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Investigation of the bacterial retting community of kenaf (*Hibiscus cannabinus*) under different conditions using next-generation semiconductor sequencing

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Abstract The microbial communities associated with kenaf (*Hibiscus cannabinus*) plant fibers during retting were determined in an effort to identify possible means of accelerating this process for industrial scale-up. Microbial communities were identified by semiconductor sequencing of 16S rRNA gene amplicons from DNA harvested from plant-surface associated samples and analyzed using an Ion Torrent PGM. The communities were sampled after 96 h from each of three different conditions, including amendments with pond water, sterilized pond water, or with a mixture of pectinolytic bacterial isolates. Additionally, plants from two different sources and having different pretreatment conditions were compared. We report that the best retting communities are dominated by members of the order Clostridiales. These bacteria appear to be naturally associated with the plant material, although slight variations between source materials were found. Additionally, heavy inoculations of pectinolytic bacteria established themselves and in addition their presence facilitated the rapid dominance of the original plant-associated Clostridiales. These data suggest that members of the order Clostridiales dominate the community and are most closely associated with efficient and effective retting. The results further suggest that establishment of the community structure is first driven by the switch to anaerobic conditions, and subsequently by possible competition for nitrogen. These findings reveal important bacterial groups involved in fiber retting, and suggest mechanisms for the manipulation of the community and retting efficiency by modifying nutrient availability.

Keywords Microbial diversity · Retting · Semiconductor · Sequencing · Bioproducts

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Introduction

The transition to a green, bio-based economy necessitates changing more than just sources for liquid transportation fuels. Plastics, as well as commodity and specialty chemicals currently derived from petroleum, must also be replaced with biologically derived alternatives. One possible component in this transition is the substitution of fiberglass/epoxy composite materials with plant fiber-based composites using bio-based resins. The first step in this process is the extraction of the plant fibers by microbial action in a process known as retting.

Kenaf (*Hibiscus cannabinus*) is a fiber-bearing plant and has been explored by the USDA as a potential cash crop for many regions of the United States. Retting of kenaf typically involves stripping the bark from the harvested

material, and then soaking the former in water (e.g., a pond or river) while endogenous microbes break down heteropolysaccharides binding the fiber bundles to facilitate their separation [16]. The resultant plant fibers find use in traditional areas such as cordage and fabrics. However, more recent applications include their incorporation into "green" composites for the auto and aerospace industries [5, 28, 33, 34, 46].

Specific applications of natural fibers in advanced and high-value materials depend upon fiber quality as well as industrial-scale production. Mechanical separation of fibers and chemical retting using NaOH or other harsh treatments are easy to scale, but result in poor-quality fibers [34, 35]. Investigations using enzymatic retting have reported high-quality fibers, but the process is likely cost-prohibitive for large-scale applications due to the high cost of purified pectinase enzymes [2, 19]. Microbial or "water" retting therefore offers the best chance for large-scale production of high-quality fibers.

In spite of many decades of research, surprisingly little is known of the microbial community involved in retting. A number of studies conducted over the last half century have focused on culturable isolates, most often identifying *Bacillus*, *Clostridium*, and *Pseudomonas* spp. [1, 3, 32, 42]. In several studies, pectinolytic retting isolates grown separately and re-introduced to the retting vats have shown the ability to accelerate the process rate and improve fiber quality [7, 17, 48]. Reports employing more modern molecular techniques to retting, however, are sparse and little is known of the actual microbes involved, the dynamic behavior of the community over time, or the suitability of microbial amendments to the retting solution.

Numerous pectinase-producing bacteria have been described, particularly members of the genera *Bacillus* [11, 24, 37, 40], *Paenibacillus* [13, 31, 38], and *Clostridium* [36, 39, 41]. Members of these bacteria have been isolated from retting solutions, as well as inoculated into retting systems for process optimization. However, the fate of these bacteria in retting solutions remains unclear. The general view of the retting process involves the colonization by *Bacillus* and *Paenibacillus* species during the initial aerobic phase of retting, followed by the subsequent displacement by *Clostridium* spp. as conditions slowly turn anaerobic [42]. In other studies, the retting solution of jute was analyzed by creating a 16S clone library and performing amplified ribosomal DNA restriction analysis (ARDRA) on the fragments [29]. Their results indicated large amounts of Proteobacteria (41 %), in addition to Firmicutes (7 %), Verrucomicrobia (5 %), Acidobacteria (5 %), Chlorobiales (5 %), and Actinobacteria (2 %) at two different jute-retting locations of Krishnanagar and Barrackpore.

Here we describe the investigation of the microbial community associated with retting kenaf bast fibers with and without a complex inoculum (pond water) as well as with the addition of isolated pectinolytic *Bacillus* and *Paenibacillus* species. Also included were test plant materials from two different growing areas in the US (Oklahoma and Mississippi) and exposed to different post-harvesting conditions. This study focused specifically on those organisms that were closely associated with or adherent to the bast fibers, rather than the bulk solution as has been described in previous research. We identify groups of bacteria associated with the plants themselves, which differed depending on the source material, as well as colonizers from the pond water. We also note that inoculation with pectinolytic microbes produces the same outcome independent of the starting material. Finally, we discuss means by which the community structure may be manipulated to achieve a desired outcome.

Materials and methods

Isolation of pectinase-producing organisms

Yeast extract pectin media were prepared in 1-l batches using the following parameters: 5 g yeast extract, 5 g pectin, pH 7.2, and adjusted with 1 N NaOH. Autoclave conditions were 15 PSI, 121 °C, and 15 min. Organisms were isolated through an enrichment process using yeast extract pectin media (YEP) as adapted from [44]. A total of 5 g of plant material, (i.e., kenaf or date palm fronds), were placed into 250 ml of sterilized YEP media in a 500-ml Erlenmeyer flask and placed in a shaking incubator at 200 rpm and 37 °C. After 24 h, 2-ml aliquots of enriched culture were inoculated into 250 ml of fresh YEP. This enrichment process was repeated three more times. The solution was serially diluted to 10^{-4} and subsequent dilutions were plated on YEP agar plates and incubated for 48 h at 37 °C. Colonies were picked based on morphological characteristics and streaked for isolation. A 1 %-cetrimide solution [8] was prepared and after a period of 48 h was applied to each of the plates with isolated colonies. After 1-h incubation at 37 °C, the production of pectinase was confirmed by zones of clearing surrounding the colonies (Online Resource I). After confirmation of pectinase production, the 16S gene sequences of each organism were amplified by colony PCR using universal bacterial primers 27F and 1492R [25]. PCR parameters were 95 °C for 5 min followed by 30 cycles of denaturing at 95 °C for 15 s, annealing at 56 °C for 30 s, and extension at 72 °C for 90 s. Each PCR reaction was confirmed by gel electrophoresis for a ~ 1.5-kb product, cleaned using a MO BIO Ultra clean 15 DNA purification kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) and sent for sequencing to Eurofins MWG Operon, Huntsville, AL, USA. After bidirectional sequencing, the two fragments

were assembled into one ~ 1.5-kb fragment and compared to the NCBI database using BLAST [4].

Controls and augmented retting conditions

Kenaf was obtained from two sources; C. Webber (USDA) denoted as KOK and S. Shi denoted as KMS. KOK was grown at the USDA Agricultural Research Station in Lane, OK. Stalks were harvested 185 days post-planting and the kenaf bark was manually stripped from the core (Online Resource 2). The first bottom meter of the bast fiber from the kenaf was used for subsequent experiments, and was cut into 2-cm pieces, and immediately used in subsequent experiments. KMS was grown at Mississippi State University North farm, and grown for approximately 6 months, dried in the field, and stored for approximately 3 years until this experiment. This harvested kenaf was stripped from the core and the bast fiber was cut up into 2-cm pieces and mixed together. Experimental setup of each of the retting environments, i.e., E1D4, C1D4, and C2D4 are shown in Table 1. Pond water was obtained from a local source for all experiments. Kenaf samples from Oklahoma (KOK) were used in all three retting environments denoted as C1D4-KOK, C2D4-KOK, and E1D4-KOK constituting the first set of experiments. A second set of experiments was performed using the kenaf samples from Mississippi (KMS) only using the E1D4 and C1D4 retting environments, but were performed as biological replicates indicated by the abbreviation B1 and B2: E1D4-KMS-B1, E1D4-KMS-B2, C1D4-KMS-B1, and C1D4-KMS-B2.

The three bacteria used to inoculate the experimental treatments (i.e., E1D4-KOK, E1D4-KMS-B1, and E1D4-KMS-B2) were isolated as described above. Fresh media was inoculated from −80 °C freezer stocks and grown overnight in 250 ml of lysogeny broth [10] in 500-ml Erlenmeyer flasks shaking at 37 °C. Cell cultures (50 ml) were pelleted by centrifugation, suspended in milliQ water (18 MΩ), and used to inoculate the appropriate experimental tanks.

Extraction of DNA from plant-associated micro flora

C1D4-KOK, C2D4-KOK, and E1D4-KOK had plant material removed at day 4 and the general extraction protocol is shown in Online Resource 3. Additional experiments were performed with C1D4 and E1D4 conditions following the same protocol, but using KMS kenaf samples. Retting tanks were set up as duplicates—E1D4-KMS-B1 and E1D4-KMS-B2; C1D4-KMS-B1 and C1D4-KMS-B2. Samples of bark fiber were removed at day 4 (96 h), rinsed in milliQ water, and used for direct DNA extraction from adherent microbial species by bead beating in suspension buffer using the Mo-BIO fecal DNA kit and Fisher Vortex Mixer, as per the manufacturer's instructions. After extraction, DNA samples were quantified by spectrophotometry, with a Nanodrop 1,000 (Thermo Fisher Scientific Inc., Waltham, MA, USA) and used for downstream processes. PCR reactions were set up using the following parameters: 10 µl of 5× HF Buffer, 200 µM of dNTPs, 250 nM of 27Fwd, 250 nM of 1492Rev, 1.5 µl of DMSO, 0.5 µl of Phusion DNA polymerase (NEB), 20 ng of gDNA, and was brought up to a final volume of 50 µl. A ~1.5-kb fragment was amplified from each of the respective sources using the following thermal cycler protocol: initial denaturation at 98 °C for 5 min, denaturation at 98 °C for 15 s, annealing of primers at 56 °C for 30 s, extension of fragments at 72 °C for 1 min, for 25 cycles, and a final extension at 72 °C for 5 min. After confirmation on a 1 % agarose gel, the PCR product was cleaned using Agencourt Ampure XP beads (Beckman Coulter Genomics, Danvers, MA) and quantified on a Nanodrop 1000. The diluted, cleaned product served as the template for the second Ion Torrent-specific nested 16S PCR amplification using IonA-E786Fwd and IonP1-E989Rev adapted from [6]. PCR reactions of 50 µl were set up using the following parameters: 10 µl of 5× HF Buffer, 200 µM of dNTPs, 250 nM of IonA-E786 Fwd, 250 nM of IonP1-E989Rev, 1.5 µl of DMSO, 0.5 µl of Phusion DNA polymerase (NEB), 20 ng full-length 16S DNA, and was brought up to a final volume of 50 µl. Ion Torrent specific primers produced a 210-bp band using the following two-step PCR thermal cycler protocol: 98 °C initial denaturation for 3 min, 98 °C denaturation for 15 s, 61 °C annealing and extension for 15 s, and repeated for 25 cycles, with a final extension for 5 min. The resultant PCR products were purified using Agencourt Ampure XP beads (Beckman Coulter Genomics, Danvers, MA) following the

Table 1 Retting conditions

Retting environment	Amount of water (MilliQ)	Pond water (autoclaved = A, not autoclaved = N)	Bacterial inoculum	Amount of plant material (g)
C1D4	750 ml	250 ml A	N/A	20
C2D4	750 ml	250 ml N	N/A	20
E1D4	750 ml	250 ml A	Yes[a]	20

[a] Inoculum consisting of 50 ml of each fresh culture pelleted by centrifugation and suspended in 50 ml of MilliQ water: *Bacillus* DP1, *Paenibacillus* DP2, *Bacillus* K1 as described in the text

protocol as outlined by Ion Torrent, and quantified on a Nanodrop 1000. Product quality was confirmed on an Experion Automated Electrophoresis System (Bio-Rad, Hercules, CA, USA).

For samples E1D4-KMS-B1, E1D4-KMS-B2, C1D4-KMS-B1, and C1D4-KMS-B2, we used custom barcoded forward primers as shown in Table 2. The PCR protocol remained the same as the previous non-barcoded primers (i.e., IonA-E786Fwd). Specific extraction, replications, and technical replicates are shown in Fig. 1.

Cleaned PCR products were diluted to the appropriate nanomolar concentration (8.4). Samples were amplified by emulsion PCR using the Ion Torrent OneTouch System per the manufacturer's instructions. The resultant beads were

Table 2 Primers used in this study

Primers	Sequences
27F	AGAGTTTGATCMTGGCTCAG
1492R	ACCTTGTTACGACTT
IonA-E786F	**CCATCTCATCCCTGCGTGTCTCCGACTCAG**GATTAGATACCCTGGTAG
IonAs1-E786F	**CCATCTCATCCCTGCGTGTCTCCGACTCAG**CTGATTAGATACCCTGGTAG
IonAs2-E786F	**CCATCTCATCCCTGCGTGTCTCCGACTCAG**TAGATTAGATACCCTGGTAG
IonAs3-E786F	**CCATCTCATCCCTGCGTGTCTCCGACTCAG**AAGATTAGATACCCTGGTAG
IonAs4-E786F	**CCATCTCATCCCTGCGTGTCTCCGACTCAG**CAGATTAGATACCCTGGTAG
IonP1-E989Rev	**CCTCTCTATGGGCAGTCGGTGAT**CTTGTGCGGGCCCCCGTCAATTC

Universal primers 27F and 1492R used for full-length 16S amplification. "Ion" refers to the Ion Torrent-specific nested PCR primers. *Bolded sequences* are the Ion Torrent specific adapter sequences. As1-4 denotes the forward barcoded 786 primer and the underlined portion are the actual barcode sequences. IonP1-E989Rev was used in all nested PCRs

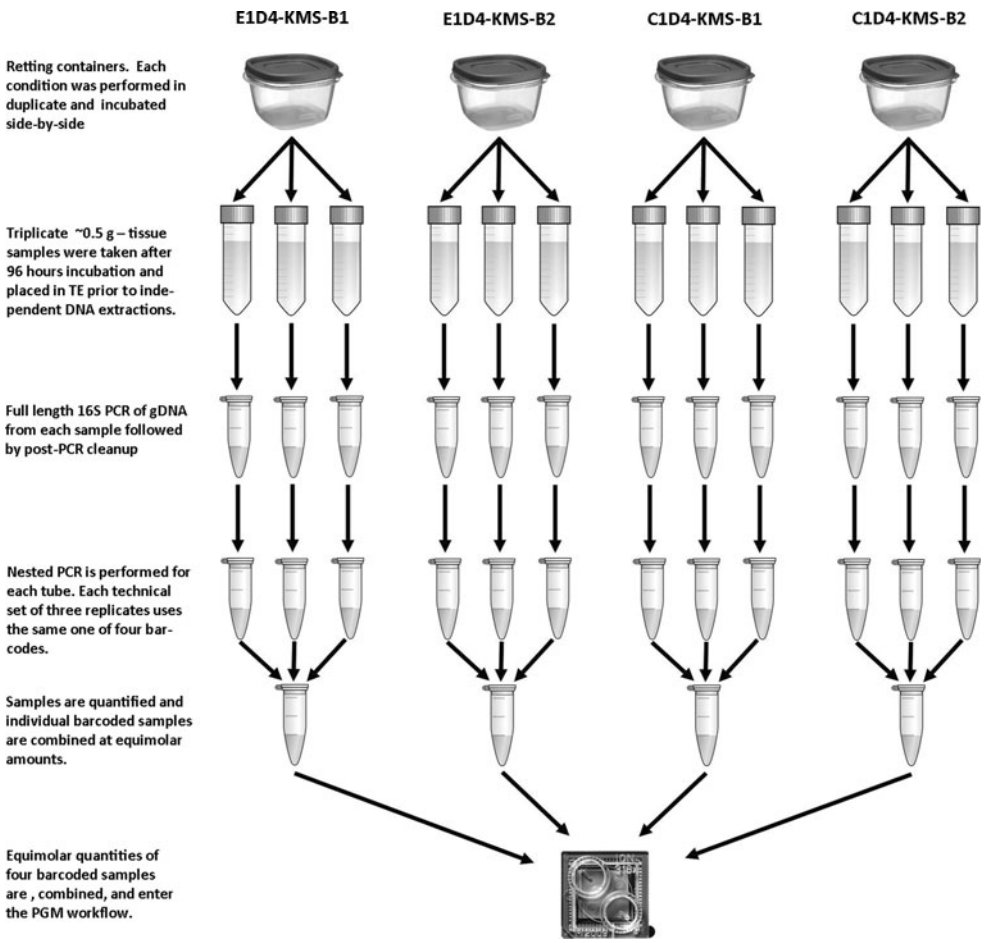

Fig. 1 Work flow of batch replicates of E1D4-KMS and C1D4-KMS showing barcoded samples prepared for the Ion Torrent Workflow. Each set was performed in triplicate and barcoded samples were pooled at the end prior to sequencing

enriched on the Ion Torrent ES prior to loading on to model 314, 10-Mb sequencing chips. This process was performed independently for each condition.

Data analysis

SFF files were converted to FASTA and quality files using Galaxy [12]. The resulting FASTA and quality files were then inserted into RDP Pyrosequencing Pipeline using Pipeline Initial Process (http://pyro.cme.msu.edu/) [15]. This program removed the forward and reverse primers from each of the sequence fragments, any sequences under 100 base pairs, and any sequences with ambiguous nucleotides (N). This quality control ensured that only sequences that had the correctly sequenced primer, both forward and reverse were included in the downstream process. The processed FASTA files were placed into RDP Classifier and set at 50 % confidence [14]. Sequences obtained during this study were deposited in the MG-RAST server under the accession numbers: 4513835.3, 4513836.3, 4513837.3, 4513838.3, 4513839.3, 4513840.3, and 4513841.3 [26].

Rarefaction curves

Sequences were aligned using Infernal aligner [30]. Aligned sequences from each retting condition were generated with their individual cluster files based on the RDP pyrosequencing pipeline. The cluster files produced were used to generate rarefaction curves that defined the number of operational taxonomic units (OTUs) at 97 % similarity level with respect to the total number of reads for each sample. Regression analysis was conducted using SigmaPlot to fit the rarefaction curves into double rectangular hyperbola curve models. On the basis of the regression curves, the number of OTUs (97 % similarity) identified based on 28,000 sequences were noted for comparison of microbial richness.

16S TA clone library of experiment day 4

PCR was repeated using the extract gDNA from experiment day 4 (E1D4-KOK). Using PuReTaq Read-To-Go PCR beads (GE Healthcare, Buckinghamshire, UK) reactions were set up using the following parameters: 250 nM of 27Fwd (Table 2), 250 nM of 1492Rev (Table 2), 20 ng of E1D4-KOK gDNA, was brought up to a final volume of 25 µl, and the following thermal cycler protocol: initial denaturation at 95 °C for 10 min, denaturation at 95 °C for 15 s, annealing at 56 °C for 30 s, extension at 72 °C for 90 s, for 30 cycles, and a final extension at 72 °C for 10 min. TA cloning was performed following instructions as outlined by Invitrogen. Colonies were picked from LB agar with 50 µg/ml kanamycin plates and grown in 10 ml LB with 50 µg/ml kanamycin. Cells were harvested by centrifugation and plasmids were extracted from each pellet using the 5 Prime FastPlasmid Mini Kit (5 Prime, Gaithersburg, MD). The DNA was quantified as described and sequenced by Eurofins MWG Operon. Sequences were identified by BLAST [4], aligned using MEGA [43], and placed into a maximum likelihood phylogenetic tree for comparison.

Results

Data from the Ion Torrent

Data yield from each of the runs ranged from 278,626 to 591,627 sequences pre-quality control. The highest run was the barcoded sequences including E1D4-B1-KMS through C1D4-B2-KMS. After applying RDP Pyrosequencing initial processor sequences were dropped for either having incorrect forward and reverse primers, having ambiguous nucleotides, being under the 100-bp cut-off, or any combination of the previous, which resulted in a range from 28,549 to 148,850 sequences (110 ± 1.86 to 113 ± 0.73).

Microbial diversity of different retting environments

The microbial communities in all three retting environments were dominated by the domain Bacteria. Three predominant phyla across all samples were the Firmicutes, Proteobacteria, and Bacteroidetes and were comprised of the following orders: Aeromonadales, Bacillales, Bacteroidales, Burkholderiales, Clostridiales, Enterobacteriales, Lactobacillales, Pseudomonadales, Rhodocyclales, Sphingobacteriales, Selenomonadales, Sphingomonadales, and Xanthomonadales. However, the predominant organisms present in most of the retting environments were represented by the orders Clostridiales, Enterobacteriales, Bacilliales, and Bacteriodetes.

"Natural" microbial retting: C2D4

C2D4-KOK was performed to characterize a "natural" microbial retting community. As expected, this pond water inoculum resulted in an environment with markedly increased diversity as compared to the other retting experiments. Phylum Bacteroidetes dominated this environment at 59 % (Fig. 2). This discrepancy was clearer at the order level where Bacteroidales comprised 58 % of the population, while the next closest retting environment C1D4-KOK had only 8 %, and none (or <0.5 %) being detected in the others. Clostridiales (26 %) and Enterobacteriales (7 %) were also present, but at substantially lower amounts as compared to the other environments (Fig. 3).

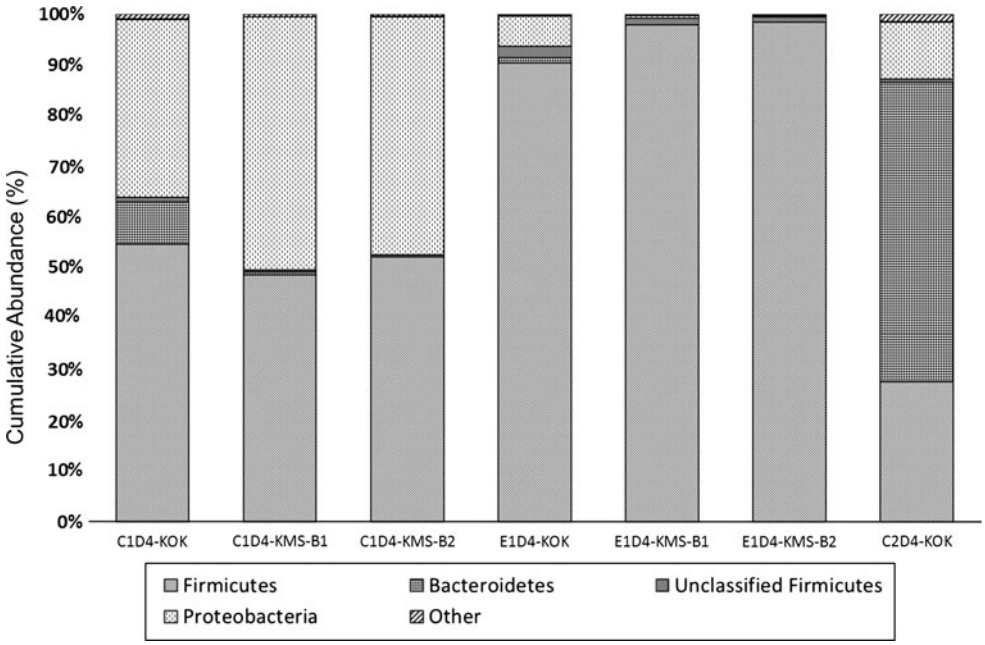

Fig. 2 Percent composition of all microbial retting environments at the phylum level. Most samples were dominated by Firmicutes except C2D4-KOK, which showed a predominance of Bacteroidetes

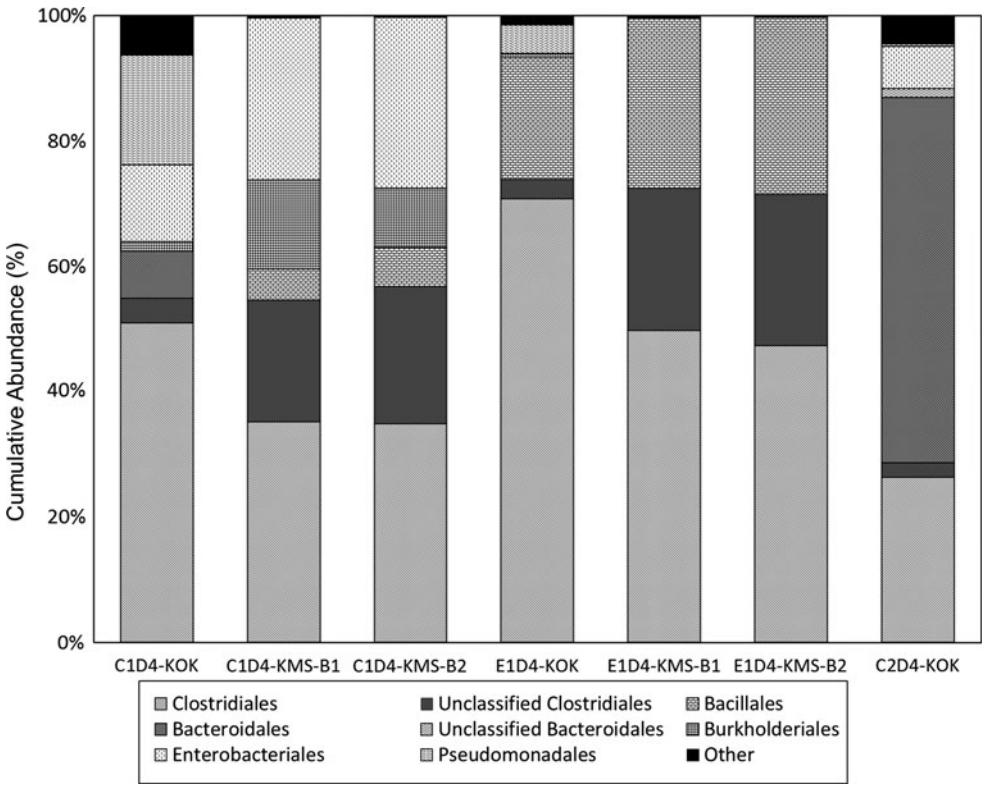

Fig. 3 Phylogenetic compositions of all retting environments at the order taxonomic level. The phylum Firmicutes is further subdivided and shows the similarities occurring between all of the E1D4 samples (KOK and KMS). Orders representing under 0.5 % of the total population were lumped into the category "other"

Composition of the plant-associated microbial flora—C1D4

All of the C1D4 experiments containing autoclaved pond water (i.e., C1D4-KOK, C1D4-KMS-B1, and C1D4-KMS-B2) had large amounts of Firmicutes: 55, 49, and 52 %, respectively, with the remainders being predominately composed of the Proteobacteria at 35, 50, and 47 % (Fig. 2). C1D4-KOK had large amounts of the order Clostridiales at 51 %, but showed additional diversity not found in the E1D4-KOK set: 18 % Pseudomonadales, 12 % Enterobacteriales, and 8 % Bacteroidales, among others (Fig. 3). The KMS samples of C1D4 had the same hierarchical order found in C1D4-KOK, but showed slight

changes in terms of Clostridiales (35 % for KMS samples), Enterobacteriales (26 and 27 %), unclassified Clostridiales (19 and 22 %), Burkholderiales (14 and 9 %), and finally one taxonomic order Bacillales (5 and 6 %) that was not present in the C1D4-KOK sample.

Augmented microbial retting with pectinolytic isolates: E1D4

E1D4 was performed to determine the effect of a large initial inoculum of pectinolytic organisms on retting efficiency as well as community structure and composition. In addition to 250 ml of autoclaved pond water, E1D4 contained 750 ml of MilliQ water and the three pectinolytic organisms: *Bacillus* DP1, *Paenibacillus* DP2, and *Bacillus* K1. All sets of E1D4 were dominated by the phylum Firmicutes ranging from 91 to 99 % of total diversity (Fig. 2). Slight changes in composition were noted and were likely due to the different origins, treatments of the plants, and fresh versus stored (Oklahoma and Mississippi). E1D4-KOK had 91 % composition Firmicutes, which were further sub-divided at the order level into Clostridiales (71 %), Bacillales (20 %), Pseudomonadales (4 %), and unclassified Clostridiales (3 %). The other set that included replicates of the plant samples from Mississippi, E1D4-KMS-B1 and E1D4-KMS-B2, had Clostridiales at 50 and 47 %, unclassified Clostridiales at 23 and 24 %, and Bacillales at 27 and 28 %, respectively (Fig. 3).

Environment-related differences in microbial richness

Microbial richness was defined based on the number of operational taxonomic units (OTUs at 97 % sequence similarity) identified in each of the different retting solutions. Sequences from each sample were subjected to alignment and then complete linkage clustering using a max distance of 3 % and placed into rarefaction curves (Online Resource 4). Additionally, the values from the rarefaction curves were placed into a double rectangular hyperbola curve model and calculated at 28,000 sequences (Online Resource 5). OTUs ranged from 832 to 2,683. Additionally, the KOK samples, i.e., Oklahoma samples, had significantly higher number of OTUs are compared to the KMS samples (independent t test, $p = 0.006$). C2D4-KOK had almost 2.5-fold higher microbial richness than found in any of the KMS samples.

TA clone library

Twenty clones generated from a near-full-length 16S clone library representing E1D4-KOK were prepared and sequenced. Sequences were analyzed using BLAST, and the full-length sequence of the closest match was used to generate a maximum likelihood phylogenetic tree (Fig. 4). Sixteen of the nineteen successful sequences were found to cluster within the genus *Clostridium*. Among these, four were identified as likely *C. beijerinckii,* and three most closely matching *Clostridium* sp. Uslt101-1. These anaerobic bacteria are notable for their ability to fix nitrogen [27]. Also present were members of the genera *Pseudomonas* and *Acinetobacter* (2 and 1, respectively).

Discussion

Understanding the microbial community in the retting environment is an essential step in improving retting process efficiency. While the crude process has been performed for thousands of years, molecular techniques have only recently been applied to understanding the microbial community involved. Selection of day four in the retting solutions was based on previous experiments that showed significant breakdown of pectin and lignin surrounding the fibers. Additionally, this was sufficient time to allow the solution to become anaerobic. A small number of studies have used Sanger sequencing of 16S clone libraries generated from plant retting solutions to explore aspects of retting microbial communities, but next-generation sequencing of 16S amplicons has not previously been reported.

The workflow here employed a nested PCR approach. This was adopted to reduce production of spurious bands resulting from direct amplification with the adapter-fused primers. Results described elsewhere suggest that PCR bias

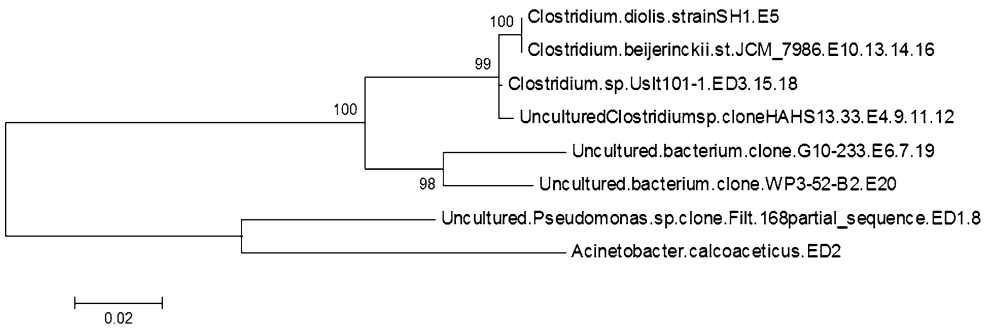

Fig. 4 Phylogenetic tree of closest matches to near-full-length 16S TA clone sequences. Sequences were aligned used MUSCLE in MEGA5 and then inserted into a bootstrapped phylogenetic tree using maximum-likelihood

introduced using the approach as described here is minimal [45]. However, it should be noted that this approach is subject to increased biases resulting from primer selectivity of a second primer set. For example, while long used in microbial community analysis, primers targeting the 8–27 bp region (*E. coli* numbering) of the 16S gene are known to exclude some groups of bacteria (e.g., TM7, Verrumicrobia) [9, 23]. As Ion Torrent amplicon length has increased, new variable regions of the 16S gene and "universal" primer sets have become available that may be more amenable to direct amplification and obviate the need for a nested approach.

Three basic retting experiments were initially conducted. C2D4 was performed to mimic a natural retting microbial process, including contributions from the plant material and pond water. Pond water was chosen as an "inoculum" due to its inherently complex nature and the presence of endogenous plant degraders. C1D4 with autoclaved pond water was performed to assess the microbial community on the plant fibers themselves and estimate their contribution to the retting process. Finally, E1D4 was performed to determine the extent to which the pectinolytic isolates could become established, their influence on the bacterial community structure and transition, and their influence on the retting rate.

The second set of the C1D4 experiments, i.e., KMS, had very similar compositions, but the overall microbial communities as compared to the original KOK experiments remained relatively unchanged, i.e., the major constituents are present, independent of the differences in source location and treatment. C2D4-KOK was the only set that used fresh plant material and fresh pond water, which most closely represented a "traditional" microbial retting environment and had the highest microbial richness, 2,683 OTUs (Online Resource 5). The microbial community was found to be markedly different from the experimental inoculation E1D4 and the autoclaved pond water C1D4 in both composition and overall diversity.

The order Clostridiales was found to be a major component across all retting environments except C2D4-KOK, which had a lesser percentage (Fig. 3). Members of this group have been found to produce numerous pectinases [36, 39, 41], which are favorable to the microbial retting process. Another large constituent in the process was the order Bacteroidales, but this group was only found in the C2D4-KOK samples. Specifically, the strongest matches indicate the predominance of members of the genus *Prevotella* (53.5 % of predicted genera), which has not typically been described as being involved in microbial retting process. However, members of this genus have been found to produce pectinases and identified as members of the ruminant gut [20, 21]. Further investigation of this group with respect to its retting capacity may be of future interest.

The phylum Firmicutes dominated samples C1D4 and E1D4 including both sets of plant materials KOK and KMS. When comparing the C1D4 and E1D4 samples, while order Clostridiales is a large component of both, in all of the E1D4 experiments the percentage of Firmicutes increased by a large margin (91–98 %) as compared to the C1D4 (KOK and KMS) samples (49–55 %). As described above, the Firmicutes are prolific pectinase producers and many have been isolated based on this property. These results were surprising given that the only difference between C1D4 and E1D4 was the addition of the isolates *Bacillus* DP1, *Paenibacillus* DP2, and *Bacillus* K1.

The typical retting solution starts as an aerobic environment, which is favorable to aerobes or facultative anaerobes like *Bacillus* and *Paenibacillus*, respectively [42]. However, as metabolism proceeds, anaerobic conditions are established. This process has been characterized in other retting experiments, which showed a later colonization by *Clostridium* by the shift from initial aerobic organisms, specifically *Bacillus licheniformis* and *B. subtilis*, to anaerobic, *Clostridium acetobutylicum* and *C. felsineum* [18]. This was confirmed by the presence of large amounts of Clostridiales in the E1D4 and C1D4 sets and to a lesser extent C2D4-KOK.

The order Bacillales (Fig. 3) represented a large amount of the diversity in the E1D4 retting environments. Typically with short-length sequences there is a loss of accuracy when going lower in the taxonomic arrangement (i.e., more sequences start being unclassified), however, looking at this specific order, i.e., Bacillalles at a more specific taxon most of the organisms are assigned to the genus *Paenibacillus*. This leads to the conclusion that this is one of our starting isolates used to inoculate the retting environment, i.e., *Paenibacillus* DP2 since it was not present in the others, i.e., C1D4 and C2D4. *Paenibacillus* has been a promising organism in the field of microbial retting due to the fact that numerous isolates have been found to produce varying kinds of pectinases showing activity against highly methylated pectin [13] including a pectate lyase [38] and other hydrolytic enzymes [31]. Additionally, *Paenibacillus* has been shown to produce antibiotics, which may have led to the changes in the microflora of the different microbial retting environments [22].

In a confirmatory experiment, a small clone library of near-full-length 16S fragments was generated and 20 random clones were sequenced for higher-level identification and as a quality control check for our methodology. The results identified an especially interesting group of organisms that clustered within the group of nitrogen-fixers related to *Clostridium* sp. Uslt101-1. This group of nitrogen-fixing bacteria was previously described by [27]. They reported that as oxygen levels decreased, the anaerobic nitrogen-fixing consortia (ANFICOs) were able to establish

and begin fixing nitrogen. Nondiazotrophic bacteria found in their experiment were *Bacillus* sp. The addition of *Bacillus* and *Paenibacillus* might have selected or supported the growth of ANFICOs in the retting solution, thus explaining why the experimental retting communities of E1D4 were so different from C1D4 to C2D4. The identification of this group and their apparent dominance in the experimental reaction, which lacked the addition of the highly eutrophic pond water (autoclaved), suggests that this retting solution was nitrogen limited. If so, this finding suggests that manipulation of nitrogen levels could serve as a primary mechanism to control and manipulate the microbial community structures during the retting process. This conclusion is consistent with the findings of Banik et al. [7] who showed that the retting process could be accelerated by the addition of nitrogen. The results further suggest that this group of bacteria is naturally associated with kenaf, either as surface-associated or potentially as endosymbionts. Given their apparent importance during retting, this line of research warrants further investigation.

Additionally, the KMS plant sets were also used to investigate the impact of PCR-based error associated with random changes within the PCR reactions, which included the biological replicates as indicated by the abbreviation B1 and B2. The general outline of our experiments is shown in Fig. 3. E1D4-KMS-B1 and E1D4-KMS-B2 were biological replicates as were C1D4-KMS-B1 and C1D4-KMS-B2. Each retting container had three pseudo-replicates removed, DNA extracted, PCR amplification of the full-length 16S, nested PCR with barcodes for each biological replicate, quantification, and final pooling for insertion into the Ion Torrent workflow. When looking at the phylogenetic diversity at the phylum level, we see a distinct similarity between the two individual biological replicates for each group, (i.e., E1D4-KMS and C1D4-KMS) (Figs. 2, 3). The results indicate that PCR-based error was effectively minimized in the process, which we found to be robust among the major group of bacteria. However, it should be noted that PCR-based *bias* was not expressly investigated here. Additionally, when comparing the original KOK samples with E1D4, there is still the large presence of the Firmicutes leading to the conclusion that regardless of the natural flora on the plant or its treatment, the detected community structure remains highly similar.

For the production of high-value, green composite materials, what is ultimately required is the cost-efficient production of fibers with consistent, uniform properties. These separated fibers can be used in downstream applications such as composites. Composites in this context require fibers be embedded in a resin. Each resin has its own properties, and therefore reacts differently with different fibers [47]. Future work will investigate if different microbes can be used to generate fibers with modified properties (e.g., surface hydrophobicity) that can be tuned for specific resins and/or applications. Inclusion of bio-based resins made from polylactic acid or polyhydroxyalkanoates would result in completely green and biodegradable composites for a number of applications [33]. Current efforts on river water retted kenaf in these polymers have been reported.

In short, we performed a 16S study on the microbial community of different retting environments using the next-generation sequencing platform Ion Torrent PGM. The work showed markedly different constituents from the different microbial retting conditions. C1D4 likely represented a community based on the microbes inherently present on the plant material, while C2D4 inoculated with pond water may reflect the constituents in a "natural" microbial retting environment. E1D4 showed how well the basal community responds to an inoculum of pectinolytic bacteria. We expected differences to occur when using different sources of plant materials given the number of different variables in their pre-treatment (i.e., growth, harvest time, location of growth, age, etc.). Surprisingly, the results were strikingly similar, suggesting that the *Clostridium* spp. in question may be more closely associated with the kenaf plant than simple surface contaminants. With a better understanding of the microbial diversity found in the different retting environments, future studies will seek to understand more about the roles of bacterial groups in the breakdown of specific heteropolysaccharides through metagenomic and metatranscriptomic studies. These studies could help facilitate discoveries of novel heteropolysaccharases that could further decrease retting time or produce fibers with different properties.

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