. These primers are "Universal Primers". The basic idea for using universal primers is that we know 16s rRNA gene is unique for all different strains but the flanking regions of 16s rRNA gene remains highly conserved over different species. So the primer will go and bind to these flanking regions of 16s rRNA gene and will enhance the extension of the gene. So we can design primers for novel species containing 16s rRNA gene. The following primers were used for the amplification and the PCR Product size was 1.4kb.

fwd_Primer: CCAGCAGCCGCGGTAATACG
Rev_Primer: TACCAGGGTATCTAATCC

Lane 1: Marker
Lane 2: PCR Product

Fig. 1: 16S rRNA PCR Product on 2% Agarose Gel.

Purification of the Amplified Product: After the amplification of the DNA in PCR we took the PCR sample in a fresh vial and added 5 µL of 3M sodium acetate solution (pH=4.6) and 100 µL of absolute ethanol in it and mixed it thoroughly. Then we vortexed the vial and left it at-20°C for 30-40 minutes to precipitate the PCR products. Then it was subjected to centrifugation for 5 minute at 10,000 rpm. To the pellet we added 300 µL of 70% ethanol, without mixing, it was again subjected to centrifugation for 5 minutes at 10,000 rpm. The produced pellet was air dried until the ethanol effervescence is removed. And lastly the pellet is suspended in 10 µL of sterile distilled water.

Sequencing of PCR Product: Primers (5'-CCAGCAGCCGCGGTAATACG-3' and 5'-TACCAGGGTATCTAATCC-3') of the 16S rRNA gene of Bacillus were used to amplify a 1471 bp fragment from the isolated DNA sample. The amplified product from three independent PCRs was gel-purified, ligated into pCR2.1 (Invitrogen Life Technologies) and transformed into Escherichia coli INVáF' (Invitrogen), as recommended by the manufacturer. Plasmid DNA was isolated using a plasmid isolation kit (Bio-Rad), digested with EcoRI and resolved by agarose gel electrophoresis. Plasmids containing appropriately sized inserts were sequenced at Eminent Biosciences, Indore, India using Sanger dideoxy sequencing. The novel isolated sequence was deposited in GenBank with accession number KC171015, maintained by the National Centre for Biotechnology Information (NCBI), at the National Institute of Health (NIH), Rockville, Maryland, USA.

Sequence Analysis: DNA Baser Sequence Assembler v. 1.0 was used to assemble both the forward and reverse sequence file. The 16S rRNA gene sequences obtained in current study together with those of *Bacillus tequilensis* strain EMBS083 and the outgroup Bacillus species were aligned and sequence similarity was assessed using DNAMan. Phylogenetic relationships between *Bacillus tequilensis* strain EMBS083 and other Gram-positive, spore-forming bacilli species were inferred from phylogenetic comparison of the 16S rRNA sequences using parsimony (dnapars) and maximum-likelihood algorithms (dnaml and dnamlk) available in phylip [11]. DNAPARS (Maximum-parsimony) were used to compare sequences, assuming that the shortest tree(s) could produce an accurate hypothesis of phylogenetic relationships. Maximum-likelihood and parsimony-derived trees were bootstrapped using 1000 random samples of the original taxon by character matrix sequences with replacement using the sequence boot procedure[11]. All resulting trees were evaluated to estimate majority rule consensus trees using the consensus procedure to produce bootstrapped phenograms[11]. Trees were treated as unrooted, although the out-group designation option was included to polarize character states.

RESULTS AND DISCUSSION

The sequence dataset obtained from the isolate has been followed for a pair wise alignment using BLAST against other species of Bacillus tequilensis which constitute to be a novel species of the same, which was named further as *Bacillus tequilensis sp. EMBS083.*

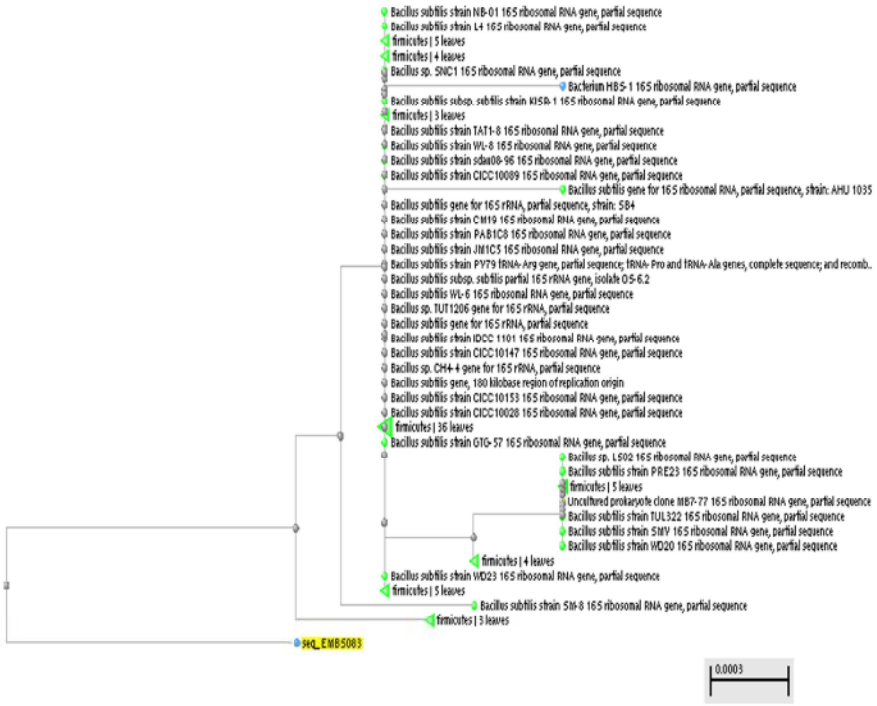

Fig. 2: Phylogenetic affiliation of *Bacillus tequilensis* sp. EMBS083 against other species of Bacillus

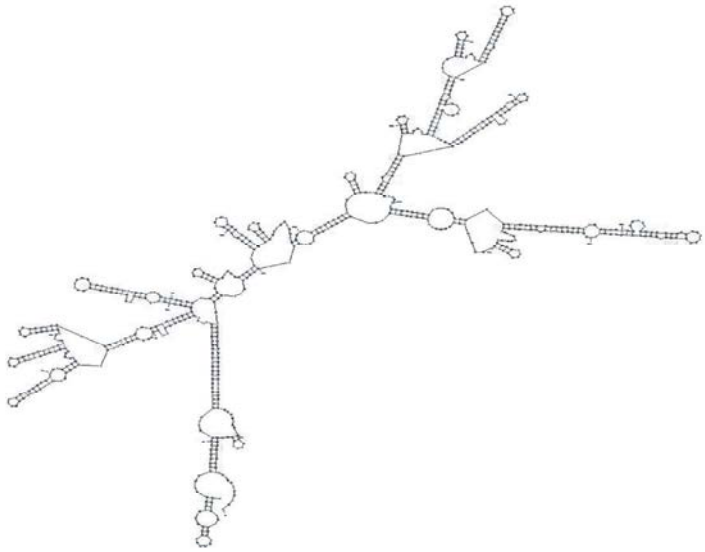

Fig. 3: RNA Secondary Structure of Bacillus tequilensis sp. EMBS083 shows the Gibb's free energy, ΔG =-282.34 kcal/mol.

The dataset *Bacillus tequilensis sp. EMBS083* consisted of 1471BP (100%) is parsimony informative. The matrix was competently and manually aligned. Coding gaps as binary characters, missing data had no affect on the topology and very affective on branch support. The 100% bootstrap consensus tree is shown Fig. 2. After the characterization of the sequence; the isolate has been deposited in GenBank with accession number KC171015.

In order to understand the significance in predicting the stability of chemical or biological molecules or entities of *Bacillus tequilensis sp. EMBS083*; RNA Secondary Structure prediction has been performed. RNA structure prediction has been carried out using UNAFOLD 4.6 in Linux Platform. Linear RNA folding has been carried out at window size 12 with max folds of 50. Folding bases 1 to 1471 of *Bacillus tequilensis sp. EMBS083* at 37°C shows the Gibb's free energy, ΔG =-282.34 kcal/mol. The thermodynamics result from each base wise of the data set shows the average of External closing pair Helix ΔG-19.70, Stack ΔG-3.40, Multi-loop ΔG-4.50, Bulge loop ΔG-2.70, Hairpin loop ΔG-1.60, Closing pair and Interior loop of ΔG-5.20 kcal/mol respectively which seems to be a very stable model Fig. 3.

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