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Isolation of Pectinase Producing Bacteria from the Rhizosphere of *Andrographis paniculata* Nees and 16S rRNA Gene Sequence Comparison of Some Potential Strains

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Abstract

Pectinases, the enzymes which break down pectic substances, have a wide range of applications in food, agriculture and environmental sectors. In the present study, attempts were made to isolate highly efficient pectinase producer from the rhizosphere of a medicinal plant, *Andrographis paniculata* Nees, known as the "King of bitters". The total heterotrophic bacterial count of the rhizosphere soil of *A. paniculata* Nees ranged from 1.53×10^9 to 2.52×10^9 cfu/g. A total of 65 bacterial colonies were randomly selected from the nutrient agar plates, purified and assessed for pectinase activity. Out of the 65 isolates, 62 (95.38%) showed varying degree of pectinase activity in plate assay using pectin as a sole source of carbon. Among the pectinase producing strains, JBST36 showed best pectinase activity which is followed by the JBST22 and JBST27. Morphological characterization, biochemical tests and 16S rRNA gene sequencing were performed to identify the three most potential strains. Based on the morphological, biochemical and molecular data, JBST22 was identified as *Bacillus flexus* and the other two were identified as *Bacillus subtilis*. Furthermore, nucleotide sequences of the 16S rRNA gene of these 3 strains were compared and a phylogenetic tree was constructed. The study reveals that there are at least 66 base differences in the 16S rRNA gene sequences of *B. flexus* JBST22 and the *B. subtilis* JBST36.

Keywords

16S rRNA Gene, *Andrographis paniculata*, Pectinase, Rhizosphere

1. Introduction

Pectinases are the enzymes that act specifically on pectic substances by decreas-

ing intracellular adhesivity and tissue rigidity [1]. This enzyme can be derived from plants, animals and microorganisms [2] [3]. The biotechnological potential of this enzyme obtained from microorganisms has drawn a great deal of attention from various researchers worldwide as likely biological catalysts in a variety of industrial processes. The largest industrial application of pectinase is in food industries for fruit juice extraction and clarification. Pectinases are also used in industries for textile processing and bioscouring of cotton fibers, for degumming of plant bast fibers, retting of plant fibers, in waste water treatment, in coffee and tea fermentation, in paper and pulp industry, for making animal feed and for citrus oil extraction. In the industrial sector, acidic pectinases are used in the extraction and clarification of fruit juices [4], whereas alkalophilic pectinases are used in the degumming of ramie fibers [5], retting of flax [6], plant protoplast formation and treatment of effluents discharged from fruit processing units [7]. Pectinases from fungal sources performs best under acidic pH and low temperature conditions and can therefore not be used in varied industrial processes where neutral to alkaline pH with high temperatures is required. It has been shown that bacteria require high pH and temperature to produce pectinase [8] [9].

The rhizosphere, a narrow adjacent zone influenced by living plant roots, is a site of high microbial activity [10]. It harbors a great diversity of microorganisms affecting plant growth and development. All plants maintain a direct interaction with soil microbes in the rhizosphere, which is the soil compartment immediately surrounding the root wherein plant root exudates directly influence the structure and function of the soil microbial community. The sugars, amino acids, flavonoids and fatty acids secreted by plant roots help to structure the associated soil microbiome [11] [12] [13] and these exudates vary among plant species and between genotypes [14]. The root exudates and the prevailing microenvironment regulates the microbial diversity of the rhizosphere soil. However, the rhizosphere is a largely unexplored frontier for discovering novel microbes having capacity to produce high amount of various enzymes.

Andrographis paniculata (Burm. f.) Wall. ex Nees (Family: Acanthaceae) is an annual herbaceous plant [15]. It is an important medicinal plant and known as "King of bitters" because it is extremely bitter in taste in all parts of the plant body. This plant is widely used around the world particularly in traditional herbal medicines produced in Bangladesh, China, Hong Kong, India, Pakistan, Philippines, Malaysia, Indonesia and Thailand [16]. Several works have been done about the phytoconstituent and activity of extracts obtained from *A. paniculata* [17] [18] [19] [20]. However, research work on the isolation and identification of biotechnologically important bacteria from the rhizosphere of *A. paniculata* is almost absent. In order to fill this lacuna, the present study was undertaken to isolate pectinase producing bacteria from the rhizosphere soil of *A. paniculata*. Furthermore, molecular phylogenetic analysis of the top 3 pectinase producing isolates was performed based on their 16S rRNA gene sequences.

2. Materials and Methods

2.1. Isolation of Bacteria

Samples of rhizosphere soil from Kalomegh (*A. paniculata*) fields were collected from 3 districts (Dhaka, Gazipur and Gaibandha) belonging to two different agroecological zones (AEZs) of Bangladesh. Among the three samples, two were collected from "Modhupur Tract" AEZ and another was from "Active Tista Floodplain" AEZ. Plant roots with adherent soil were taken from healthy plants, placed in sterile containers and transported to the laboratory as soon as possible. Before isolation, the roots were gently shaken to remove excess soil and vortexed for 10 min in 0.9% sterile saline. Samples were serially diluted with sterile saline. Aliquots from the diluted samples were plated onto nutrient agar. After incubation for 24 h at 30˚C, colonies formed on the nutrient agar plates were counted. The number of heterotrophic bacteria was calculated using the formula:

cfu/ml = (No. of colonies × dilution factor)/volume added on of culture plate.

Finally, the cfu/ml was converted to cfu/g of soil.

Morphologically dissimilar colonies were selected randomly from different plates for each sample and marked. A single touch of sterilized loop was taken and streaked on agar plate for pure culture. The streaked plates were incubated at 30˚C for 24 h. Well isolated single colony was selected for purification. The well isolated single colony was subjected to further purification through repeated plating by streaking method. Purified single colony was preserved and used for determining colony characteristics and identification.

2.2. Screening for Pectinase Production

The isolates were screened for pectinase activity by plate assay method. This was done by inoculating the organisms on modified MS medium [21] containing 0.3% KH_2PO_4, 0.6% Na_2HPO_4, 0.2% NH_4Cl, 0.5% NaCl, 0.01% $MgSO_4 \cdot 7H_2O$, 1.5% agar and 0.2% pectin. The pectin containing MS medium was incubated at 30˚C for 48 h. Pectin utilization was detected by flooding the culture plates with freshly prepared Iodine-Potassium Iodide solution [22]. This solution forms a translucent halo zone where pectin depolymerization has occurred. A clear zone around the growth of the bacteria was indicated to pectinase activity. The enzyme activity was observed by measuring the diameter of clear zone around the colony in millimeter. Clear zone of each isolates were measured and recorded [23].

2.3. Morphological and Biochemical Characterization

The top 3 pectinase producing bacterial strains were provisionally identified based on morphological and biochemical characteristics. The colony characteristics (margin, elevation, form, surface, color, opacity and size) on the nutrient agar, growth pattern on selective media, microscopic characteristic after staining (cell shape, Gram reaction and spore formation) were recorded. A series of bio-

chemical tests were performed to identify the bacteria [24] [25] [26] [27].

2.4. Growth at Different pH and NaCl Concentrations

The effect of pH for pectinase producing isolates was determined by culturing the bacterium in the media with different pH. The experiment was carried out at various pH 6, 7, 8 and 9. Optical density was measured at 600 nm by a Spectrophotometer (Specord 205, Analytik Jena, Germany). To assess the ability to tolerate higher salt concentration, overnight culture of test isolates were inoculated in nutrient broth containing 0%, 1%, 2%, 4%, 6% and 8% NaCl, respectively. The growth of isolated bacteria on different concentration of NaCl was measured by a Spectrophotometer at 600 nm.

2.5. Extraction and Purification of Genomic DNA

Genomic DNA of the 3 most promising strains was isolated using the Maxwell DNA purification kit and Maxwell 16 extraction platform (Promega, USA). In brief, 50 µl of fresh bacterial sample of each strain was mixed with the lysis buffer and mixed thoroughly by vortexing. After incubation, the whole mix was transferred in the Maxwell 16 DNA purification cartridge. The cartridge was then loaded into Maxwell16 instrument and DNA was isolated following manufacturer's instructions. Concentration of purified DNA samples was quantified by using NanoDrop (Thermo Scientific, USA).

2.6. Amplification of 16S rRNA Gene by PCR

The genomic DNA extracted from the bacterial strains was used to amplify the 16S rRNA gene. The primer pair 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') were used to amplify the gene by PCR. Amplification was carried out in an automatic thermocycler (Astec, Japan). Briefly, the 25 µl PCR reaction mixture contained 12.5 µl 2× GoTaq G2 hot start master mix, 0.1 µl of each primer together with 1.0 µl of DNA template. The PCR conditions were as follows: Initial denaturation at 95˚C for 4 min followed by 30 thermal cycling consisting of denaturation at 95˚C for 30 s, annealing at 49˚C for 30 s, extension at 72˚C for 90 s and a final extension at 72˚C for 5 min.

2.7. Agarose Gel Electrophoresis of PCR Product and DNA Sequencing

PCR products were loaded onto 0.8% agarose gel containing ethidium bromide solution (Promega, USA). DNA ladder (Promega, USA) was used as a DNA size marker. Electrophoresis was done at constant voltage of 100 V for 1.5 hour in a horizontal electrophoresis system (CBS Scientific, USA) following manufacturer's protocol. After electrophoresis the gel was carefully removed and the DNA bands were observed under UV-transilluminator (Alpha Imager, USA) to confirm the presence of amplicon. Following electrophoresis, DNA band of desired

size was excised from gel and placed in a 1.5 ml microcentrifuge tube. The PCR product was purified using the SV mini column according to the manufacturer's instruction. The sequence of PCR products were determined by Sanger dideoxy chain termination method using an ABI 3700 Genetic Analyzer (1st Base Laboratory, Malaysia).

2.8. DNA Sequence Comparison and Phylogenetic Analysis

The resulting DNA sequence was compared by basic local alignment search tool [28]. Based on the highest degree of similarity, identity for the bacterial isolates was assigned. For phylogenetic analysis, the 16S rDNA sequences were aligned with the Clustal W program [29] and the tree was constructed with the maximum likelihood method based on the Tamura-Nei model [30] integrated in the MEGA7 software [31]. The phylogenetic tree was tested with 1000 bootstrap replicates.

3. Results and Discussion

3.1. Enumeration of Bacteria

In the present study, bacteria were isolated from the rhizosphere of *A. paniculata* growing in three different districts of Bangladesh. The total heterotrophic bacterial counts were 1.56×10^9, 1.53×10^9 and 2.52×10^9 cfu/g in the rhizosphere soil sample AP1, AP2 and AP3, respectively. The variation in the count of heterotrophic bacteria due to difference in location was not statistically significant. The total heterotrophic bacterial count observed in this study corroborates with previous report that the bacterial population in the rhizosphere ranges from 10^8 to 10^9 per gram of rhizosphere soil [32].

3.2. Cultural and Morphological Characteristics of the Isolates

From the nutrient agar plates 65 bacterial colonies were purified for further study. The colony characteristics of the purified isolates varied in margin, elevation, form, surface appearance, color, opacity and size. The isolates also varied in their growth pattern in nutrient agar slant. Out of the 65 isolates, 52 (80%) were Gram positive and 13 (20%) were Gram negative bacteria. Among the isolates 24 (36.9%) were spore forming and 41 isolates (63.1%) were non-spore forming bacteria. Appearance of diverse type of bacterial colonies on the nutrient agar plate indicates that the rhizosphere soil of *A. paniculata* promotes the survival and proliferation of both Gram positive and Gram negative as well as both spore forming and non-spore forming bacteria.

The isolates were also tested for their ability to grow on different types of selective and differential media (MacConkey agar, Mannitol Salt agar, Cetramide agar, Triple sugar agar etc.). Furthermore, isolates were subjected to different type of biochemical tests (Catalase test, MR-VP test, citrate utilization test, sugar fermentation test, nitrate reduction test etc.) for identification. Based on the morphological, cultural and biochemical characteristics isolates were provisio-

nally identified as *Bacillus*, *Pseudomonas* and *Micrococcus*.

3.3. Screening of the Bacterial Isolates for Pectinase Activity

All of the 65 isolates were tested for their pectin degradation ability by plate assay method in the modified MS medium containing 0.2% pectin as sole source of carbon (**Figure 1**). Clear zone in plate were measured and recorded (**Figure 2**). Pectinase producing bacteria were present in all three soil samples. Among the 65 isolates of bacteria evaluated in this study, 62 (95.38%) isolates were pectinase enzyme producer. Tariq and Latif [33] isolated 62 bacterial isolates from soil, water, rotten fruit and vegetables of which 16 were pectinase producer. The findings of the present research indicate that in addition to fruit and vegetables, rhizosphere soil of *A. paniculata* also harbors a good number of pectinase producing bacteria. This also justifies the selection *of A. paniculata* rhizosphere soil for isolating pectinase producing bacteria for the present study.

The pectinase producing isolates were classified based on [34] as very good pectinase producer (clear zone ≥ 15 mm), good pectinase producer (clear zone ≥ 10 mm), weak pectinase producer (clear zone ≥ 5 mm) and poor pectinase producer (clear zone ≤ 5 mm). Among the 65 isolates, 41 isolates were very good pectinase producer indicating that not only the pectin rich fruit peels but also the rhizosphere soil of *A. paniculata* harbors good number of pectinase producer (**Table 1**). Among the very good pectinase producers, the top three strains (JBST22, JBST27 and JBST36) were selected for further study.

3.4. Stress Tolerance

The top three pectinase producing strains were inoculated in nutrient broth containing different concentrations of NaCl (1%, 2%, 4%, 6% and 8%) to test their ability to survive under different osmotic pressure. All the three strains

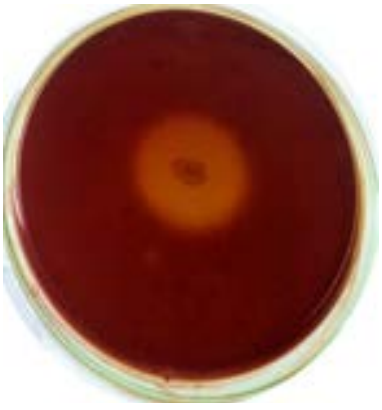

Figure 1. Assessment of pectinase activity of a bacterial isolate. The pectin containing MS medium was incubated at 30˚C for 48 h. Pectin utilization was detected by flooding the culture plates with freshly prepared Iodine-Potassium Iodide solution. This solution formed a translucent halo zone where pectin depolymerization has occurred.

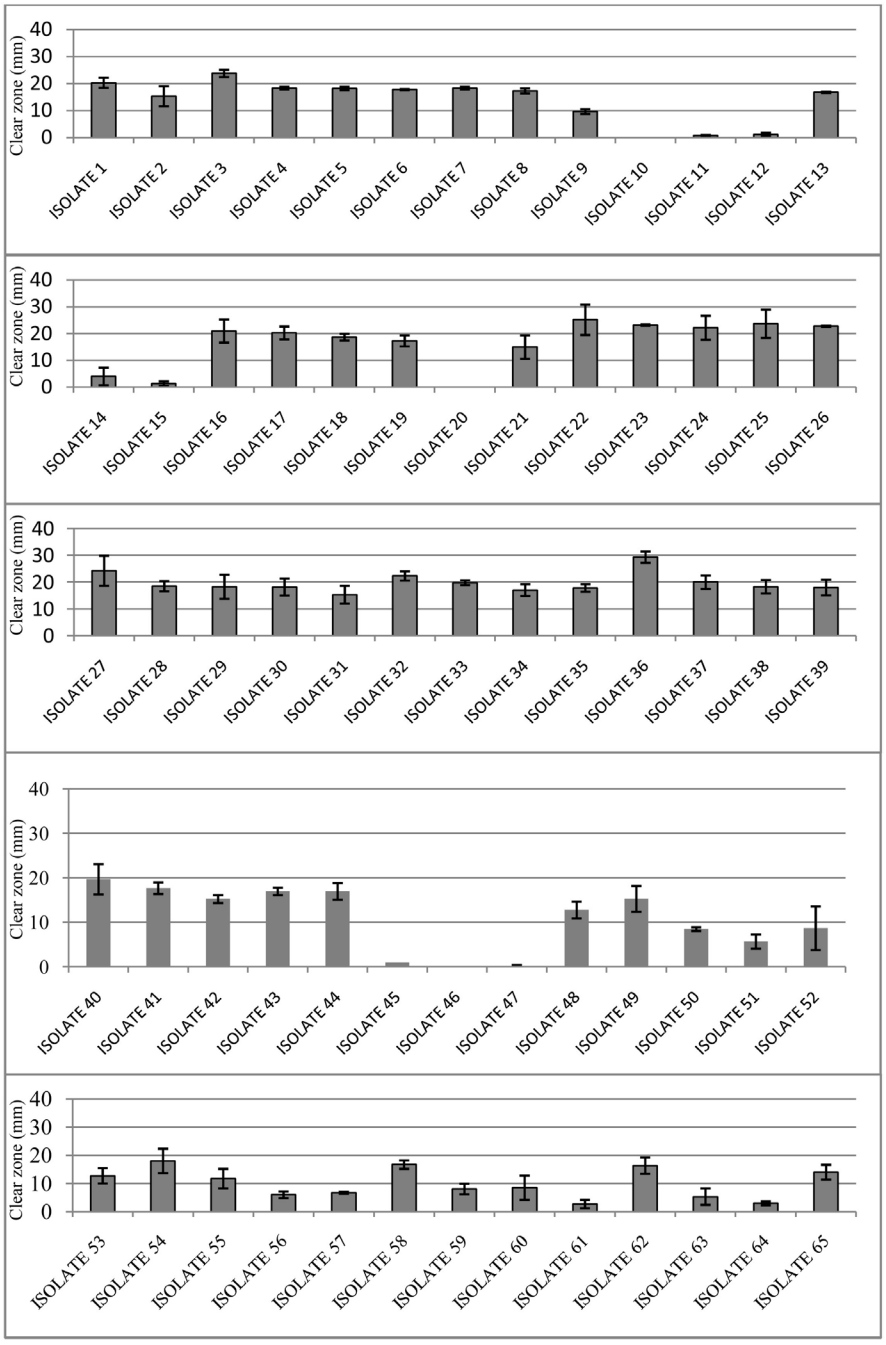

Figure 2. Zone of pectionolytic activity (mm) produced by the isolated bacteria.

were able to grow at 8% NaCl concentration. Among the three isolates, JBST36 grew better at pH 8.0 and JBST22 grew better in a more alkaline condition (pH 9.0). All the three strains were able to survive well at 40˚C temperature.

3.5. Comparison of 16S rRNA Gene Sequences and Phylogenetic Analysis

The 16S rRNA gene is a segment of prokaryotic DNA which codes for an rRNA, and this rRNA in turn makes up part of the small subunit of a ribosome. The 16S

Table 1. Grouping of pectinase producers on the basis of zone of pectinolysis.

Group	Clear zone size (mm)	No. of isolates
Very good producer	≥15	41 (JBST1-JBST8, JBST13, JBST16-JBST19, JBST21-JBST44, JBST49, JBST54, JBST58 and JBST62)
Good producer	≥10	4 (JBST48, JBST53, JBST55 and JBST65)
Weak producer	≥5	9 (JBST9, JBST50-JBST52, JBST56-JBST57, JBST59, JBST60 and JBST63)
Poor producer	<5	8 (JBST11, JBST12, JBST14, JBST15, JBST45, JBST47, JBST61 and JBST64)

rRNA gene sequence is about 1.5 kb long and is composed of both variable and conserved regions. This gene has enough interspecific polymorphisms to be used as a molecular marker to identify bacteria. Universal primers are usually chosen as complementary to the conserved regions at the beginning of the gene and at end of the whole sequence and the sequence of the variable region in between is used for the comparative taxonomy [35] [36]. 16S rDNA sequencing has played an important role in the accurate identification of bacterial isolates and discovery of many novel bacteria [37].

As expected, the PCR amplification of the 16S rRNA gene yielded a single amplicon of about 1.5 kb for each of the three selected strains used in this study (**Figure 3**). The obtained DNA sequences were edited and preliminary identification was carried out by comparing the sequences with those in the nucleotide database of National Center for Biotechnology Information (NCBI) using BLAST. The partial 16S rRNA gene sequences obtained for JBST22, JBST27 and JBST36 were 100% identical to the published sequence for *Bacillus flexus* strain ML-27 isolated from *Populus euphriatica*, *Bacillus subtilis* strain PARZ2 isolated from *Prosopis julifora* and *Bacillus subtilis* strain CS10 isolated from wild rice, respectively. Based on the morphological, biochemical and molecular data, JBST22, JBST27 and JBST36 were identified and designated as *Bacillus flexus* strain JBST22, *Bacillus subtilis* strain JBST27 and *Bacillus subtilis* strain JBST36, respectively. The sequence of these 3 strains were submitted to the GenBank of NCBI and the accession numbers KY407785 (*Bacillus flexus* JBST22), MK085080 (*Bacillus subtilis* JBST27) and MK085082 (*Bacillus subtilis* JBST36) were obtained. The study reveals that there are at least 66 base differences between the 16S rRNA gene sequences of *B. flexus* JBST22 and *B. subtilis* JBST36 (**Table 2**). Among these 66 differences, 65 were base substitution. However, no such difference was present in the 16S rRNA gene sequences of *B. subtilis* JBST27 and *B. subtilis* JBST36. To confirm the position of these 3 strains in phylogeny, a number of 16S rRNA gene sequences of some representative *Bacillus* species were selected from the GenBank database for the construction of phylogenetic tree. As shown in **Figure 4**, the phylogenetic tree indicated that the strain JBST22 and *B. flexus* share one cluster whereas the other two strains (JBST27 and JBST36) and

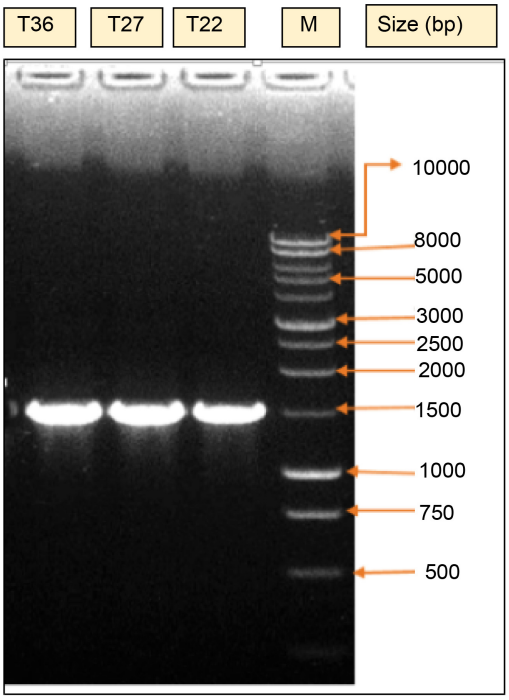

Figure 3. Agarose gel electrophoresis of the PCR product. The 16S rRNA gene was amplified as described in the Materials and Methods. The PCR product was electrophoresed in 0.8% agarose gel containing ethidium bromide and visualized under UV-light. M represents molecular size marker. T22, T27 and T36 represent PCR product of JBST22, JBST27 and JBST36 strains, respectively.

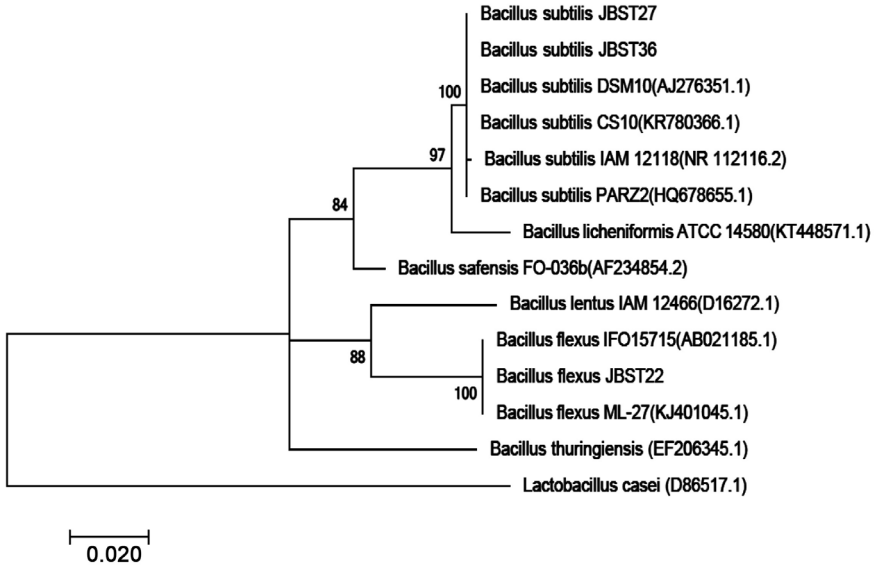

Figure 4. Phylogenetic position of *Bacillus* strains (JBST22, JBST27 and JBST36) based on their 16S rRNA gene sequences. Sequences were aligned with previously published 16S rRNA gene sequences. The GenBank accession numbers of the sequences are indicated in parentheses. The scale bar represents a 2% nucleotide sequence divergence.

Table 2. Nucleotide sequence variation in the 16S rRNA gene among pectinase producing *Bacillus* strains (JBST22, JBST27, JBST36). Numerals indicate the nucleotide positions corresponding to the complete 16S rRNA gene sequence of *Bacillus subtilis* strain IAM 12118 (Accession No. NR_112116).

Nucleotide position →	**125**	**153**	**164**	**178**	**179**	**181**	**182**	**183**	**187**	**188**	**189**	**190**	**191**	**196**	**197**	**198**	**199**	**200**	**204**	**205**	**207**	**212**
B. subtilis IAM 12118	T	T	G	G	G	T	T	G	G	A	A	C	C	G	G	T	T	C	C	A	A	G
B. flexus JBST22	C	C	A	A	A	C	A	T	C	T	C	T	T	A	A	G	A	G	A	T	G	A
B. subtilis JBST27	T	T	G	G	G	T	T	G	G	A	A	C	C	G	G	T	T	C	C	A	A	G
B. subtilis JBST36	T	T	G	G	G	T	T	G	G	A	A	C	C	G	G	T	T	C	C	A	A	G
Nucleotide position →	**216**	**226**	**240**	**248**	**294**	**426**	**433**	**441**	**468**	**469**	**470**	**471**	**472**	**473**	**476**	**477**	**479**	**480**	**481**	**483**	**562**	**585**
B. subtilis IAM 12118	C	C	A	C	G	T	A	G	C	G	T	T	C	G	T	A	G	G	C	G	G	G
B. flexus JBST22	T	T	G	T	A	C	G	A	A	A	G	A	G	T	C	T	C	T	T	-	A	C
B. subtilis JBST27	C	C	A	C	G	T	A	G	C	G	T	T	C	G	T	A	G	G	C	G	G	G
B. subtilis JBST36	C	C	A	C	G	T	A	G	C	G	T	T	C	G	T	A	G	G	C	G	G	G
Nucleotide position →	**588**	**623**	**635**	**677**	**679**	**682**	**744**	**747**	**749**	**772**	**790**	**847**	**860**	**1019**	**1020**	**1031**	**1032**	**1036**	**1040**	**1042**	**1049**	**1149**
B. subtilis IAM 12118	T	C	G	G	G	T	A	C	C	A	G	G	C	T	C	G	A	C	T	C	G	C
B. flexus JBST22	G	A	T	A	A	C	G	T	T	C	A	A	T	C	T	A	G	T	C	T	A	T
B. subtilis JBST27	T	C	G	G	G	T	A	C	C	A	G	G	C	T	C	G	A	C	T	C	G	C
B. subtilis JBST36	T	C	G	G	G	T	A	C	C	A	G	G	C	T	C	G	A	C	T	C	G	C

Bacillus subtilis share another cluster.

4. Conclusion

The study reveals that the rhizosphere of *A. paniculata* harbors highly potential pectinase producing bacteria. The most potential pectinase producing strain was identified as *Bacillus subtilis* JBST36. This strain may be used for production and purification of the enzyme for industrial purpose. Another highly potential strain was identified as *Bacillus flexus* JBST22. This is probably the first report on the isolation of *Bacillus flexus* from the rhizosphere of *A. paniculata* growing in Bangladesh.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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