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Screening and Molecular Characterization of Pectinolytic Bacterial Strains from Jute Retting Water Bodies

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ABSTRACT

Retting largely determines jute fibre quality. It is traditionally carried out in water bodies and basically a microbiological process. Efficient pectinolytic microbial inocula with low cellulolytic activity can improve fibre quality, reduce the time of retting and evade environmental pollution that arises due to retting. Thus, pectinolytic bacteria were isolated from retting water bodies occurring in two notable jute-growing belts in West Bengal, India. Thirty-eight pure bacterial strains were isolated and screened for polygalacturonase (PG), pectin lyase (PNL) and cellulase activity. PG, PNL and cellulase activity of the isolates were within the ranges of 8.74 - 41.00 IU g^{-1} cell wet weight, 2.23-75.7 U mL^{-1} and 0-0.316 μmol glucose mL^{-1} of culture filtrate min^{-1}, respectively. Eight organisms, selected as future retting inocula based on their balanced proportion of pectinolytic activity and low or no cellulolytic activity, were identified. Majority of the isolates were found to be rod shaped, Gram positive, spore former and they could utilize starch, sucrose, mannitol, lactose and were catalase positive. The 16S rDNA sequences of the isolates were similar to the species: *Agrobacterium* sp. BN-2A (GQ891102), *Bacillus pumilus* strain Geo-03-422 (GQ891103), *Microbacterium* sp. PVC8 (GQ891104), *Bacillus pumilus* strain IK-MB12-518F (GQ891105), *Bacillus* sp. L6 (GQ891097), *Bacillus pumilus* strain EK-17 (GQ891098), *Bacillus* sp. YACS30 (GQ891099), *Bacillus pumilus* strainIK-MB13-518F (GQ891100).

Key words: Jute retting, pectinolytic bacteria, polygalacturonase, pectin lyase, cellulase

INTRODUCTION

One of the least expensive and versatile textile fibre jute (*Corchorus* sp.) is almost exclusively cultivated in East Asia and some parts of Latin America. India, Bangladesh and Thailand account for 90% of the world jute production. Out of the 8 lakhs ha of jute acreage in India, 5.8 lakhs ha is concentrated in the districts of North 24-Parganas, Murshidabad, Nadia, Hoogly and Howrah in West Bengal. In recent times, jute production is severely constrained by the competitiveness relative to synthetic fibres at the consumer's end and more remunerative crops at the grower's end

(Ahmed and Akhter, 2001; Haque *et al.*, 2003). In order to overcome the dual challenges confronting the production and marketing of jute, the primary concern is to improve fibre quality. The retting process is the major limitation to efficient and high quality fibre production, thus being the key feature in any future expansion of jute and allied fibres as industrial crops (Pallesan, 1996; Tamburini *et al.*, 2003). The industrial retting process needs to be enhanced by reducing the time and cost while maintaining the desired fibre quality.

A plethora of microorganisms, naturally present in jute retting water bodies degrade pectin and other gummy materials that hold fibres with the stalks (Haque *et al.*, 2003). The enzymes PG (Zhang *et al.*, 2000) and PNL (Soriano *et al.*, 2005) are the primary agents involved in retting. PNL show specificity for methyl-esterified substrates (pectin), while PG is specific for unesterified polygalacturonate (pectate). Information pertaining to the nature and activity of the microorganisms isolated from flax retting environments are available (Tamburini *et al.*, 2003, 2004). In-depth studies on the microorganisms involved and their enzyme activities in the jute retting process are limited.

The main focus of this study was to screen out the efficient bacterial isolates with high pectinolytic activity and low or no cellulolytic activity for future use as retting inocula. The work also entails characterization of the screened isolates by conventional and molecular (16S rDNA sequence analysis) techniques to generate database for future research.

MATERIALS AND METHODS

Sample collection: Water samples were collected from jute-retting ponds (temperature range 32 and 34°C) located in two villages of N-24 Parganas, viz. Sonatikari (22°41'27"N; 88°35'44"E) and Baduria (22°44' 24"N, 88°47' 24"E), West Bengal, India. For each retting pond, five replicated samples were collected from areas in close proximity to the submerged mat of jute bundles, at a depth of 1 to 3 ft, to get maximum coverage of the retting consortia. The samples were then immediately placed on ice for transport to the laboratory and then preserved at 4°C.

Isolation and purification of pectinolytic bacteria: The organisms were isolated initially on yeast extract-pectate agar medium (1% pectin, 1% yeast extract, 0.5% NaCl, 1.5% agar, pH 7), followed by streaking in the same agar plates to obtain single isolated colonies, which were further grown on replica plates. After growth, one replica plate was flooded with 1% solution of cetyltrimethyl ammonium bromide (Donaghy *et al.*, 1990). Clear zones around colonies indicated pectinolytic activity. In this way, 38 pure cultures were obtained and maintained in same agar slants.

Phenotypic and biochemical characterization of the isolates: Colony characteristics of the isolates were determined on yeast extract-pectate agar slants and cellular morphology by negative staining method. Gram character, spore formation and different biochemical characteristics of the isolates were determined (Holt *et al.*, 1993).

Enzyme activity of the isolates: The strains were grown in the modified yeast extract-pectate broth for 2 days at 30°C with shaking (250 rpm) (Kashyap *et al.*, 2000). After centrifugation (7000 g, 10 min, 4°C), cell-free supernatant was used for the assay of pectinolytic activities.

The PG activity of the organisms was tested by the Dinitrosalicylic acid (DNS) method as described by Kobayashi *et al.* (2001). One unit (IU) corresponds to the release of 1 μmol of reducing

groups produced in 1 min at the assay temperature by 1 mL of supernatant. The IU was corrected by the culture wet weight (IU g^{-1} cells). The analyses were carried out in duplicate.

The PNL activity of the isolates was assayed according to Pitt (1988). One unit (U) of activity was the amount of enzyme that caused a change in absorbance of 0.01 under the condition of the assay. The assays were done in duplicate.

Cellulase activity (Sadasivam and Manickam, 2008) of the isolates was determined by growing them in carboxymethyl cellulose (CMC) broth (1% CMC, 1% yeast extract, 0.5% NaCl, pH 7) for 2 days at 30°C with shaking (250 rpm). The enzyme activity was carried out in duplicate and data expressed as the μmol glucose released mL^{-1} of culture filtrate min^{-1}.

Isolation of genomic DNA from the isolates: The DNA samples isolated by sodium dodecyl sulfate proteinase K- cetyltrimethylammonium bromide (CTAB) method (Sambrook and Russell, 2001) were treated with RNase A. DNA concentrations were estimated by visual examination of ethidium bromide-stained agarose gels as well as by spectrophotometric examination.

Amplification of 16S rDNA: The PCR of the genomic DNA of the isolates were conducted in a final volume of 50 μL. The reaction mixture included 20-50 ng of isolated genomic DNA, 2U taq polymerase (Promega, USA), 1×PCR buffer with 1.5 mM $MgCl_2$, 200 μM each dNTP and 10 pmol of each primer (IDT, USA). The primers were chosen to amplify partial 16S rDNA sequence. The forward primer 515F used was (5'-3') GTGCCAGCAGCCGCGGTAA and the reverse primer 1492R was (5'-3') TACGGYTACCTTGTTACGACTT. Before amplification cycle, DNA was denatured for 5 min at 94°C and after amplification an extension step (7 min at 72°C) was performed. The cycling parameter consisted of 29 cycles at: denaturation at 94°C for 30 sec, primer annealing at 56°C for 1 min, extension at 72°C for 1 min. The samples were held at 4°C until analysis by agarose gel electrophoresis. All the amplified PCR products were agarose gel eluted using Promega gel elution kit.

Sequencing of the 16SrDNA fragment and BLAST search: The amplified and gel-eluted PCR fragments of the rDNA were sequenced in ABI 3100 Genetic Analyzer with the primer 515F. Sequencing reaction was performed by using the Big Dye terminator cycle sequencing Kit V3.1 (Applied Biosystems, Foster City, USA) following the manufacturer's protocol. The partial 16S rDNA sequences of the isolated strains were compared with those available in the public databases. Identification to the species level was determined as a 16S rDNA sequence similarity of >99% with that of the prototype strain sequence in the GenBank.

Phylogenetic analysis: Phylogenetic analyses were conducted in Molecular Evolutionary Genetics Analysis software version 4.0 (MEGA4) (Tamura *et al.*, 2007).

RESULTS AND DISCUSSION

Enzyme activities of the bacterial isolates: Out of 16 isolates from Sonatikari and 22 isolates from Baduria, 5 isolated showed PG activities greater than 30 IU g^{-1}, while 17 isolates exhibited PG activities within the range of 15 to 30 IU g^{-1} and the rest showed less than 15 IU g^{-1} (Table 1). The PG activity of most aerobic bacteria isolated from hemp and flax retting water was between 10 and 39 IU g^{-1} (Tamburini *et al.*, 2003), which is comparable to our findings.

Table 1: Enzyme activities of bacterial isolates from Sonatikari and Baduria

Sample code	Polygalacturonase activity (IU g^{-1} cell wet weight)	Pectin lyase activity (U mL^{-1})	Cellulase activity (μmol glucose mL^{-1} of culture filtrate min^{-1})
Sonatikari			
SO1	38.32	25.96	0.049
SO2	14.04	22.55	0.16
SO3	13.35	33.72	0.122
SO4	15.47	34.68	0.176
SO5	8.74	46.85	0.13
SO6	22.05	43.77	0.137
SO7	12.45	75.7	0.168
SO8	14.22	45.59	0
SO9	26.42	55.28	0.256
SO10	37.26	22.18	0.176
SO11	16.12	15.49	0.014
SO12	17.5	11.35	0.056
SO13	19.95	37.68	0
SO14	37.26	39.79	0
SO15	11.66	27.98	0
SO16	12.91	30.98	0.184
Baduria			
BA1	16.98	71	0.056
BA2	22.54	11.92	0
BA3	13.43	13.67	0.092
BA4	25.32	6.55	0.042
BA5	14.27	29.24	0.056
BA6	12.16	18.71	0.115
BA7	22.54	26.36	0.13
BA8	13.69	02.69	0.122
BA9	13.12	02.69	0.056
BA10	15.96	04.58	0.273
BA11	21.87	22.91	0.1
BA12	11.58	14.27	0.028
BA13	10.74	02.23	0.316
BA14	14.75	02.46	0.021
BA15	41	23.66	0.13
BA16	26.42	29.67	0.028
BA17	20.27	22.18	0
BA18	22.67	29.67	0.1
BA19	18.43	31.43	0.215
BA20	14.75	23.66	0
BA21	23.64	20.25	0.107
BA22	34.67	22.55	0.049

The PNL activities (Table 1) of the isolates showed that the organisms producing higher PG activities failed to produce higher PNL activities. Limited report in this respect (Soriano *et al*., 2005) showed that *Paenibacillus* sp. BP23, *Bacillus* sp. BP7 and *Bacillus* sp. DT7 produced PNL activities of 47, 1.51 and 52 U mL^{-1}, respectively.

The range of cellulase activities of the isolates from Sonatikari and Baduria respectively were 0 to 0.256 and 0 to 0.316 μmol glucose mL^{-1} of culture filtrate min^{-1} (Table 1). Baduria isolates

Table 2: Morphological characteristics of the isolated bacteria

Sample code	Colony characteristics	Morphology	Cellular arrangement	Gram character	Endospore formation
SO1	White, moist	Rod	Single and in cluster	- ve	Non-spore former
SO7	White, moist, gummy	Rod	Single and in chain	+ve	Spore former
SO8	Green, moist, gummy	Rod	Single and in cluster	+ve	Non-spore former
SO14	White, dry, wrinkled	Rod	Single and in chain	+ve	Spore former
BA1	White, dry, wrinkled	Rod	Single and in chain	+ve	Spore former
BA15	White, dry, wrinkled	Rod	Single and in chain	+ve	Spore former
BA16	White, dry, wrinkled	Rod	Single and in chain	+ve	Spore former
BA22	White, dry, wrinkled	Rod	Single and in chain	+ve	Spore former

showed lower mean cellulase activity (0.093 μmol glucose mL^{-1} of culture filtrate min^{-1}) than Sonatikari isolates (0.102 μmol glucose mL^{-1} of culture filtrate min^{-1}).

Based on balanced proportions of pectinolytic and low/no cellulolytic activities (Table 1), 8 pure cultures (SO1, SO7, SO8, SO14, BA1, BA15, BA16 and BA22) were selected for detailed characterization.

Morphological and biochemical characteristics of the bacterial isolates: Most of the organisms were found to produce white colonies with dry or moist surface (Table 2). They were rod shaped (Fig. 1), arranged singly and in chain or clusters. Except SO1, all the organisms were Gram positive and majority of them were spore formers.

The organisms could utilize starch, sucrose, mannitol, lactose and were catalase positive (Table 3). Except SO7 and BA1, rest of the organisms did not produce gas on lactose broth. Regarding nitrate and citrate utilization, methyl red test and Voges-Proskauer test some organisms showed positive and some showed negative results. None of the organisms produced indole, H_2S and could liquefy gelatin. Except SO7, all the isolates showed alkaline reaction on Triple Sugar Iron (TSI) agar medium. The isolate SO7 showed acid formation.

Identification of the strains on the basis of 16S rDNA sequences: Based on the sequencing of 16S rDNA of the isolates and subsequent comparison with GenBank, the isolates were similar to *Agrobacterium* sp. BN-2A (SO1), *Bacillus pumilus* strain Geo-03-422 (SO7), *Microbacterium* sp. PVC8 (SO8), *Bacillus pumilus* strain IK-MB12-518F (SO14), *Bacillus* sp. L6 (BA1), *Bacillus pumilus* strain EK-17 (BA15), Bacillus sp. YACS30 (BA16) and *Bacillus pumilus* strain IK-MB13-518F (BA22). Out of the 8 isolates, 6 belonged to the genus *Bacillus,* which agrees with the previous report that *Bacillus* spp. dominated in jute retting (Tamburini *et al*., 2003). *Agrobacterium* sp. BN-2A and *Microbacterium* sp. PVC8 as isolated in this study was not previously reported as jute retting bacteria.

Phylogenetic analysis: The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 0.57967622 is shown (Fig. 2). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 554 positions in the final dataset.

Six of the screened isolates belong to phylum Firmicutes and one each belong to Alphaproteobacteria and Actinobacteria.

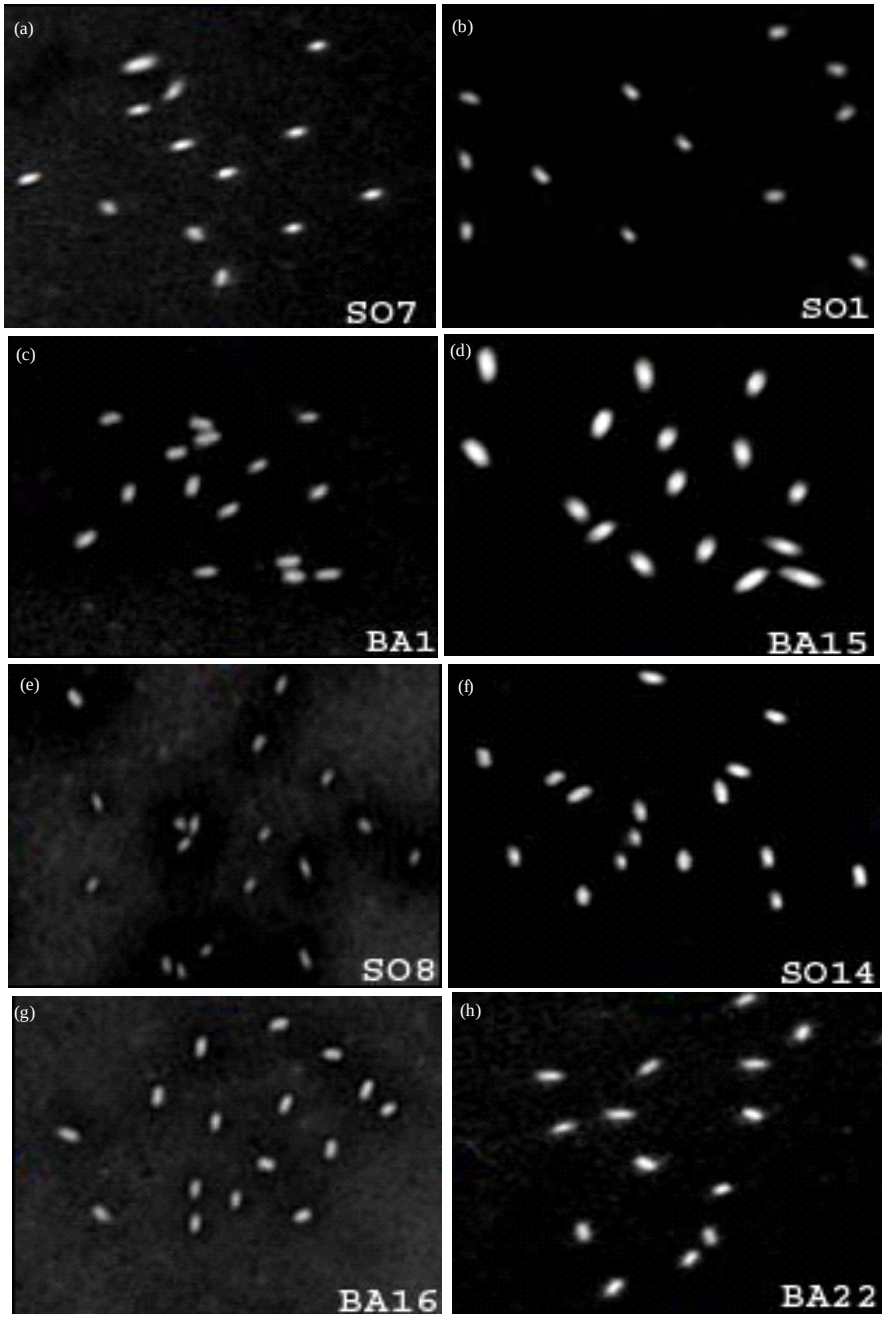

Fig. 1(a-h): Light microscopic appearance (1000X) of the efficient strains of microorganisms isolated from jute retting water bodies by negative staining. SO1: *Agrobacterium* sp. BN-2A (GQ891102); SO7: *B. pumilus* strain Geo-03-422 (GQ891103); SO8: *Microbacterium* sp. PVC8 (GQ891104); SO14: *Bacillus pumilus* strain IK-MB12-518F (GQ891105); BA1: *Bacillus* sp. L6 (GQ891097); BA15: *B. pumilus* strain EK-17 (GQ891098); BA16: *Bacillus* sp. YACS30 (GQ891099); BA22: *B. pumilus* strainIK-MB13-518F (GQ891100)

Table 3: Biochemical characteristics of the isolated bacteria

Organism code	Starch utilization	Sucrose utilization	Mannitol utilization	Lactose utilization	Gas production	Nitrate utilization	Citrate utilization	Catalase test	MR test	VP test	Indole production	H_2S production	Gelatin liquefaction	Growth on TSI agar
SO1	+ ve	+ ve	+ ve	+ ve	- ve	- ve	+ ve	+ ve	- ve	- ve	- ve	- ve	- ve	Showed alkaline reaction
SO7	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	- ve	- ve	- ve	- ve	Showed acid formation
SO8	+ ve	+ ve	+ ve	+ ve	- ve	- ve	- ve	+ ve	- ve	- ve	- ve	- ve	- ve	Showed alkaline reaction
SO14	+ ve	+ ve	+ ve	+ ve	- ve	- ve	+ ve	+ ve	+ ve	+ ve	- ve	- ve	- ve	Showed alkaline reaction
BA1	+ ve	+ ve	+ ve	+ ve	+ ve	- ve	+ ve	+ ve	+ ve	+ ve	- ve	- ve	- ve	Showed alkaline reaction
BA15	+ ve	+ ve	+ ve	+ ve	- ve	- ve	+ ve	+ ve	+ ve	+ ve	- ve	- ve	- ve	Showed alkaline reaction
BA16	+ ve	+ ve	+ ve	+ ve	- ve	- ve	+ ve	+ ve	+ ve	+ ve	- ve	- ve	- ve	Showed alkaline reaction
BA22	+ ve	+ ve	+ ve	+ ve	- ve	- ve	+ ve	+ ve	- ve	+ ve	- ve	- ve	- ve	Showed alkaline reaction

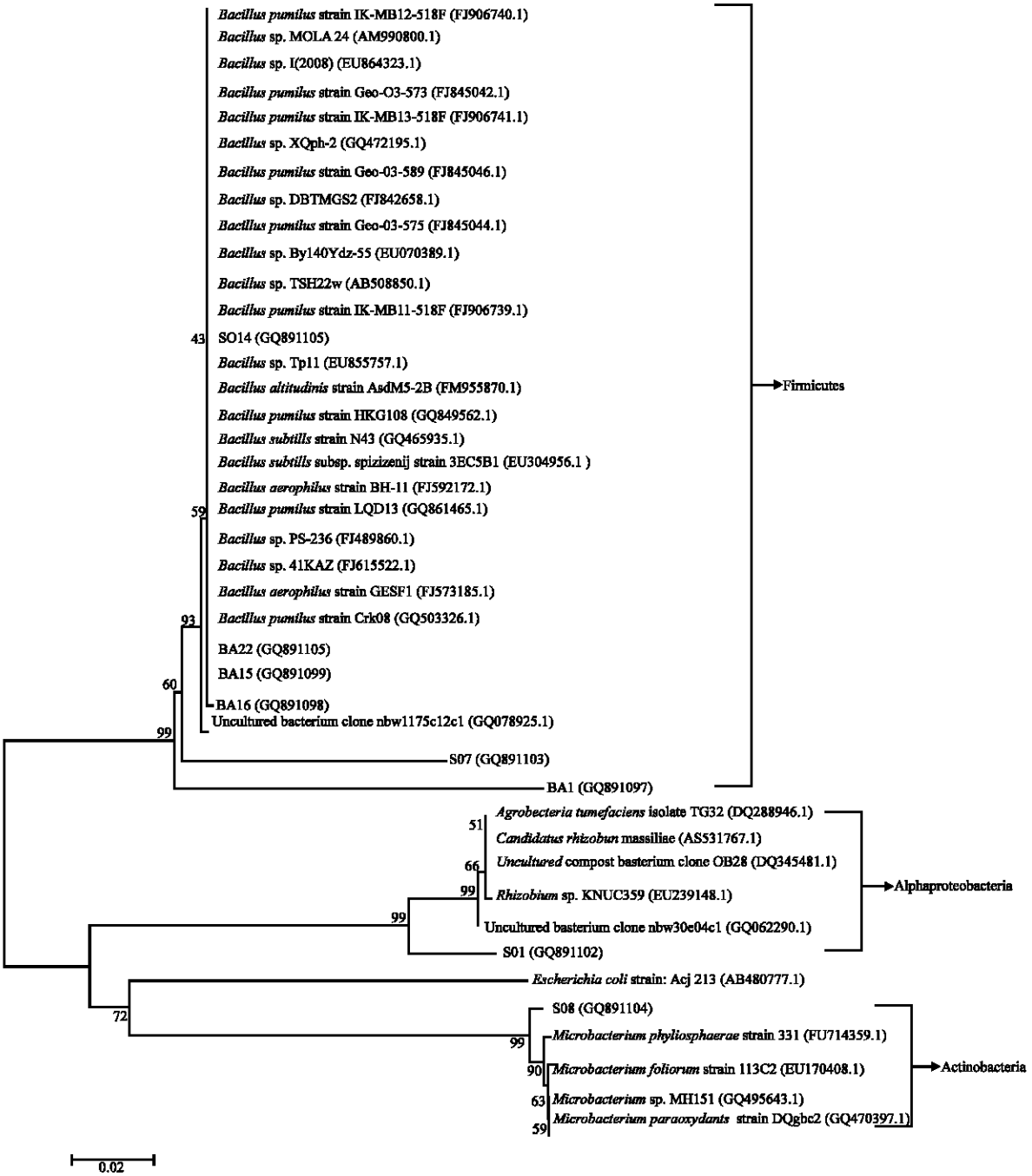

Fig. 2: Phylogenetic tree based on 16S rDNA comparisons showing the position of pectinolytic isolates and related species

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