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ORIGINAL ARTICLE

Bacterial Population Structure of the Jute-Retting Environment

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Abstract Jute is one of the most versatile bast fibers obtained through the process of retting, which is a result of decomposition of stalks by the indigenous microflora. However, bacterial communities associated with the retting of jute are not well characterized. To investigate the presence of microorganisms during the process of jute retting, full-cycle rRNA approach was followed, and two 16S rRNA gene libraries, from jute-retting locations of Krishnanagar and Barrackpore, were constructed. Phylotypes affiliating to seven bacterial divisions were identified in both libraries. The bulk of clones came from *Proteobacteria* (~37, 41%) and a comparatively smaller proportion of clones from the divisions—*Firmicutes* (~11, 12%), *Cytophaga–Flexibacter–Bacteroidetes* group (CFB; ~9, 7%), *Verrucomicrobia* (~6, 5%), *Acidobacteria* (~4, 5%), *Chlorobiales* (~5, 5%), and *Actinobacteria* (~4, 2%) were identified. Percent coverage value and diversity estimations of phylotype richness, Shannon–Weiner index, and evenness confirmed the diverse nature of both the libraries. Evaluation of the retting waters by whole cell rRNA-targeted flourescent in situ hybridization, as detected by domain- and group-specific probes, we observed a considerable dominance of the beta-*Proteobacteria* (25.9%) along with the CFB group (24.4%). In addition, 32 bacterial species were isolated on culture media from the two retting environments and identified by 16S rDNA analysis, confirming the presence of phyla, *Proteobacteria* (~47%), *Firmicutes* (~22%), CFB group (~19%), and *Actinobacteria* (~13%) in the retting niche. Thus, our study presents the first quantification of the dominant and diverse bacterial phylotypes in the retting ponds, which will further help in improving the retting efficiency, and hence the fiber quality.

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Introduction

Natural fibers have become more prevalent over the last 10 years with various utilities in textiles, composites, handicrafts, packaging, etc. Jute, also known as the "golden fiber," is one of the most versatile bast fibers, occupying second position among the natural industrial fibers of the world and accounting for nearly 15% of the total output of natural fibers, thus making it economically significant [26]. India and Bangladesh account for 56 and 30% of the world area and production, respectively. Jute fibers are obtained from *Corchorus olitorius* (Tossa jute) and *Corchorus capsularis* (white jute) through the process of retting [1, 26], which is essentially a biochemical process where the jute bundles are steeped in water and subjected to controlled decomposition of biopolymers such as pectins, celluloses, and hemicelluloses that hold the bast cells to the rest of the stem [26]. Biological retting is the cheapest and a universally practiced method for the commercial extraction of jute fibers. It utilizes a complex microbial community, which include hydrolytic, cellulolytic, fermenting, homoacetogenic, syntrophic, and acetate-utilizing bacteria, necessary for dissolution and decomposition of these biopolymers [1, 32]. However, if retting is continued for a very long time, fiber starts to rot and is of little value. As retting is one of the most important factors responsible for determining the quality and yields of fiber, other than type of bast fiber plant, age of plant, temperature and pH of

retting water, type and depth of water and activators, controlling the quality of water along with improving microorganisms used in the process are the keys to improved fiber quality [1, 26]. Thus, a better understanding of the microbial community involved in the retting process could substantially improve the extraction and yields of jute fiber in terms of quality and quantity and also help averting pollution brought about by the release of large quantities of organic matter and chemicals into the environment. Microorganisms have been isolated from the retting environments of bast fibers like flax [42, 46] and jute [1, 32]. However, no study of the microbial population structure of the jute-retting environment has been made using molecular-based approaches nor have there been any reports on unculturable organisms active in jute-retting environment.

The current understanding of jute-retting environment is based almost exclusively on culture-based studies. These studies have suggested that most retting microbes belong to a restricted number of cosmopolitan taxa and are largely aerobic, with only few reported anaerobic isolates. Large numbers of Firmicutean bacteria of the genus *Bacillus*, viz., *B. subtilis*, *B. polymyxa*, *B. mesentericus*, *B. cereus*, and *B. macerans*, and anaerobic bacteria of the genus *Clostridium*, viz., *C. tertium*, *C. aurantibutyricum*, and *C. felsineum* have been isolated from retting water, along with large numbers of Gram-negative genera such as *Erwinia* and *Pseudomonas* [1]. Although some information is available regarding the nature and activity of microorganisms involved in retting, the purpose of the present study is to report the dominance of other groups of bacteria present in the jute-retting environment.

Approximately 99% of the microorganisms cannot be cultivated by using traditional cultivation techniques [23, 24]. The application of molecular-phylogenetic methods to study natural microbial ecosystems without the traditional requirement for cultivation [3, 13, 14] has resulted in the discovery of many unexpected evolutionary lineages. Among many, methods based on the amplification of fragment coding for 16S rRNA have emerged as a powerful tool [28, 44]. Thus, in the present study, we examined two jute-retting ecosystems located in Barrackpore and Krishnanagar, as they are the major sites of jute fiber production in West Bengal, India. Using the full-cycle rRNA approach along with whole-cell flourescent in situ hybridization (FISH) analysis [4], we determined the bacterial community associated with the retting process. Furthermore, we identified dominant cultivable bacterial microbiota by sequencing 16S rDNA and a comparative sequence analysis. Results from application of culture-independent and culture-dependent methods documented the presence of a diverse bacterial population.

Materials and Methods

Sample Collection

Retting water samples were collected from jute-retting ponds ~100 km apart, located in Krishnanagar (23°24′N, 88°33′E) and Barrackpore (22°46′N, 88°22′E; Fig. 1). Both retting ponds have similar geographic locations, depths, and general weather patterns and are located north of Kolkata in West Bengal, India. The water samples were collected during the retting season (August–September) from ponds where retting was in progress. For each retting pond, subsamples were collected at evenly spaced locations, around and at close proximity to the submerged mat of jute bundles, at a depth of 7 to 9 ft, to get maximum coverage of the retting consortia. The subsamples were then pooled to create a single composite sample. The composite samples were mixed well and immediately placed on ice for transport. The temperature and pH of the retting ponds during the retting season was 32–34 and 5.0–5.3°C, respectively, whereas the dissolved concentration ranged between 1.9–2.8 mg/l. The retting water enriched with microbial cells was separated from the rough debris by repeated filter treatments. For microbiological analysis, 500 ml of retting water was filtered using 0.45-μ filter and was used for extraction of community DNA.

DNA Extraction, PCR Amplification, and Cloning of 16S rRNA Genes

Total cellular nucleic acids were extracted according to the procedure described by Saano *et al.* [41]. Polymerase chain reaction (PCR) amplification of 16S rRNA gene was carried out using the universal *Eubacteria*-specific primers 19F (5′-AGAGTTTGATCCTGGCTCA-3′) corresponding to *E. coli* positions 8 to 26 and reverse primer 20R (5′-GCTCGTTGCGGGACTTATCC-3′) corresponding to *E. coli* positions 1088 to 1107. The PCR conditions used were 94°C, 30 s; 56°C, 30 s; 72°C, 1 min; for 30 cycles in a Minicycler (MJ Research, Waltham, MA, USA). Amplified PCR products (~1.1 kb) were cloned into pBluescript KS + vector for construction of clone libraries. These libraries were assigned the prefix JRCK and JRCB for ‘Jute-Retting Clones’ from ‘Krishnanagar’ and ‘Barrackpore’, respectively, followed by the clone number.

ARDRA of 16S rRNA gene clones

All 16S rRNA gene clones were completely digested with *Msp*I, *Hae*III, and *Taq*I [16]. Resulting fragments were subsequently resolved by agarose gel (2%) electrophoresis and stained using ethidium bromide. Restriction digestion

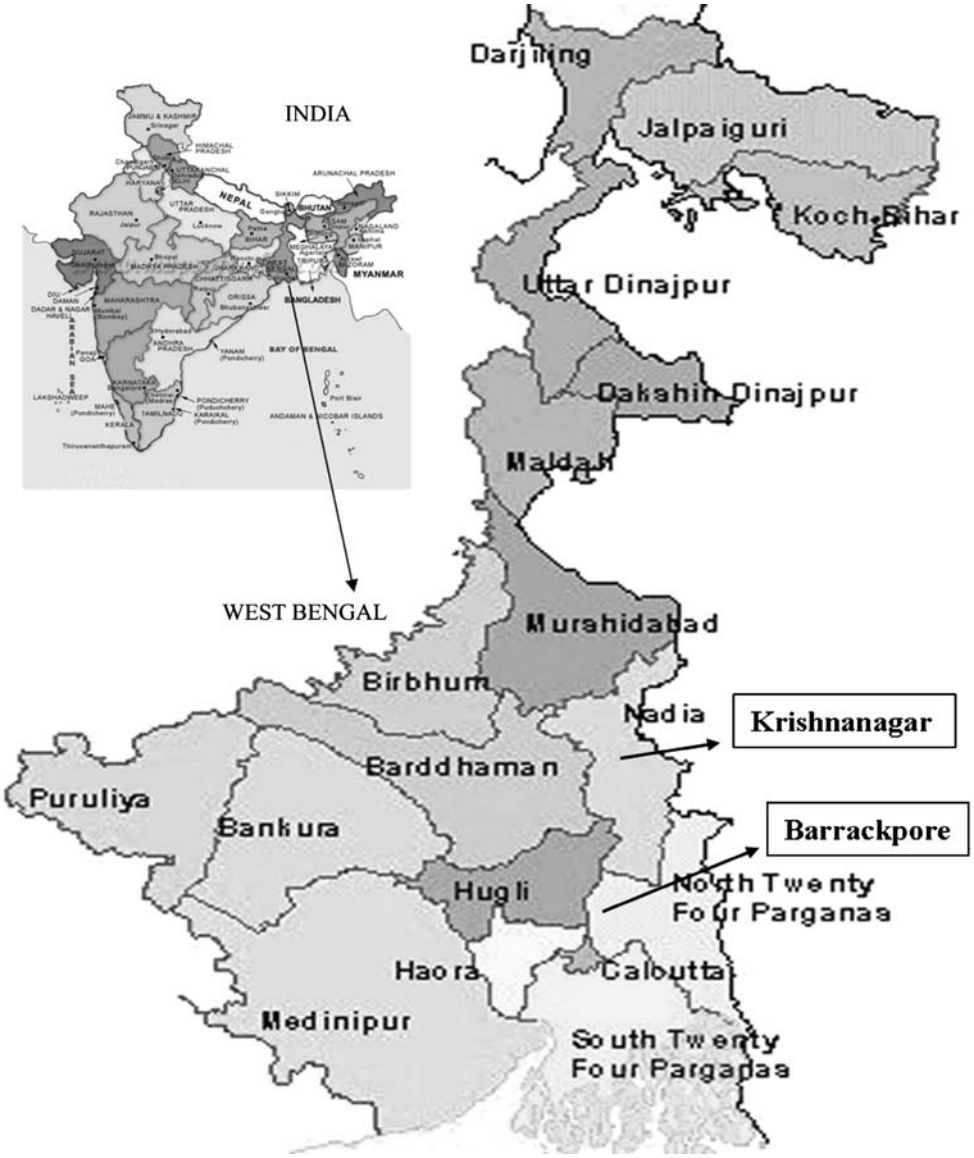

Figure 1 Map of the jute-retting locations of Krishnanagar and Barrackpore in West Bengal, India

profiles were compared using NTSYSpc, Numerical Taxonomy System (Version 2.1) [38], to avoid sequencing redundant clones.

Community Richness and Composition Analysis

Coverage analysis of the sample sites was calculated as $[1-(n/N)]\times$ 100, where n is the number of singleton sequences and N is the total number of sequences for the analyzed sample [17]. Phylotype richness, S, was calculated as the percentage of the total number of distinct amplified rDNA restriction analysis patterns to clones. Shannon–Weiner diversity index [17] was calculated as follows: $H=-\sum (p_i)(\log_2 p_i)$, where p_i is the proportion of the ith phylotype. Evenness [17] was calculated from the Shannon–Weiner diversity function as follows: $E=H/H_{max}$ where $H_{max}=\log_2(S)$.

Sequencing and Phylogenetic Analysis

Based on ARDRA profile, representative clones were selected for sequencing with M13 primers using a CEQ 8000 sequence analyzer (Beckman Coulter, USA). The sequences were identified using the online program of the Ribosomal Database Project II (RDP-II; accessed at http://rdp.cme.msu.edu/index.jsp) [12] and the advanced basic local alignment search tool (BLAST) search program [2]. Integrity of the sequence data and detection of chimeras was analyzed using the RDP-II CHECK_CHIMERA program [12]. The distances of sequences sampled from both the libraries were plotted using the PATRISTICv1.0 [21]. For more detailed phylogenetic analyses, 16S rRNA gene sequences were aligned against selected sequences extracted from GenBank, using the program ClustalX,

version 1.83 [45]. The evolutionary distances between pairs of microorganisms were determined by the Jukes and Cantor method [27], and trees were constructed using the neighbor-joining and maximum-likelihood methods [40]. The significance levels of interior branch points obtained in neighbor joining analysis were determined by bootstrap analyses (1000 data resamplings).

Whole-Cell rRNA-Targeted FISH

The microbial community composition and abundance was determined by FISH using the rRNA-directed oligonucleotides probes [4]. A set of flourescein isothiocyanate (FITC)- and Cy3-labeled oligonucleotide probes with reported group specificity for the domains *Bacteria* and *Archaea*, as well as for some phyla within the domain *Bacteria* and some classes within the *Proteobacteria*, was used in this study (Table 1; Hysel India Pvt., India). The filtered and fixed subsamples (100–250 ml) from each of the freshly collected original samples were treated with chloramphenicol (100 μg/ml) and incubated at approximate retting water temperature (32°C) for1 h. Whole-cell hybridization was performed as described by Manz *et al.* [34].

Microscopy and Digital Image Analysis

The hybridized and 4′,6-diamidino-2-phenylindole (DAPI)-stained samples were examined under an epifluorescence microscope at ×400 magnification (Eclipse 80i, 40X, Nikon, Japan) equipped with 100-W high-pressure mercury lamp (Osram HBO 103W/2 N) and filter sets for DAPI (UV-2A, Nikon, Japan), FITC (B-2A, Nikon), and Cy3 (Y-2E/C, Nikon). Three replicate samples were counted for each hybridization procedure. Around 10 randomly chosen microscopic fields were counted per well, and the data for the three parallel counts were averaged. Quantification of cells hybridized with specific probes relative to the number of Eub338-hybridized cells was done; for each grid, up to 300 DAPI stained cells were counted.

Recovery and Identification of Bacterial Isolates

Populations of viable bacteria were recovered from the water samples by first blending the sample in phosphate-buffered saline, and appropriate dilutions were spread onto Nutrient agar (3%), TSA (2%), and YEPD agar plates. Inoculated plates (in triplicates) were incubated for 5–7 days at 32°C. Morphologically distinct isolates were streak-purified and identified genetically by 16S rRNA gene sequence analysis, using the RDP database. The isolates were designated as JRI for ‘Jute-Retting Isolates’, followed by the isolate number.

Nucleotide Sequence Accession Numbers

The accession numbers for the sequences submitted to GenBank are DQ252375 to DQ252481, DQ238814 to DQ238826 and EF067808 to EF067839.

Results

Clone Library Screening

Two 16S rRNA gene clone libraries having 323 clones (147 and 176 clones from JRCK and JRCB libraries, respectively) were constructed. ARDRA profiles generated using restriction enzymes, identified a total of 110 unique patterns of small subunit (SSU) rRNA gene clones, 52 and 58 clones from the Krishnanagar and the Barrackpore libraries, respectively. To test the reliability of the ARDRA analysis, a series of clones showing identical ARDRA patterns were sequenced. All clones from unique ARDRA clades showed identical sequences. The estimated proportion of phylo-

Table 1 Oligonucleotide probes used in this study

Probe	Specificity	Sequence (5-3′) of probe	5′-label	Target[a] site (rRNA positions)	% FA[b] *in situ*	Reference
EUB338	*Bacteria*	GCTGCCTCCCGTAGGAGT	FITC	16S (338–355)	0–35	[5]
EUB338II	*Bacteria*	GCAGCCACCCGTAGGTGT	FITC	16S (338–355)	0–35	[15]
ALF968	Alpha subclass of *Proteobacteria*	GGTAAGGTTCTGCGCGTT	Cy3	16S (968–985)	35	[35]
BET42a	Beta subclass of *Proteobacteria*	GCCTTCCCACTTCGTTT	Cy3	23S (1027–1043)	35	[34]
GAM42a	Gamma subclass of *Proteobacteria*	GCCTTCCCACATCGTTT	Cy3	23S (1027–1043)	35	[34]
CF319a	CFB group	TGG TCC GTG TCT CAG TAC	Cy3	16S (319–336)	42	[33]
EUB338 III (SBACT V 338)	*Verrucomicrobia*	GCT GCC ACC CGT AGG TGT	Cy3	16S (338–355)	42	[15]
ARCH915	*Archaea*	GTG CTC CCC CGC CAA TTC CT	Cy3	16S (917–934)	35	[31]

[a] *Escherichia coli* numbering [7]
[b] Percentage of formamide (*FA*) in *in situ* hybridization buffer

Table 2 Comparison of phylotype richness, diversity, and evenness values for the jute retting water environment bacterial communities

Diversity estimations	16S rRNA gene clone libraries	
	JRCK	JRCB
Percent coverage value	61.22	65.91
Phylotype richness '*S*'	52	58
Shannon–Weiner index '*H*'	3.6	3.7
Evenness '*E*'	0.92	0.93

types in the retting environment represented in the library was remarkably high, and other diversity estimation like richness, Shannon–Weiner index and, evenness, of the two libraries, as shown in Table 2, were found to encompass the heterogeneity of the bacterial community of the jute-retting environment.

16S rRNA Gene Sequence Analysis and Phylotype Assignment

Phylotypes associated with seven bacterial divisions were identified from 110 partial 16S rRNA gene sequences (700 to 850 bp) representing all 323 clones (Fig. 2). The bulk of clones in JRCK and JRCB libraries came from *Proteobacteria* and a vast majority of the *Proteobacterial* group, in both libraries, belonged to the beta- (~17, 15%) and the gamma- (~10, 16%) subclass of *Proteobacteria*. Delta- (~6, 4%) and alpha-(~4, 4%) subclasses accounted for a very small number of clones. *Firmicutes* formed a second most abundant component (~19, 16%) of bacterial assemblages in both the libraries belonging mainly to the genera *Bacilli* and *Clostridia*. Comparatively smaller proportion of clones from the divisions—*Cytophaga–Flexibacter– Bacteroidetes* group (CFB; ~11, 13%), *Verrucomicrobia* (~8, 11%), *Acidobacteria* (~4, 6%), *Chlorobiales* (~2, 8%), and *Actinobacteria* (~7, 1%) were also identified in both libraries. Of the 110 bacterial phylotypes, ~22% were completely unidentified displaying >5% sequence dissimilarity from publicly available sequences, ~27% of the sequences were identified at the phylum level only, i.e., genera could not be assigned to them, and ~51% represented sequences from species that have been cultivated previously. In addition, 11 phylotypes belonging to the genera *Achromobacter*, *Zoogloea*, *Erwinia*, *Pseudomonas*, *Bacilli*, *Clostridia*, *Cytophaga*, and *Verrucomicrobia* were found to be common between JRCK and JRCB libraries. The distance matrix plot, comparing the distances between the two libraries using PATRISTICv1.0, showed that majority of the points, representing the sequences, were dispersed throughout the plot around the regression line (r=0.367), indicating sequences of diverse nature (Fig. 3).

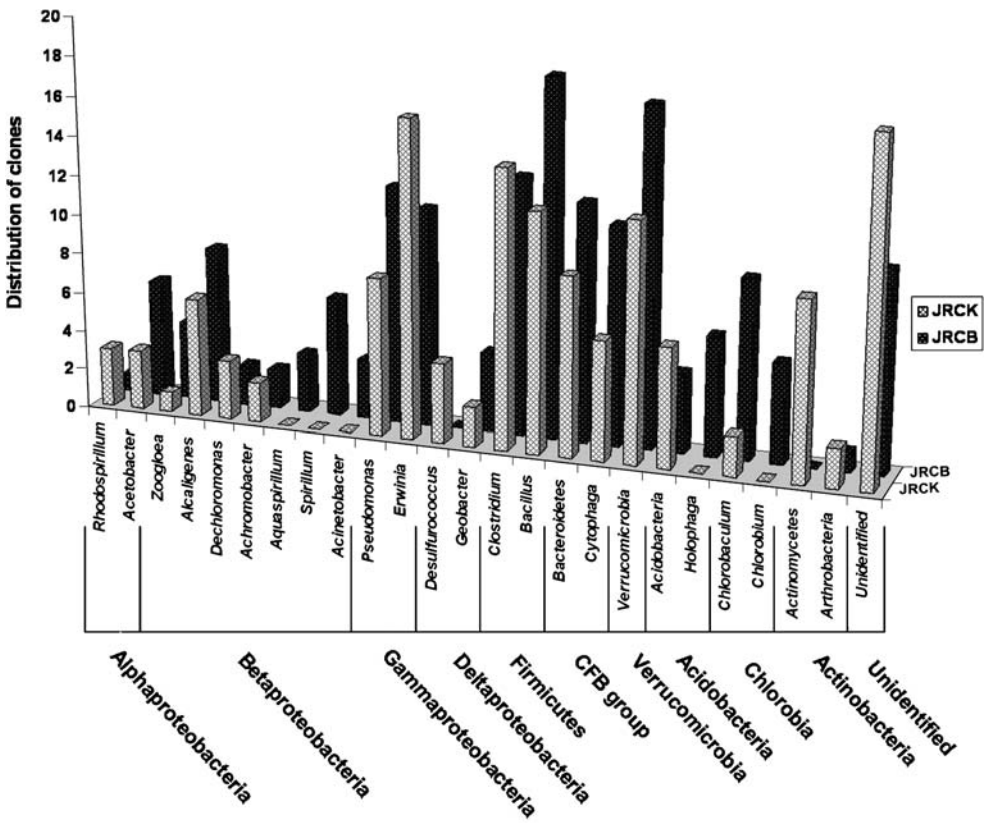

Figure 2 Relative distribution of clones represented by seven major phylogenetic groups found in the 16S rRNA gene libraries of jute-retting water samples from Krishnanagar (*JRCK*) and Barrackpore (*JRCB*) in West Bengal

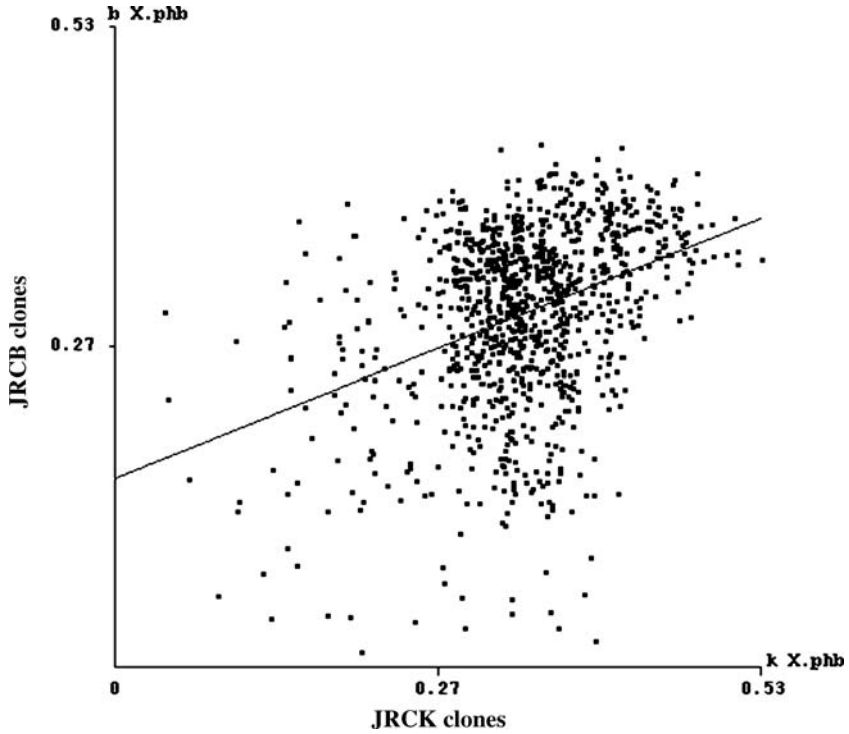

Figure 3 Distances matrix of sequences sampled from both the libraries plotted using the PATRISTICv1.0

Phylogenetic Analysis

The phylogenetic relationships of these cloned sequences were indicated in a character-based evolutionary tree, based on the partial length of the 16S rRNA gene sequences, and constructed using the neighbor-joining algorithm (Fig. 4). Of the 22% of completely unidentified sequences, 50% formed separate groups (clades A and B) in the phylogenetic tree representing members of new or not-described divisions, and the remaining 50% of the sequences represented deeply branching members of previously described bacterial divisions. Approximately, 5, 10, 2, and 2% of unidentified phylotypes from the two libraries showed close branching with known members of the Green non-sulfur bacteria, *Proteobacteria*, *Firmicutes*, and *Chlorobial* phylotypes, respectively. A few additional sequences that fell on the same branch and were 99% similar were not included in the tree.

Whole-Cell rRNA-Targeted FISH

To discriminate major groups of jute-retting bacteria, whole-cell FISH was performed on a selection of relevant groups from those listed in Table 1. All probe-specific cells were presented as the percentage of cells visualized by DAPI. EUB338-labeled cells represented approximately 75–90% of the DAPI-stained cells, whereas ARCH917-labeled cells represented a low but detectable count of about 4%. The result indicates that microorganisms affiliated with the domain *Bacteria* are the major constituents in the jute-retting ponds. Members of the alpha-*proteobacteria* were detected at substantially lower *in situ* abundance of 8.52%, whereas members of the beta and gamma-*proteobacteria* accounted for 25.9 and 20.9% of bacterial cells fluorescing with the EUB probe mix. The high abundance of beta- and gamma-*proteobacteria* was in accordance with the results of the gene library survey. Probes targeting the members of CFB and *Verrucomicrobia* groups also indicated comparatively high abundance of 24.4 and 16.6% (Fig. 5), which is interesting, as these groups have never been previously linked with jute retting.

Phylogenetic Distribution in Bacterial Culture Collection

Thirty-two bacterial species were isolated on culture media from the two retting environments and identified by 16S rDNA analysis. The isolates showing >95% identity to sequences in the database and were distributed majorly between the four phyla—the *Proteobacteria* (mainly beta- and gamma-*Proteobacteria*; ~47%), the *Firmicutes* (~22%), the CFB group (~19%), and the *Actinobacteria* (~13%; Table 3). A phylogenetic tree was constructed with the identified bacterial isolates and the sequences of known organisms from the database (Fig. 6). The overlap between the culture collection and the clone libraries was almost ~5%. Isolates JRI02, JRI04, JRI06, JRI11, JRI15, and JRI31 showed closest identity with sequences belonging to *Dechlorosoma* sp., *Pseudomonas* sp., *Bacillus* sp., *Cytophaga* sp., *Arthrobacter* sp., and *Erwinia* sp., which were also present in the 16S rDNA retting water libraries.

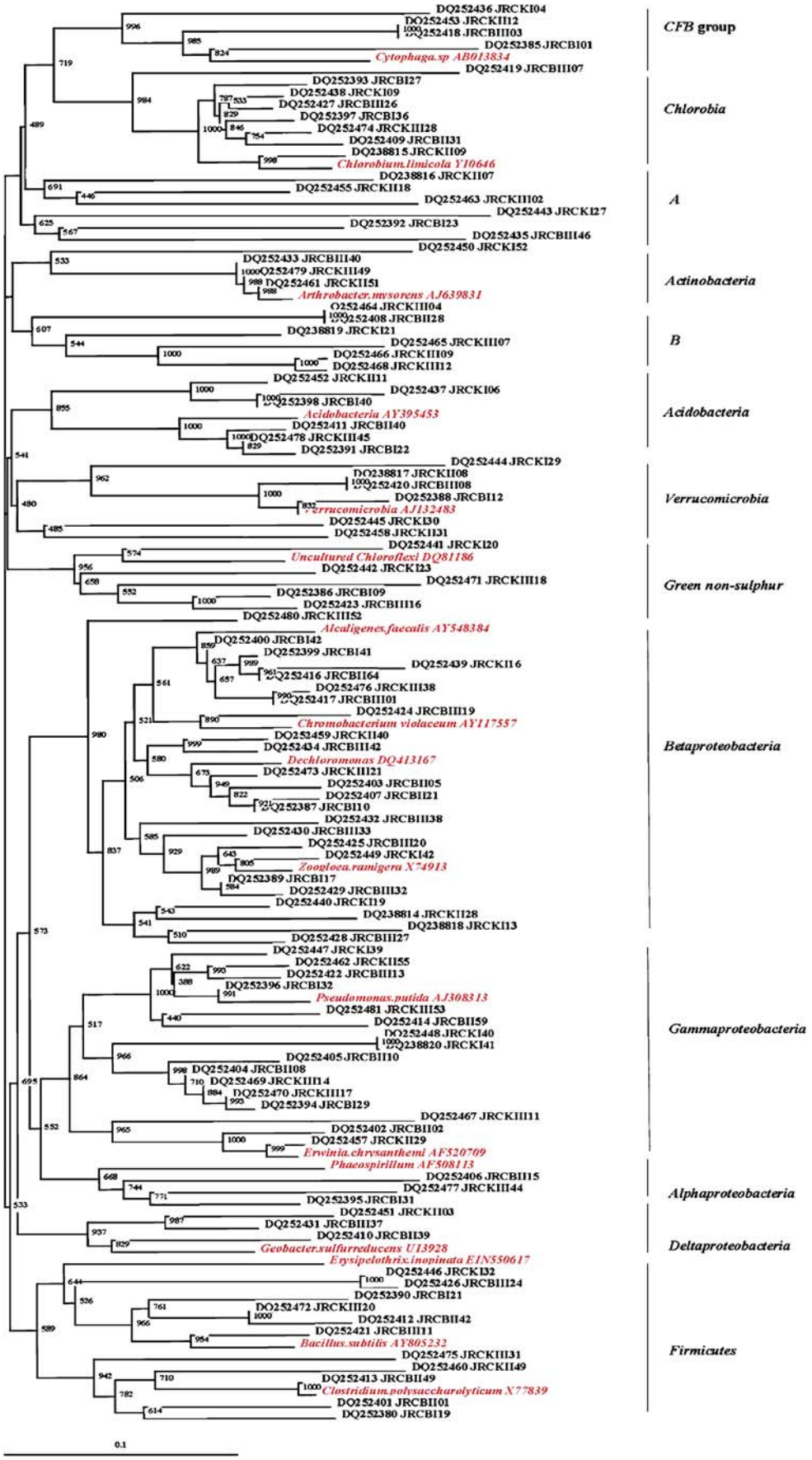

Figure 4 Neighbor-joining phylogenetic tree constructed by using bacterial partial SSU rDNA sequences retrieved from Krishnanagar (*JRCK*) and Barrackpore (*JRCB*) retting water sample. GenBank accession numbers followed by the library sequence name represents the identified clones. The reference sequences selected to span the phylogenetic breadth are indicated in *red* with their accession numbers. Clades A and B are groups of sequences that cannot be assigned to any known group of organisms within the domain. Names of the major taxa are shown in *boldface*. *Numbers* at nodes represent the percentages of occurrence of nodes in 1000 bootstrap trials. The *scale bar* represents the expected number of substitutions per nucleotide position

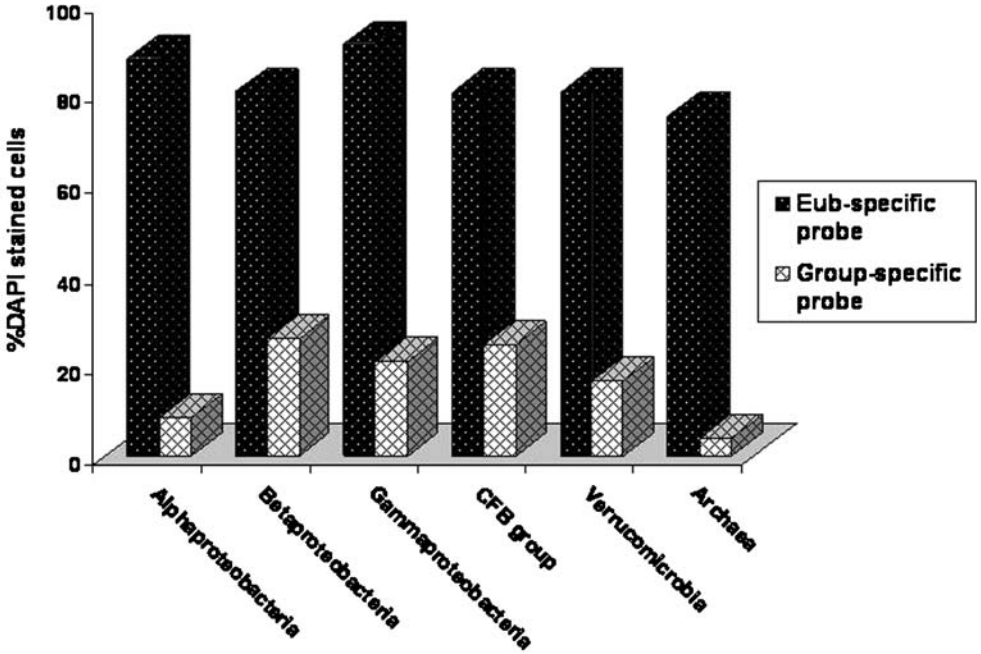

Figure 5 Graphical presentation of bacterial community by whole-cell rRNA-targeted FISH. The values obtained with group-specific probes indicate the relative percentage of specific groups to that of *Eubacteria* in the jute-retting ecosystem

Discussion

Retting, brought about by the cellulolytic and pectinolytic activities of microorganisms, is a major limitation to efficient production of jute fibers [1]. The associated microbiota, which can have dramatic impacts on retting efficiency and fiber quality, are not well characterized. The knowledge and understanding of bacterial ecosystem in the retting niche is not only a step towards improving the quality of fiber but will also help in confining the process to closed spaces and preventing pollution brought about by the activity of microorganisms on jute stems, liberating large quantities of organic matter and chemicals into the environment, leading to decrease in pH and dissolved oxygen concentration, and thus causing extensive damage to the living, aquatic resources in the region. The present study, to the best of our knowledge, represents one of the first attempts to estimate natural bacterial communities in jute-retting environment, using culture-independent and culture-dependent approaches. As jute production and fiber extraction is one of the major industries in Krishnanagar and Barrackpore, hence, retting ponds from these two locations were specifically chosen for analysis of the microbial population present in the retting ecosystem.

Comparative sequence analysis of clones obtained from these locations revealed a rich spectrum of bacterial diversity. About 7 phyla and 110 phylotypes were identified in the 16S rRNA gene clone libraries. Although the clones identified by sequence analysis were distributed among the phyla *Proteobacteria*, *Firmicutes*, CFB group, *Verrucomicrobia*, *Acidobacteria*, *Chlorobia*, and *Actinobacteria* in JRCK and JRCB libraries, however, the proportion and diversity of clones belonging to different genera or species varied in both the clone libraries (Fig. 2). The phylotypes common in both libraries mainly belonged to the phyla *Proteobacteria*, *Firmicutes*, CFB group, and *Verrucomicrobia*. The similarity between the two retting ponds, with respect to the groups (phyla) of bacteria observed, could be attributed to the fact that both ponds were having similar geographic locations and fell in the same agroclimatic zone. Calculations of diversity indices (Table 2) and distance matrix plot (Fig. 3) also supported previous suggestions that both sample sites harbor relatively high species diversity [24].

The majority of clones obtained were dominated by phylotypes from three major bacterial divisions—*Proteobacteria*, *Firmicutes*, and CFB group—also present in the culture collection (Table 3). Interestingly, most of the isolates belonging to these phyla revealed enzyme activities for key retting enzymes viz., xylanase, pectinase, and cellulase (manuscript in preparation). Members of phyla *Acidobacteria* and *Verrucomicrobia* are largely recalcitrant to culturing, so although they formed a distinct proportion of clones in the library, their absence in our culture collections was not surprising [25, 36]. Molecular analysis based on the 16S rRNA gene revealed some overlap with cultured strains at the phylum or family level but much less correspondence at the genus or species level. Only six isolates showed sequence similarity >97% to clones in the library. In similar studies, where workers have specifically compared cultured isolates and 16S rRNA gene clone libraries derived from the same samples, 0 to 40% overlaps between the two have

Table 3 Closest phylogenetic affiliation of isolates based on BLASTn comparison to the GenBank database

Isolate designation and accession no.	Sequence length (bp)	Closest relative in databank (accession no.)	Similarity (%)	Affiliation phylum/class
JRI-05 (EF067830)	761	*Cellulomonas* sp. (AJ292035)	99	*Actinobacteria*
JRI-07 (EF067838)	817	*Rhodococcus* sp. (AY837749)	96	*Actinobacteria*
JRI-15 (EF067818)	550	*Arthrobacter* sp. (DQ252479)	98	*Actinobacteria*
JRI-25 (EF067837)	614	*Micrococcus luteus* (AM285006)	98	*Actinobacteria*
JRI-08 (EF067839)	871	*Flavobacterium denitrificans* (AJ318907)	98	CFB group
JRI-11 (EF067824)	746	*Cytophaga* sp. (DQ252385)	98	CFB group
JRI-23 (EF067825)	716	*Cytophaga arvensicola* (AM237311)	98	CFB group
JRI-13 (EF067808)	580	*Chryseobacterium* sp. (AM159535)	98	CFB group
JRI-24 (EF067826)	680	*Bacteroides fragilis* (M11656)	99	CFB group
JRI-27 (EF067829)	738	Rumen bacterium (AF544207)	99	CFB group
JRI-01 (EF067822)	679	*Exiguobacterium* sp. (AJ846291)	97	*Firmicutes*
JRI-06 (EF067810)	624	*Bacillus* sp. (DQ252421)	98	*Firmicutes*
JRI-14 (EF067812)	551	*Bacillus subtilis* (AB048252)	99	*Firmicutes*
JRI-26 (EF067813)	847	*Bacillus licheniformis* (AY842871)	98	*Firmicutes*
JRI-22 (EF067811)	741	*Bacillus cereus* (DQ459876)	97	*Firmicutes*
JRI-19 (EF067828)	807	*Clostridium* sp. (DQ252460)	99	*Firmicutes*
JRI-21 (EF067823)	694	*Staphylococcus arlettae* (AB009933)	96	*Firmicutes*
JRI-09 (EF067827)	753	*Agrobacterium* sp. (AM181759)	99	*Alphaproteobacteria*
JRI-02 (EF067820)	447	*Dechlorosoma* sp. (DQ252434)	97	*Betaproteobacteria*
JRI-10 (EF067819)	793	*Achromobacter xyloxidans* (AF531768)	97	*Betaproteobacteria*
JRI-30 (EF067831)	670	*Alcaligenes sp.* (Y14908)	95	*Betaproteobacteria*
JRI-03 (EF067821)	690	*Pantoea agglomerans* (AY691544)	97	*Gammaproteobacteria*
JRI-12 (EF067834)	664	*Shewanella sp.* (AM286803)	99	*Gammaproteobacteria*
JRI-16 (EF067835)	768	*Escheriachia coli* (DQ360844)	98	*Gammaproteobacteria*
JRI-04 (EF067817)	808	*Pseudomonas sp.*(DQ252422)	99	*Gammaproteobacteria*
JRI-17 (EF067816)	717	*Pseudomonas putida* (AB109777)	98	*Gammaproteobacteria*
JRI-20 (EF067815)	710	*Pseudomonas fluorescens* (AY271793)	99	*Gammaproteobacteria*
JRI-29 (EF067814)	724	*Pseudomonas aeroginosa* (CP000438)	99	*Gammaproteobacteria*
JRI-32 (EF067836)	766	*Pseudomonas syringiae* (AM184090)	98	*Gammaproteobacteria*
JRI-18 (EF067832)	758	*Erwinia carotovora* (AF373189)	98	*Gammaproteobacteria*
JRI-31 (EF067833)	714	*Erwinia sp.* (DQ252457)	98	*Gammaproteobacteria*
JRI-28 (EF067809)	638	*Enterobacter sakazakii* (AY803190)	99	*Gammaproteobacteria*

been reported [8, 17, 19, 43, 44]. Although 16S rDNA cloning (Fig. 4) and cultivation (Fig. 6) provided slightly inconsistent descriptions of relative phylotype, these two methods described roughly similar phyla dominance in samples from both the locations.

In the clone libraries, the high frequency of beta-*Proteobacteria* phylotype signals suggests that this group of heterotrophic bacteria contributes a significant fraction of the retting bacterial population. Most bacteria, with high degradation capacities, assimilating acetate, and other organic acids under oxic, anoxic, nitrate-present anoxic, and sulfate-present conditions are reported to be members of beta- and gamma-*Proteobacteria* [6]. As degradation of the jute stems leads to high percentage of organic carbon and anoxic conditions in the retting zones, hence, abundance of phylotypes belonging to beta-*Proteobacteria* is noteworthy. Interestingly, most members of beta-*Proteobacteria* found in our libraries were heterotrophs like *Zoogloea*, *Achromobacter*, *Dechloromonas*, and *Alcaligenes*, which are important in degradation of organic matters and in maintenance of the biofilm structure. Besides these, members of the organotrophic CFB family are also recognized to have a potentially unique role in the utilization of complex organic molecules like pectin [30], xylan [20, 29], and cellulose [20]. Previous studies have revealed that beta-*Proteobacteria* are believed to share the ability to degrade complex organic macromolecules with the *Bacteroidetes* [30]. Thus, the appearance of *Proteobacteria* and CFB phylotypes as major contributors to the libraries, along with the *Verrucmicrobia* group, some species of which are also reported to ferment sugars and utilize cellulose [11], indicates the possibility of their role in the decomposition of jute stems.

Identification of species belonging to the genera *Pseudomonas*, *Erwinia*, *Clostridium*, and *Bacillus* as abundant organisms in the retting ponds, appears to correlate well

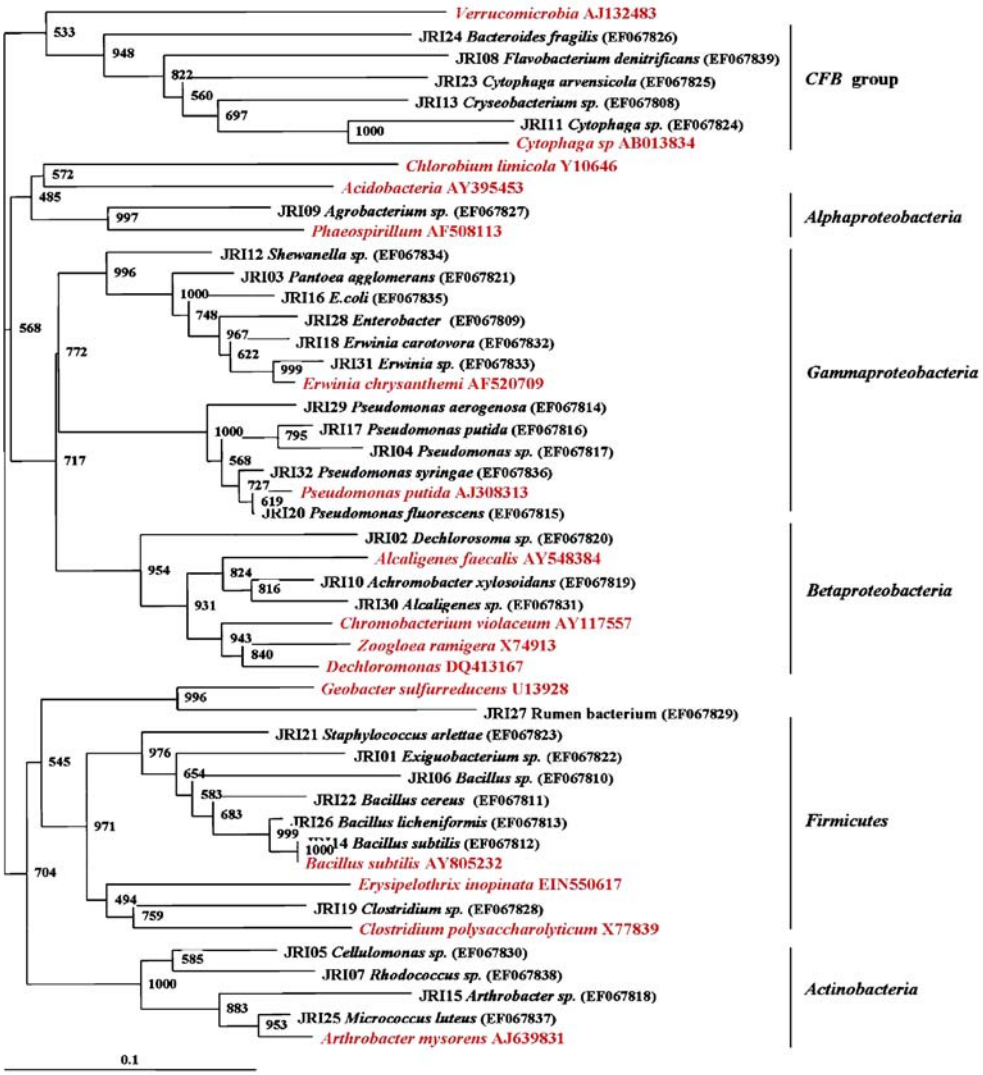

Figure 6 Neighbor-joining phylogenetic tree constructed by using bacterial partial SSU rDNA sequences of the culturable isolates retrieved from the retting water samples. Library sequence number followed by the name of the bacteria and their accession number in parenthesis represents the identified isolates. The reference sequences selected to span the phylogenetic breadth are indicated in *red* with their accession numbers. Names of the major taxa are shown in *boldface*. *Numbers* at nodes represent the percentages of occurrence of nodes in 1000 bootstrap trials. The *scale bar* represents the expected number of substitutions per nucleotide position

with the major environmental factors that determine the levels of defined microbial groups in retting ponds, including anoxic conditions, and the fact that the main portion of the substrates is in the form of polymers, such as xylan, pectin, and cellulose. In addition, saccharolytic *Clostridium* sp. are widely regarded as typical fermenting microorganisms in anoxic habitats, such as sediments and sludge digesters [9, 10], whereas, *Pseudomonas* sp. and *Erwinia* sp. have been associated with the synthesis and secretion of a set of enzymes that degrade plant cell wall components, such as pectinases, cellulases, and proteases [37]. Therefore, the abundance these species in our clone libraries and culture collections is in accordance with the earlier culture-based studies, which implicate their role in the retting of bast fibers like jute [1, 32] and flax [39, 42].

Although major microbial activities in retting waters are confined close to the submerged jute stems, however, organisms involved indirectly in the retting process may be found distributed in the retting ponds; thus a spatial organization of the microbial populations has to be expected. The retting niches are characterized by elevated levels of organic acids and hydrogen sulfide in the associated degradation of non-fibrous tissues of the jute stem; hence, presence of green-sulfur bacteria like *Chlorobia* in this sulfide-rich niche was not unexpected. Conversely, it is not clear why we could not isolate the organism belonging to the phyla *Chlorobia*. It may be that some species make the sample-media transition better than others due to the bias imposed by isolation of bacteria on laboratory media [18]. On the other hand, although members of the class *Actinobacteria* have been isolated from and detected in a wide range of soils and fresh water [48], surprisingly however, they formed the smallest group in our clone libraries as well as culture collections. This may be due to (1) the presence of a truly small population of *Actinobacteria* compared to the more abundant groups, such as the *Proteobacteria* or *Firmicutes*, (2) bias in the PCR against 16S rRNA genes because of high GC

content, or (3) failure of the lysis methods used to extract DNA from these gram-positive *Actinobacterial* cells in the retting water [47].

In addition to these groups, a large proportion of sequences identified, grouped with the so-called "unculturable" class. A significant proportion of the sequences obtained showed relatively low homology to extant sequences (<95%), suggesting that these retting ponds represent a substantial pool of novel species and/or genera. Members of these lineages are not only distantly related to known organisms but are sufficiently abundant, giving an impression of their unlimited variability. As little is known about the closest relatives of these unidentified organisms, the biological significance of these putative organisms was unclear.

Furthermore, to eliminate bias due potential experimental artifacts, the microbial community composition in the jute-retting water was analyzed by whole-cell FISH (Figs. 4 and 5). To get an idea of the comparative abundance of *Archaea* to the *Eubacterial* domain, we targeted the *Archaeal* population with specific FITC-labeled probe. As the members of the *Archaea* domain are predominantly extremophiles, hence, one of the reasons for low abundance of *Archaea* could be attributed to the moderate temperatures (32–35°C) of the retting waters during the retting seasons. This result suggested that the majority of the microbial communities associated with retting ecosystem are members of the domain *Bacteria*. Several studies have reported that the beta-*Proteobacteria* constitute a dominant fraction in freshwater systems [22, 50, 51]. In contrast, in the retting environment, we observed a considerable dominance of the CFB group, along with beta-*Proteobacteria*, whereas gamma-*Proteobacteria* constituted the second most abundant group. *Verrucomicrobia*, however, accounted for a comparatively lower number. In addition, poor representation of the alpha-subclass as compared to beta- and gamma-subclass of *Proteobacteria* in FISH analysis of the retting water samples is in accordance with our sequencing results and confirms that, with the protocol followed, these probes do not simply light up cells nondiscriminately and may in fact reflect the actual community composition.

Thus, the nutrient-enriched environment due to excess organic matter provides a rich medium to initiate profuse growth of aerobic, facultatively aerobic, and anaerobic microorganisms belonging to the phyla *Proteobacteria*, *Firmicutes*, the CFB group, the *Verrucomicrobia* group, *Acidobacteria*, *Chlorobia*, and *Actinobacteria*, which bring about retting of jute. Of particular note was the striking similarity between the bacterial communities in jute-retting environment and that colonizing and decomposing rice straw, which was also dominated by members of the Gram-positive bacteria with low GC-content, including the genera *Clostridium* and *Bacillus*, the alpha, beta, and gamma subdivisions of the division *Proteobacteria*, and the CFB group [49]. Thus, concerning the possible impact of these groups in the degradation of rice straw, also rich in hemicellulose, cellulose, pectin, lignin, and water-soluble polysaccharides, and considering their abundance in the jute-retting ponds, we hypothesized that they may also be important colonizers and degraders of jute stems during the retting process.

In conclusion, the 16S rRNA gene clone libraries provided a comprehensive sampling of the phylogenetic diversity in the two environments along with the traditional isolation techniques and clearly demonstrated that large diversity is present within the examined jute-retting environment. This suggests that the bacteria represented by these sequences may be primary colonizers of jute surfaces in retting waters and noteworthy as retting organisms. As jute retting is greatly influenced by the microbial population present in the retting environment, consequently, these organisms deserve further study for their potential in retting of jute fibers in industrial applications. Thus, our investigation led to a better understanding of the microbial population structure and ecology of the retting ponds.

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