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ORIGINAL RESEARCH ARTICLE

Isolation, screening and molecular identification of antagonistic bacteria against *Colletotrichum gloeosporioides* in mango

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ABSTRACT

The study was conducted at the Postharvest and plant Biotechnology laboratory, Department of Horticulture, Patuakhali Science and Technology University, Patuakhali, Bangladesh during the period from January to July 2017 to isolate, screening and molecular identification of antagonistic bacteria against anthracnose of mango. All treatments were arranged in a completely randomized design (CRD) with replications and repeated twice. Epiphytic bacteria, isolated from leaf and fruit surfaces of mango, were tested as biocontrol agent against anthracnose disease caused by *Colletotrichum gloeosporioides* wherein 20 strains were confirmed as antagonistic. Molecular characterization of the three potential strains of bacteria were done by the amplification of 16S rDNA gene following the extraction of genomic DNA, polymerase chain reaction (PCR) amplification, gel electrophoresis and gel documentation. The PCR amplified products and the genomic DNA samples were sent to the Macrogen Company through Sunchon National University, Seoul, South Korea for molecular identification by sequence analysis. Among the 20 antagonistic bacteria screened *in vitro* by dual and concomitant tests, two isolates, namely GB6 (PSTU-Hort-8), and GB19 (PSTU-Hort-14) were recognized as antagonistics to the test fungus. Using the molecular identification systems, isolated bacterial strains PSTU-Hort-8 was identified as *B. subtilis* with National Center for Biotechnology Information (NCBI) accession numbers MW659188; on the other hand, strain PSTU-Hort-14 was identified as *Stenotrophomonas rhizophila* with NCBI accession number MW659190.

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INTRODUCTION

Mango is one of the most common and popular fruit and often mentioned as the 'King of fruits' due to its excellent flavor, attractive color, delicious taste and high nutritive value. In Bangladesh, it occupies an area of 37,830 hectares of land with an annual production of 1161685 metric ton (MT) (BBS, 2016). Mango is cultivated mainly in regions with tropical and subtropical climates with high relative humidity (Levin *et al.*, 2018), conditions that also favor the development of diverse fungal phyto-pathogens. One of the most problematic in negatively affecting production is *Colletotrichum gloeosporioides*, the causal agent of anthracnose, considered the most common disease for fruit worldwide. This disease harms mango fruit, limiting sales both nationally and internationally (Bally *et al.*, 2009). At world level, anthracnose has caused losses of up to 60% in mango production (Madhu and Pradeep, 2016).

Conventional methods of control of anthracnose are based

mainly on the application of synthetic fungicides; nonetheless, their common use has caused new generations of phytopathogens resistant to treatment. In addition to this limitation on effectiveness of synthetic fungicide, there is growing interest of consumers for healthier food without dangerous chemical residues. Thus, there is growing interest in the development of non-fungicide control methods for reducing postharvest fruit losses caused by phyto-pathogens (Zhang *et al.*, 2017). Biological control with microbial antagonists has become a promising alternative for controlling fungi causing diseases at the postharvest level (Dukare *et al.*, 2018). Microorganisms with this potential are very diverse, among which epiphytic bacteria stand out specifically, those isolated on fruit surface or fruit lesions. Nonetheless, there are other environments, such as the marine ecosystem that represents an important biological resource for searching for new antagonistic microbial agents toward phyto-pathogens. However, some studies have demonstrated that microorganisms from other environments, such as the marine ecosystem, could be also highly efficient for the control of fungal plant pathogens (Wang *et al.*, 2010). Therefore, this study was carried out with the following specific objective to isolate, screening and molecular identification of antagonistic bacteria against anthracnose of mango.

MATERIALS AND METHODS

Isolation of antagonistic bacteria

Leaves and fruits of mango were collected from PSTU Germplasm centre and local area of Dumki upazila. Antagonistic bacteria were isolated from healthy and diseased mango fruits of locally grown plant. A total of 100 isolates of bacteria were obtained by the dilution method from leaf and fruit surfaces of mango. These isolates were evaluated for *in vitro* antagonism toward *C. gloeosporioides* by dual incubation method (Fokkema, 1978). Isolates with inhibitory characteristics against the test fungus were selected for further screening by means of dual and concomitant test.

Preparation of conidial suspension of *C. gloeosporioides*

C. gloeosporioides, the causal organism of anthracnose of mango were taken from naturally infected mango fruits. Small portions of symptomatic tissue were placed on petri plates containing potato dextrose agar and maintained on PDA (PDA, Merck Specialties Private Limited, Mumbai, JB2JF62036) at 28 ± 2°C for seven days. Spores were harvested from the media by flooding the surface with sterilized distilled water and gently agitating the plate with a sterilized bent glass rod to displace the spores. The resulting suspensions were filtered through two layers of sterile muslin cloth. Conidia were examined under a light microscope and identified by comparing their morphological and cultural characteristics with descriptions published in specialized literatures (Sutton, 1992). The concentration of conidia in the filtered suspension was adjusted to 5×10^5 conidia mL^{-1} with sterile distilled water using a haemacytometer (Obagwu and Korsten, 2003).

Dual culture assay

A mycelial plug of six mm diameter were taken from four days old culture of *C. gloeosporioides* and placed on the centre of a 9 cm diameter petri dish containing PDA. A loop full bacterial isolates from 24 hours culture were then streaked on PDA 1.5 cm from the edge of plate. As a control, agar discs of the same fungus were placed on a PDA culture plate without the bacteria. Plates were then incubated at room temperature (28 ± 2°C) for seven days. After the incubation period, percent inhibition of radial growth (PIRG) was recorded based on the following formula (Sivakumar *et al.*, 2002).

$$\text{PIRG (\%)} = \frac{R1 - R2}{R1} \times 100$$

(R_1 = Radial growth of *C. gloeosporioides* in control plate, R_2 = Radial growth of *C. gloeosporioides* interacting with antagonistic bacteria)

Preparation of aqueous antagonist suspension

Isolates were selected based on their antagonistic activity. In preparing aqueous antagonist suspension, isolates were grown on NA at 28 ± 2°C for 24 hours. A loop of each culture were then transferred to a 250 mL conical flask containing 50 mL of nutrient broth and incubated on a rotary shaker at 150 rpm for 72 hours at 31°C. To enumerate the colony forming units (CFU), cultures were serially diluted in sterile distilled water and plated on nutrient agar. The number of CFU were counted after 48h incubation at 28 ± 2°C. At the time of use, the sus-

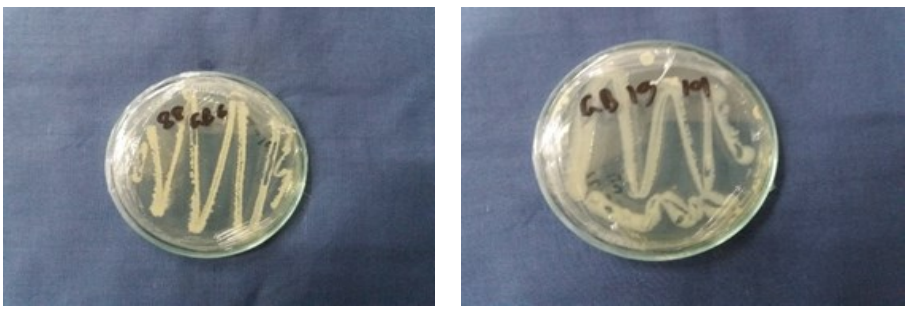

Plate 1. *Growth of B. subtilis PSTU-Hort-8 (A) and S. rhizophila PSTU-Hort-14 (C) on NA medium after 24 hours of incubation at 28 ± 2°C.*

pensions were adjusted to approximately 10^8 CFU mL^{-1} by spectrophotometer standard growth curve. To obtain filter sterilized culture filtrate, 72 hours old cultures were centrifuged at 8,000 rpm for 10 minutes. The cellular pellets were discarded and the supernatant were filtered through sterilized filter (0.45 µm) (Yoshida *et al.*, 2001). This supernatant were designated as sterilized culture filtrate of bacteria and used in this experiment to determine their effect on fungal growth.

Molecular identification of antagonistic bacteria

Based on colonies colors, sizes and shapes, initially 100 different bacterial strains were isolated. Among them 50 were creamy white and shiny, 30 dry and creamy white, and 20 were white. Pure culture of each isolate was maintained for screening test against *C. gloeosporioides*. Among the bacterial strains, three were most potential and used for molecular characterization. DNA was extracted using DNAzol reagent (Molecular Research Center Inc, Cincinnati, OH) and Cenis method simultaneously. The extraction using DNAzol reagent was performed according to the manufacturer's protocol. Initially all the bacterial strains were cultured in liquid medium PDB (NB, HiMedia Laboratories Pvt. Ltd. Mumbai, REF- M002).

PCR for amplification of bacterial 16S rDNA

Bacterial 16S rDNA were amplified by PCR using following universal forward and reverse primer:

Forward primer # 101 F 5′- AGAGTTTGATCCTGGCTCAG-3′

Reverse primer # 102 R 5′-TACGGCTACCTTGTTAGCGAC-3′

The PCR product was run into and agarose gel contain at 100 volt for 40 minutes. The agarose gel contained 1.5 g agarose per 100 ml distilled water and 6 µL ethidium bromide (EtBr). At the end the agarose gel was placed in gel documentation system and the DNA bands of were visualized using ultra violate radiation. The DNA bands approximately 1500 bp were cut by a sterilized blade and transferred into an eppendorf tube. DNA was purified using DNA purification kit. The anticipated product of approximately 1500 bp was extracted after agarose gel electrophoresis of PCR product by a Gel Purification Kit (FavorPrep GEL/PCR Purification Mini Kit, Favorgen Biotech Corp).

Cloning of bacterial 16S rDNA

Bacterial 16S rDNA were cloned into the pGEM-T Easy vector (Promega, WI, USA) according to Islam *et al.*, 2010. Initially the 16S rDNA were cloned into pGEM-T Easy vector with the following ligation mixture:

Transformation

The ligated vector with insert was transformed into *E. coli* Dh5α according to Islam *et al.*, 2010. Initially competent *E. coli* Dh5α was prepared and stored at -80°C. For the transformation *E. coli* Dh5α was kept in ice for toughing for 10 min. The ligation mixture was mixed with competent *E. coli* Dh5α cells and kept for 30 min. Then the mixture was transfer into water bath of 42°C for heat shock transformation of the DNA into competent *E. coli* Dh5α cells for 1.5 minute and then immediately transferred into ice for 10 minutes. After that 1 ml LB broth was added into the eppendorf tube and kept into the shaker of 37°C for 1.5 hour. Then the transformed *E. coli* Dh5α cells were spreaded into the LAXI plate having Luria Berteni (LB) broth, Ampicillin, X-gal (Bromo-chloro-Indolyl-Galacoside), Dimethyl Salfoxide (DMSO), IPTG (Isopropyl Thio-galactoside). The plates were kept at 37°C for overnight. Next day blue and white colonies were appeared in the LAXI plate. The white colonies were approximately having pGEM-T easy + 16S rDNA and blue colonies were having only self-ligated pGEM-T easy. White and few colonies were picked into LAXI plate. All cloning related techniques were used as described by Sambrook and Russel (2001).

Plasmid isolation and conformation for recombination

Plasmid DNAs of recombinant colonies were extracted using a plasmid extraction kit (FavorPrep Plasmid Extraction Mini Kit, Favorgen Biotech Corp.). The recombinant white colonies were cultured in LB broth having Ampiciline and IPTG.

Sequencing and analysis

Nucleotide sequencing of 16S rDNA was conducted using the dideoxy-chain termination method from Macrogen Company through Sunchon National University, Seoul, South Korea. The DNA sequences were analyzed using DNAMAN analysis system. Searches for similarity of 16S rDNA sequences were performed using the BLASTn on the NCBI website (https://www.ncbi.nlm.nih.gov).

Deposition of nucleotide sequences in Gene Bank of NCBI

DNA sequences of 16S rDNA of bacterial isolates were submitted to National Center for Biotechnology Information (NCBI) for deposition in the Gene Bank and the accession number.

Development of phylogenetic tree

Phylogenetic analysis was performed using neighbor-joining methods (Saitou and Nel, 1987). Bootstrap analysis was performed using data resampled 1,000 times using the DNAMAN analysis system. All reference sequences were obtained from the National Center for Biotechnology Information (NCBI) database.

Experimental design and statistical analysis

All treatments in this study were arranged in a completely randomized design (CRD) with eight replications. The recorded data on different parameters of the experiment were tabulated and analyzed with appropriate design of experiment (Gomez and Gomez, 1984) adopting a statistical programme MSTAT-C. All the treatment means were calculated and analyses of variances (ANOVA) of different parameters considered were done by 'F' variance test. The means were separated by Least Significant Difference (LSD) test at 5% level of significance.

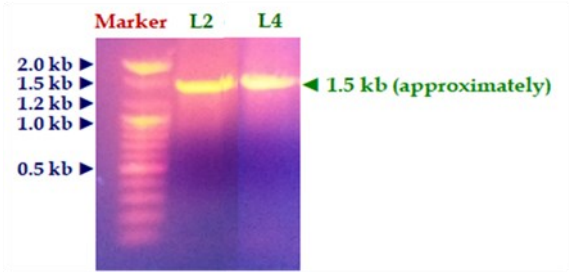

Figure 1. *Agarose gel electrophoresis picture of PCR amplified of 16S rDNA of antagonistic bacteria isolated from mango. Here, Molecular marker (100 bp ladder), L2 and L4 = amplified 16S rDNA of antagonistic bacteria (PSTU-Hort-8 and PSTU-Hort-14) isolated from mango.*

ORIGIN

1 CTCAGGACGA ACGCTGGCGG CGTGCCTAAT ACATGCAAGT CGAGCGGACA GATGGGAGCT
61 TGCTCCCTGA TGTTAGCGGC GGACGGGTGA GTAACACGTG GGTAACCTGC CTGTAAGACT
121 GGGATAACTC CGGGAAACCG GGGCTAATAC CGGATGGTTG TTTGAACCGC ATGGTTCAAA
181 CATAAAAGGT GGCTTCGGCT ACCACTTACA GATGGACCCG CGGCGCATTA GCTAGTTGGT
241 GAGGTAACGG CTCACCAAGG CAACGATGCG TAGCCGACCT GAGAGGGTGA TCGGCCACAC
301 TGGGACTGAG ACACGGCCCA GACTCCTACG GGAGGCAGCA GTAGGGAATC TTCCGCAATG
361 GACGAAAGTC TGACGGAGCA ACGCCGCGTG AGTGATGAAG GTTTTCGGAT CGTAAAGCTC
421 TGTTGTTAGG GAAGAACAAG TACCGTTCGA ATAGGGCGGT ACCTTGACGG TACCTAACCA
481 GAAAGCCACG GCTAACTACG TGCCAGCAGC CGCGGTAATA CGTAGGTGGC AAGCGTTGTC
541 CGGAATTATT GGGCGTAAAG GGCTCGCAGG CGGTTTCTTA AGTCTGATGT GAAAGCCCCC
601 GGCTCAACCG GGGAGGGTCA TTGGAAACTG GGGAACTTGA GTGCAGAAGA GGAGAGTGGA
661 ATTCCACGTG TAGCGGTGAA ATGCGTAGAG ATGTGGAGGA ACACCAGTGG CGAAGGCGAC
721 TCTCTGGTCT GTAACTGACG CTGAGGAGCG AAAGCGTGGG GAGCGAACAG GATTAGATAC
781 CCTGGTAGTC CACGCCGTAA ACGATGAGTG CTAAGTGTTA GGGGGTTTCC GCCCCTTAGT
841 GCTGCAGCTA ACGCATTAAG CACTCCGCCT GGGGAGTACG GTCGCAAGAC TGAAACTCAA
901 AGGAATTGAC GGGGGCCCGC ACAAGCGGTG GAGCATGTGG TTTAATTCGA AGCAACGCGA
961 AGAACCTTAC CAGGTCTTGA CATCCTCTGA CAATCCTAGA GATAGGACGT CCCCTTCGGG
1021 GGCAGAGTGA CAGGTGGTGC ATGGTTGTCG TCAGCTCGTG TCGTGAGATG TTGGGTTAAG
1081 TCCCGCAACG AGCGCAACCC TTGATCTTAG TTGCCAGCAT TCAGTTGGGC ACTCTAAGGT
1141 GACTGCCGGT GACAAACCGG AGGAAGGTGG GGATGACGTC AAATCATCAT GCCCCTTATG
1201 ACCTGGGCTA CACACGTGCT ACAATGGACA GAACAAAGGG CAGCGAAACC GCGAGGTTAA
1261 GCCAATCCCA CAAATCTGTT CTCAGTTCGG ATCGCAGTCT GCAACTCGAC TGCGTGAAGC
1321 TGGAATCGCT AGTAATCGCG GATCAGCATG CCGCGGTGAA TACGTTCCCG GGCCTTGTAC
1381 ACACCGCCCG TCACACCACG AGAGTTTGTA ACACCCGAAG TCGGTGAGGT AACCTTTTAG
1441 GAGCCAGCCG CCGAAGGTGG GACAGATGAT TGGGGTGAAG TCGTAACAAG GTAGCCGTAT
1501 CGGAAGGTGC GGCTGGATCA CCTCC

Figure 2. *Nucleotide sequence and composition of 16S rDNA of B. subtilis strain PSTU-Hort-8(MW659188).*

RESULTS AND DISCUSSION

The results on the different parameters are presented in figure and table for ease of discussion under the following sub-headings and possible interpretations have been given whenever necessary.

Isolation of antagonistic bacteria from leaf and fruit surfaces of mango

Initially, 100 different bacterial isolates were collected from the surface of mango based on colonies colors, sizes and shapes. Among them 20 were white, 30 were dry and creamy white and 50 were creamy white and shiny. Pure culture of each isolate was maintained for screening test against *C. gloeosporioides*.

Screening, selection and molecular identification of antagonistic bacteria

Out of 100 bacterial isolates, 20 were inhibitory towards *C. gloeosporioides* on PDA. Among 20, two isolates namely strains PSTU-Hort-8 and PSTU-Hort-14 had significantly ($p \leq 0.05$) higher inhibitory effect than the others. The PIRG of strains PSTU-Hort-8 and PSTU-Hort-14 were 78.00 and 80.00%, respectively with respect to the control after seven days of incubation. On PDA medium, these two bacterial strains strongly inhibited the mycelial growth of *C. gloeosporioides*. In addition, no mycelial growth was observed after four days of incubation, when PDA plugs from the interaction zone were re-cultured on fresh PDA plates.

Molecular identification of antagonistic bacteria

The most potential three antagonistic bacteria were selected for molecular characterization and phylogenetic analysis.

PCR amplification of 16S rDNA

PCR amplification of 16S rDNA was conducted with the universal forward primer # 101F 5′- AGA GTT TGA TCC TGG CTC AG-3′ and reverse primer # 102R 5′-TAC GGC TAC CTT GTT AGC GAC-3′. The PCR products were observed by the agarose gel electrophoresis and visualized by the ultra-violate radiation. The DNA bands approximately 1500 bp were observed (Figure 1). Similarly, Islam *et al.* (2010) studied to determine different endophytic bacterial strains by 1500 bp PCR amplicon of 16S rRNA.

Cloning of bacterial 16S rDNA and transformation into *E. coli* Dh5α

The DNA bands approximately 1500 bp were purified. The purified DNA was cloned into the pGEM-T Easy vector using T4 ligase enzyme. The construct of pGEM-T Easy vector with insert of bacterial 16S rDNAs were presented in the Figure 1. The pGEM-T Easy + 16S rDNA was transformed into *E. coli* Dh5α. The transformed *E. coli* Dh5α were plated on LAXI (LB broth, ampicillin, X-gal and IPTG) plate and the plates were kept overnight at 37°C for the growth of the recombinant *E. coli* DH5α colonies. There were blue and white colonies on the LAXI plates. The white colonies were having pGEM-T Easy + 16S rDNA and blue colonies were having only self-ligated pGEM-T Easy vector. Some white colonies and few blew colonies were picked in LAXI plates for further studies.

Plasmid isolation and conformation for recombination

The plasmids were isolated from the white colonies having pGEM-T Easy + 16S rDNA. The pGEM-T Easy + 16S rDNA were confirmed by the endonuclease digestion with *Eco*R1. Two DNA bands were observed after digestion with *Eco*R1, one band was approximately 3 kb, which was most probably for pGEM-T Easy another was approximately 1.5 kb, which was most probably for 16S rDNA. The plasmid having these two DNA bands were confirmed that the plasmid having 16S rDNA.

Sequencing and analysis

The conformed pGEM-T Easy + 16S rDNAs were sent to the Macrogen Company, Seoul, South Korea for molecular identification by sequence analysis. The sequencing was conducted with the T7 promoter primer 5'-TAA TAC GAC TCA CTA TAG GGG-3' and SP6 promoter primer 5'-TAC GAT TTA GGT GAC ACT ATA G-3'. For each bacterial strain the obtained sequences by T7 and SP6 promoter primer were aligned and analyzed using DNAMAN analysis system. The 16S rDNA of PSTU-Hort-8 (GB6) was 1525 bp length, where 25% A; 24% C; 32% G; 20% T and molecular weight was 473.65 kDa for ssDNA and 940.2 kDa for dsDNA (Figure 2). On the other hand, the 16S rDNA of PSTU-Hort-14 (GB19) were 1530 bp length, where 25% A; 23% C; 32% G; 21% T and molecular weight 475.38 kDa for ssDNA and 943.3 kDa for dsDNA (Figure 3).

Nucleotide sequence and composition of PSTU-Hort-8

Sequence length: 1525 bp
Composition 380 A; 361 C; 482 G; 302 T; 0 OTHER
Percentage: 25% A; 24% C; 32% G; 20% T; 0%OTHER
Molecular Weight (kDa): ssDNA: 473.65 dsDNA: 940.2

Nucleotide sequence and composition of PSTU-Hort-14

Sequence length: 1530 bp
Composition 379 A; 352 C; 485 G; 314 T; 0 OTHER
Percentage: 25% A; 23% C; 32% G; 21% T; 0%OTHER
Molecular Weight (kDa: ssDNA: 475.38; dsDNA: 943.3

Similarity search for matching

Searches for similarity of 16S rDNA sequences were performed using the BLASTn on the NCBI website (https://

ORIGIN

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1    CTGGTTCAGA GTGAACGCTG GCGGTAGGCC TAACACATGC AAGTCGAACG GCAGCACAGT
61   AAGAGCTTGC TCTTATGGGT GGCGAGTGGC GGACGGGTGA GGAATACATC GGAATCTACC
121  TTTTCGTGGG GGATAACGTA GGGAAACTTA CGCTAATACC GCATACGACC TTCGGGTGAA
181  AGCAGGGGAC CTTCGGGCCT TGCGCGGATA GATGAGCCGA TGTCGGATTA GCTAGTTGGC
241  GGGGTAAAGG CCCACCAAGG CGACGATCCG TAGCTGGTCT GAGAGGATGA TCAGCCACAC
301  TGGAACTGAG ACACGGTCCA GACTCCTACG GGAGGCAGCA GTGGGGAATA TTGGACAATG
361  GGCGCAAGCC TGATCCAGCC ATACCGCGTG GGTGAAGAAG GCCTTCGGGT TGTAAAGCCC
421  TTTTGTTGGG AAAGAAAAGC AGTCGATTAA TACTCGGTTG TTCTGACGGT ACCCAAAGAA
481  TAAGCACCGG CTAACTTCGT GCCAGCAGCC GCGGTAATAC GAAGGGTGCA AGCGTTACTC
541  GGAATTACTG GGCGTAAAGC GTGCGTAGGT GGTTGTTTAA GTCTGTTGTG AAAGCCCTGG
601  GCTCAACCTG GGAATTGCAG TGGATACTGG GCGACTAGAG TGTGGTAGAG GGTAGTGGAA
661  TTCCCGGTGT AGCAGTGAAA TGCGTAGAGA TCGGGAGGAA CATCCATGGC GAAGGCAGCT
721  ACCTGGACCA ACACTGACAC TGAGGCACGA AAGCGTGGGG AGCAAACAGG ATTAGATACC
781  CTGGTAGTCC ACGCCCTAAA CGATGCGAAC TGGATGTTGG GTGCAATTTG GCACGCAGTA
841  TCGAAGCTAA CGCGTTAAGT TCGCCGCCTG GGGAGTACGG TCGCAAGACT GAAACTCAAA
901  GGAATTGACG GGGGCCCGCA CAAGCGGTGG AGTATGTGGT TTAATTCGAT GCAACGCGAA
961  GAACCTTACC TGGTCTTGAC ATGTCGAGAA CTTTCCAGAG ATGGATTGGT GCCTTCGGGA
1021 ACTCGAACAC AGGTGCTGCA TGGCTGTCGT CAGCTCGTGT CGTGAGATGT TGGGTTAAGT
1081 CCCGCAACGA GCGCAACCCT TGTCCTTAGT TGCCAGCACG TAATGGTGGG AACTCTAAGG
1141 AGACCGCCGG TGACAAACCG GAGGAAGGTG GGGATGACGT CAAGTCATCA TGGCCCTTAC
1201 GACCAGGGCT ACACACGTAC TACAATGGTA GGGACAGAGG GCTGCAAACC CGCGAGGGCA
1261 AGCCAATCCC AGAAACCCTA TCTCAGTCCG GATTGGAGTC TGCAACTCGA CTCCATGAAG
1321 TCGGAATCGC TAGTAATCGC AGATCAGCAT TGCTGCGGTG AATACGTTCC CGGGCCTTGT
1381 ACACACCGCC CGTCACACCA TGGGAGTTTG TTGCACCAGA AGCAGGTAGC TTAACCTTCG
1441 GGAGGGCGCT TGCCACGGTG TGGCCGATGA CTGGGGTGAA GTCGTAACAA GGTAGCCGTA
1501 TCGGAAGGTG CGGCTGGATC ACCTTCCTTT
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Figure 3. *Nucleotide sequence and composition of 16S rDNA of S. rhizophila strain PSTU-Hort-14 (MW659190).*

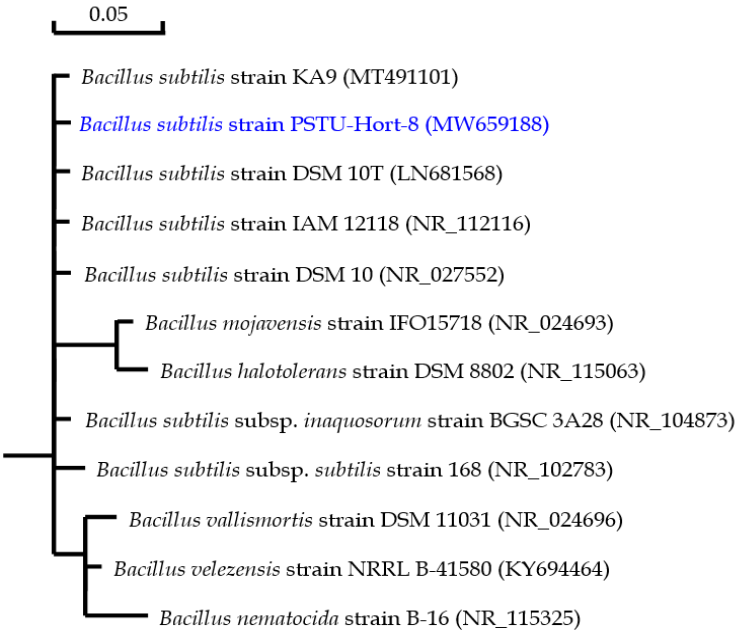

Figure 4. *Phylogenetic tree of B. subtilis strain PSTU-Hort-8 (MW659188) on the basis of 16S rDNA sequence and generated from 1,000 bootstrap trees. The scale bar is in fixed nucleotide substations per sequence position.*

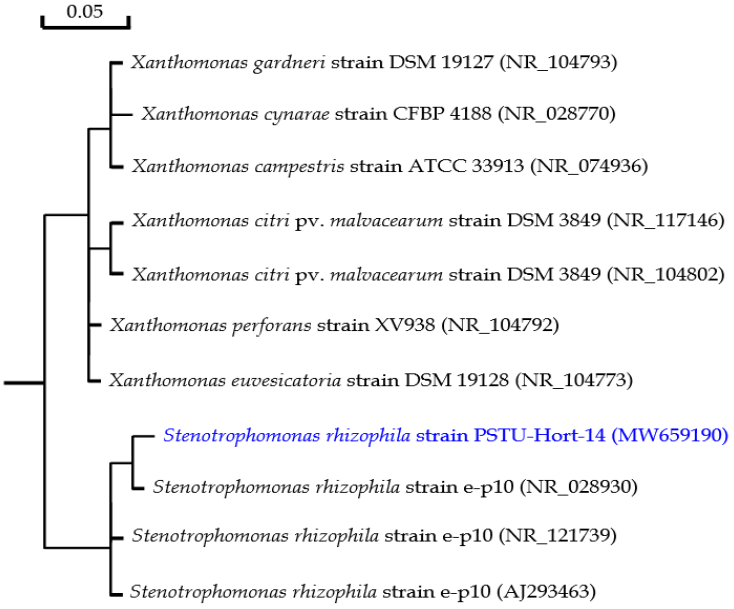

Figure 5. *Phylogenetic tree of S. rhizophila strain PSTU-Hort-14 (MW659190) on the basis of 16S rDNA sequence and generated from 1,000 bootstrap trees. The scale bar is in fixed nucleotide substations per sequence position.*

www.ncbi.nlm.nih.gov) for matching with existing DNA sequences in the NCBI Gene Bank to identify the bacterial strains. The 16S rDNA of the both the isolated strain PSTU-Hort-8 has been showed highest matching with *B. subtilis* strain KA9 (accession number: MW659188) with 98.75% similarity. On the other hand, the 16S rDNA of the isolated strain PSTU-Hort-14 were showed highest matching with *S. rhizophila* strain e-p10 (accession number: MW659190) with 99.84% similarities. Similarly, different researcher identified bacterial strains using 16S rDNA (Islam *et al*., 2010; Yeasmin *et al*., 2015; Math *et al*., 2010).

Development of phylogenetic tree and multiple sequence alignment

Phylogenetic analysis was performed using neighbor-joining methods. Bootstrap analysis was performed using data resampled 1000 times using the DNAMAN analysis system (Figure 4 and 5). From the phylogenetic tree, it has been observed that isolated strain PSTU-Hort-8 closely related to *B. subtilis* strain KA9 (accession number: MW659188). On the other hand, PSTU-Hort-14 has been closely related to *S. rhizophila* strain e-p10 (accession number: MW659190).

Deposition of nucleotide sequences in gene bank of NCBI

DNA sequences of 16S rDNA of isolated bacterial strains were submitted to National Center for Biotechnology Information (NCBI) for deposition in the Gene Bank and the accession number. The received accession number MW659188 is for *B. subtilis* strain PSTU-Hort-8 and accession number MW659190 is for *S. rhizophila* strain PSTU-Hort-14. In this study, one strain

of *S. rhizophila* and two strains of *B. subtilis* as biocontrol agents had been isolated and identified. However, to our knowledge, it is the first time that *S. rhizophila* strain PSTU-Hort-14 is isolated from mango fruit surface in Bangladesh. A wide range of antifungal properties were shown by these strains and from this point of view these bacterial strains were selected as promising bacteria to be tested for biological control of anthracnose of mango. The application of bacteria as biological control agents has been considered one of the most promising alternatives to protect fruit against infection by phytopathogens, minimizing the use of synthetic fungicides (Di Francesco *et al.*, 2016). The capacity of *S. rhizophila* isolated from plant rhizosphere to antagonize different pathogens in vitro, such as *Verticillium dahliae*, *Pythium ultimum*, *Rhizoctonia solani*, *Botrytis cinerea*, *Sclerotinia sclerotiorum*, among others (Kai *et al.*, 2007) and from marine isolations for biological control of anthracnose in mango (Hernandez-Montiel *et al.*, 2017). Searches for similarity of 16S rDNA sequences were performed using the BLASTn on the NCBI website (https://www.ncbi.nlm.nih.gov) for matching with existing DNA sequences in the NCBI Gene Bank to identify the bacterial strains. The 16S rDNA of the both the isolated strain PSTU-Hort-8 has been showed highest matching with *B. subtilis* strain KA9 (accession number: MW659188) with 98.75% similarity. On the other hand, the 16S rDNA of the isolated strain PSTU-Hort-14 were showed highest matching with *S. rhizophila* strain e-p10 (accession number: MW659190) with 99.84% similarities. Similarly, different researcher identified bacterial strains using 16S rDNA. DNA sequences of 16S rDNA of isolated bacterial strains were submitted to National Center for Biotechnology Information (NCBI) for deposition in the Gene Bank and the accession number. The received accession number MW659188 is for *B. subtilis* strain PSTU-Hort-8 and accession number MW659190 is for *S. rhizophila* strain PSTU-Hort-14.

The identification of an effective antagonistic bacterium was the most important step for the application of a biocontrol agent. Bacterial biocontrol agent can be identified by 16S rDNA gene sequence. The strain identification was done by 16S rDNA sequencing which is considered as a 'golden index' for strain detection (Ma *et al.*, 2008). On the basis of 16S rDNA, Moore *et al.*, (1997) mentioned a 3% difference between the genera *Stenotrophomonas and Xanthomonas*, which is equivalent to 45–68 nucleotide positions. Here, we obtained 44 nucleotide differences between strains *S. maltophilia* DSM 50170T and *X. campestris* DSM 3586T, which is exactly 2±96%. Normally, this level of sequence difference suggests strains of different species. If 3% is used as the yardstick between the two genera *Stenotrophomonas* and *Xanthomonas*, and after the transfer of the high 16S rDNA similarity values among various *Xanthomonas* species of 98±9–100% to *S. maltophilia*, the isolates of cluster E1 must be regarded as a different species. The bacterial species definition should be based on both DNA–DNA hybridization and 16S rDNA sequence analysis (Stackebrandt & Goebel, 1994).

Conclusion

PCR amplification of 16S rDNA was conducted using the universal forward primer # 101F 5′- AGA GTT TGA TCC TGG CTC AG-3′ and reverse primer # 102R 5′-TAC GGC TAC CTT GTT AGC GAC-3′. The DNA bands approximately 1500 bp were purified and cloned into the pGEM-T Easy vector. The pGEM-T Easy + 16S rDNA was transformed into *E. coli* Dh5α. The plasmids were isolated from the white colonies having pGEM-T Easy + 16S rDNA. The sequencing was conducted with the T7 promoter primer 5'-TAA TAC GAC TCA CTA TAG GGG-3' and SP6 promoter primer 5'-TAC GAT TTA GGT GAC ACT ATA G-3'. For each bacterial strain the obtained sequences by T7 and SP6 promoter primer were aligned and analyzed using DNAMAN analysis system. The 16S rDNA of Pstu-Hort-1 was 1525 bp length, where 25% A; 24% C; 32% G; 20% T and molecular weight was 473.65 kDa for ssDNA and 940.2 kDa for dsDNA. On the other hand, the 16S rDNA of Pstu-Hort-3 were 1530 bp length, where 25% A; 23% C; 32% G; 21% T and molecular weight 475.38 kDa for ssDNA and 943.3 kDa for dsDNA.

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