

Article

Effects of Pectinase on Bacterial Succession during Hemp Retting

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Abstract: Pectinase accelerates hemp retting. An elevated temperature during the enzymatic action is favorable for bacterial colonization. Industrial hemp (*Cannabis sativa* L.) bast fiber was retted in a 40 °C water bath under four different conditions: water retting, pectinase retting, bacterial retting, and bacterial retting with the presence of pectinase. Bacterial communities were sampled from the retting liquid of each condition at the beginning of retting and on days 1, 3, and 5. The bacterial successions were identified by 16S rRNA gene metagenomic sequencing. The results showed that Bacillaceae dominated the hemp retting conditions containing 1% (*m/v*) pectinase, suggesting that pectinase can manipulate the bacterial community succession by changing the nutrients available to bacteria through the breakdown of pectin. Micromorphological analysis also observed the degradation of a gum-like substance and the aggregation of bacteria with the addition of pectinase.

Keywords: hemp; retting; next-generation sequencing; bacterial succession

1. Introduction



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Natural fibers, which are renewable and sustainable, have become potential alternatives to petroleum-based fibers in the field of automotive components and composites. Interest in using industrial hemp (*Cannabis sativa* L.) bast fiber has been increasing in the USA since the 2018 Farm Bill legalized the commercial production of hemp. The USDA National Agricultural Statistics Service released its first National Hemp Report in 2022 [1] (USDA, 2022), indicating that hemp has been explored as a potential cash crop in many states of the United States. While the cultivation of industrial hemp has increased, the potential of hemp fiber in the development of a green biomass-based economy has not been widely explored due to decades of prohibition.

Retting is an essential process for fiber extraction. It is a process used to separate the bast fibers from the core, or to obtain clean lignocellulosic fibers from raw biomass [2,3]. The fiber quality is highly related to the retting process. The main components of hemp bast are cellulose (53–91%), hemicellulose (4–18%), lignin (1–21%), and pectin (1–17%) [4]. The retting process loosens the cellulosic fibers and fiber bundles from pectin and other cementing compounds, such as hemicellulose and lignin, thereby separating the fibers from the non-cellulosic material. It has been a challenge to efficiently produce long and high-quality fibers [5]. Water, dew, chemical, physical, and biological retting are the major types of retting processes [6]. Dew and water retting are the two traditional types of retting, which usually take several weeks. Chemical retting is relatively controllable in terms of the fiber quality; however, it is high-cost and creates water pollution and other environmental issues [7,8]. Physical retting, such as thermal mechanical retting, can separate a large amount of fiber with a fiber refiner in a short time. This method uses hot steam, high pressure, ultrasound, as well as mechanical pulping to extract lignocellulosic fiber from woody biomass. Biological retting utilizes certain microorganisms, such as bacteria or

fungi and/or their products (enzymes), to break down the non-fiber components that hold the cellulosic fiber together. The common approach for bacterial retting is that bacteria are cultured in a well-controlled laboratory environment and then inoculated into the fiber retting process. In this retting process, the inoculated bacteria may not be optimal to the biomass type being retted. The inoculated bacteria species may not survive in an environment where the microbial communities are complex, especially in industrial applications. The advantages of enzymatic retting are offset by the additional costs and requirements of incorporating enzymes into the production environment [9]. Over the past few decades, research on microbial communities involved in retting has led to an increasing interest in the more environmentally friendly bacterial retting process.

Studies have been conducted on the isolation of bacteria from retting solutions and the inoculation of specific bacteria into the retting process. Di Candilo et al. [10] found that the anaerobic *Clostridium felsineum* bacterial strains reduced the duration of the water retting process from 12 to 6 days at a temperature of 20 °C. Samples in water containing the bacterial strain also had higher fiber moduli than those retted in plain water. The bacterial retting process is greatly influenced by the temperature, and it has been concluded that the temperature for bacterial growth is between 25 to 35 °C for most bacterial species, which also helps to maintain the fiber quality [11]. Di Candilo et al. [12] introduced an aerobic pectinolytic bacterial strain (*Bacillus* sp. ROO40B) and an anaerobic pectinolytic bacterial strain (*Clostridium* sp. L1/6) into the water retting process of hemp stems. It was found that ROO40B did not show cellulolytic activity. This has an important implication for strain selection since cellulolytic activity has been found in all anaerobic pectinolytic strains so far [12,13].

The relative abundances of different bacterial populations change during the retting process. Several studies on microbial succession during the retting process have demonstrated that microbial communities change depending on the retting conditions. The bacterial succession of water retting begins with the proliferation of aerobic bacteria (often *Bacillus* spp.), and as oxygen is gradually consumed, anaerobic bacteria (e.g., *Clostridium* spp.) begin to multiply [13–15]. The incubation of additional pectinolytic bacteria, such as *Bacillus cereus* HDYM-02, also changed the bacterial succession during retting [16]. Higher enzyme activity in the bacteria-inoculated retting process was observed, as evidenced by the increase in galacturonic acid and reducing sugars in the bacterial retting liquid and the decrease in fiber-associated gum compared with regular water retting. It has also been shown that the addition of specific enzymes can accelerate the release of fiber and shorten the retting time to 24 h [17–19]. The most studied enzyme in the retting process is pectinase, which degrades the non-cellulosic polymer pectin in hemp fibers. Bernava et al. [20] compared the hemp fiber tensile strength after 2, 5, 24, and 48 h of pectinase retting at 25 °C and 50 °C. The maximum tensile strength of the hemp fiber was obtained after 48 h of retting at 25 °C with a 4% pectinase water solution.

The isolation and purification of enzymes is an expensive process; therefore, the cost of enzymatic retting alone is high. It is not economically friendly to apply enzymes to each batch of retting, especially for large-scale industrial applications. Natural fibers inevitably carry bacteria from the natural environment during the planting and harvesting processes. Bacteria can use these carbon-based polymers as their food source. Additionally, retting conditions suitable for enzymatic action are sufficient for bacteria to proliferate. However, little is known about bacterial succession after a small amount of enzyme application during retting. Here, we investigated bacterial communities associated with the retting of hemp bast fibers with or without the addition of the pectinase enzyme, as well as with the addition of residue from a long-term-pectinase-retted hemp core. The objective of this research is to provide a bacterial retting strategy that can be scaled up by enriching a portion of the bacteria with a small amount of pectinase, which does not require culturing the bacteria in a tightly controlled laboratory environment (Figure 1).

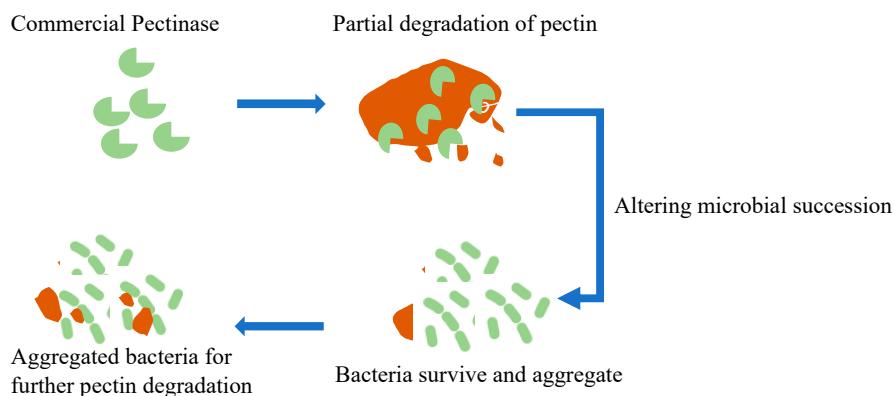


Figure 1. Schematic diagram of the bacterial retting strategy of hemp bast.

2. Materials and Methods

2.1. Sample Preparation and Testing

Industrial hemp (*Cannabis sativa L.*) from Canada was obtained through EcoEnvision, LLC (Frisco, TX, USA). Pectinase (containing pectinase from *Aspergillus niger* and maltodextrin as the carrier) was purchased from LD Carlson. This product is used in commercial winemaking with the advantages of low prices and easy accessibility.

Hemp bast fiber was cut into 1/4-inch (0.635 cm) lengths and retted in a 40 °C water bath using four different retting schedules (R1–R4, Table 1). The experimental plan is graphically shown in Figure 2. The retting solution was collected at the beginning of each retting schedule (day 0) and on the 1st, 3rd, and 5th days during retting and stored at –20 °C for bacterial sequencing analysis (R1d0–R4d5, Table 1). The pH in the retting liquid was monitored using a Mettler Toledo pH meter (SevenCompact™ S210, Mettler-Toledo, LLC, Columbus, OH, USA). Reducing sugar analysis with the dinitrosalicylic acid (DNS) method [21] was conducted during the bacteria enrichment process. Samples were diluted 100 times before testing. 3,5-DNS, potassium sodium tartrate tetrahydrate, and galacturonic acid from Sigma Aldrich were used for the analysis. Micromorphological fiber analysis was conducted on fibers collected randomly from day 1 to day 5 of the four retting schedules. The samples were dried and mounted to the aluminum stub using conductive adhesive and observed under an FEI Quanta 200 Environmental Scanning Electron Microscope (ESEM) with a spot size of 3.0 and an accelerating voltage of 15 kV.

Table 1. Summary of four retting experiments.

Schedule	Description of the Retting Schedule	Sample Labeling			
		Day 0	Day 1	Day 3	Day 5
R1	Hemp fiber (5 g) was retted with tap water (200 mL) only	R1d0	R1d1	R1d3	R1d5
R2	Hemp bast fiber (5 g) was retted with 1% (m/v) pectinase in tap water (200 mL)	R2d0	R2d1	R2d3	R2d5
R3	Hemp bast fiber (5 g) was retted with 1% (m/v) pectinase in tap water (200 mL) and 1 g solid residues from hemp core retting (with cultured bacteria)	R3d0	R3d1	R3d3	R3d5
R4	Hemp bast fiber (5 g) was retted with tap water (200 mL) and 1 g of solid residues from hemp core retting (with cultured bacteria)	R4d0	R4d1	R4d3	R4d5

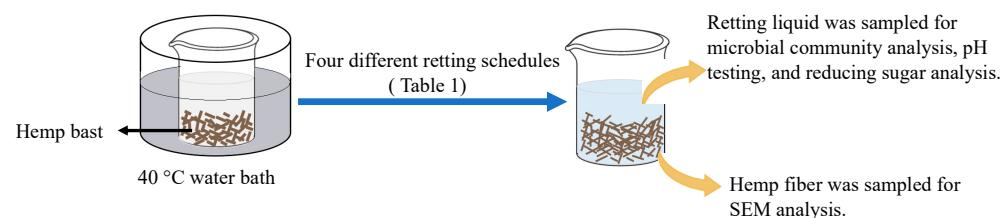


Figure 2. Experimental plan.

Solid residues (with cultured bacteria) from hemp core retting in schedules R3 and R4 were prepared by retting hemp core with 1% pectinase enzyme at ambient laboratory temperature for two months. The cultured bacteria were the bacterial communities developed after inoculation with the hemp core under the conditions described above.

Pectinases from *Aspergillus niger* have activity over a wide range of temperatures (from 20 °C to 70 °C) [22]. The optimum pectinase activity is at 40 °C [23] or 50 °C [24]. The reason for choosing 40 °C here was to preserve the greater activity of pectinase while saving energy. The reason for using pre-cultured bacteria was to test whether the addition of pectinase would change the environment, which already had a dominant bacteria species in a controlled manner.

2.2. Microbial Community Analysis by Illumina MiSeq Sequencing

Microbial cells from 3 mL of the retting solution were harvested by centrifugation for 5 min at 10,000 × g. Genomic DNA was recovered from the pellet using the DNeasy PowerSoil Pro Kit and the automated QIAcube Connect robot (Qiagen, Carlsbad, CA, USA). The 16S rRNA gene was amplified using universal bacterial primers targeting the V4 hypervariable region [25,26]. The PCR reaction contained the following: DNA template (0–100 ng); 0.5 µL of each primer (10 µM); 2.5 µL 10× AccuPrime PCR Buffer II; 2.5 µL BSA (1.6 mg/µL); 1.5 µL MgCl (50 mM); 0.1 µL AccuPrime Taq High Fidelity (5 U/µL); and PCR-grade water to a final volume of 25 µL. PCR amplification was carried out as follows: denaturation at 94 °C for 2 min, 25 cycles of 94 °C for 30 s, 52 °C for 30 s, 68 °C for 40 s, and then 68 °C for 5 min and held at 4 °C. PCR products were viewed using gel electrophoresis (1.5% agarose). Successful PCR amplification products were cleaned using AMPure XP (Beckman Coulter, Chaska, MN, USA) magnetic bead-based purification. After cleanup, the PCR products were indexed using the Illumina Nextera XT Index Kit v2 (Illumina, San Diego, CA, USA) following the manufacturer's instructions, and they were purified again using AMPure XP magnetic beads. Each 50 µL index PCR reaction contained the following: 5 µL 10× AccuPrime PCR Buffer II; 5 µL Nextera XT indexing primers 1; 5 µL Nextera XT indexing primers 2; 0.2 µL AccuPrime Taq High Fidelity (5 U/µL); 5 µL purified DNA; and PCR-grade water. The PCR recipe was as follows: denaturation at 94 °C for 3 min, 8 cycles of 94 °C for 30 s, 55 °C for 30 s, 68 °C for 30 s, and then 68 °C for 5 min and held at 4 °C. PCR products were quantified using the Qubit dsDNA HS Assay Kit (Invitrogen, Carlsbad, CA, USA) and pooled in equimolar amounts. The pooled sample was then denatured, diluted, loaded, and sequenced using the MiSeq Reagent V2 (500 cycles) kit (Illumina Part #15044223, Rev B.) on the Illumina MiSeq sequencing instrument (Illumina, San Diego, CA, USA).

2.3. Bioinformatics Analysis

Sequences generated from the MiSeq System were processed through the Mothur v.1.36.1 pipeline [27]. Paired-end sequences were assembled, primers and barcodes were removed, and short sequences (<100 bp) and low-quality sequences (homopolymers > 8) were excluded from the dataset. Sequence alignments were performed using reference sequences from the SILVA database [28]. The sequences that could not be aligned were removed, and the aligned sequences were further filtered to remove gaps. Redundant sequences were reduced using the Unique.seqs command and a precluster (diffs = 2) algorithm, and chimeras were removed after identification using UCHIME [29]. Taxonomic classification was conducted using the Ribosomal Database Project (RDP) classifier with a minimum confidence of 80% [30], and the up-to-date, curated EzBioCloud database as a reference [31]. Sequences classified as mitochondria, chloroplast, archaea, and eukaryote, as well as unknown sequences, were removed from the dataset. Principle coordinate analysis (PCoA) based on UniFrac distances was used to investigate differences in the microbial communities among the different retting schedules [32].

3. Results and Discussion

The pH change in the liquid during the retting process is shown in Figure 3. The pH value of the retting conditions involving pectinase (schedules R2 and R3) decreased drastically during the retting process. By comparison, the pH of the retting solutions without pectinase increased during the retting processes. The gradual decrease in the pH in the pectinase-amended conditions was most likely associated with the release of acetic acid and galacturonic acid [33]. Galacturonic acid is the main monosaccharide unit of pectin and is an uncommon substrate for microbial fermentations [34], and its release is used to define pectinase activity [35,36]. Pectinases can be broadly classified into pectin esterase, hydrolase, and lyase according to their modes of action [37]. Depending on the optimal pH of the enzyme activity, pectinases can be classified as acidic and alkaline enzymes [38]. It has been observed that pectinases produced by different bacteria or fungi have different optimal pH values. Alqahtani et al. [39] found that isolated *Bacillus subtilis* 15A-B92 produced bacterial pectinase extracellularly, and the pectinase activity was highest in submerged fermentation conditions at pH 4.5. The highest activity of pectinase from *Aspergillus* was found at pH 6.5 [40]. Fahmy et al. [41] reported that the optimal activity of pectinase from *Aspergillus niger* was at pH 5.0. Zhao et al. reported that the pectinase produced by *B. cereus* HDYM-02 retained more than 50% of its activity when the pH was between 4.0 and 7.0 [36]. Previous reports have shown that *Bacillus* species can also produce alkaline pectinases [42,43]. In this study, the application of small amounts of pectinase altered the pH of the retting environment. The succession of bacterial populations was also observed during the retting process. The relationship between pH and the multiplication of certain bacteria in the retting process needs to be further investigated. Pakarinen et al. observed an increase in galacturonic acid in the hydrolysate when pectin was removed from the hemp fiber by adding pectinases [44]. The galacturonic acid then serves as a carbon source for bacteria, facilitating their proliferation. Additionally, certain Firmicutes members use complex carbohydrate metabolism to produce short-chain fatty acids under anaerobic conditions, resulting in further reductions in pH [45,46].

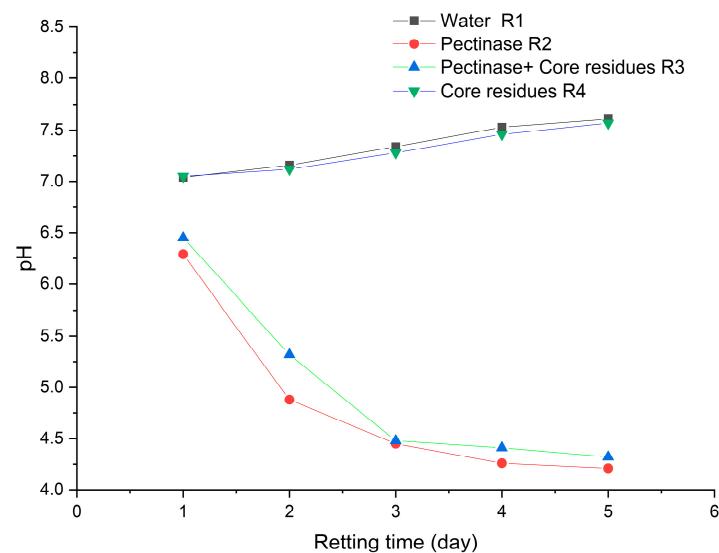


Figure 3. pH changes during four retting processes. The retting conditions with the involvement of pectinase rapidly turn acidic.

In the water retting (schedule R1; see Table 1), the fibers were covered with gummy substances at the beginning of the retting (Figure 4a) and on the fifth day of the retting (Figure 4b). The gummy substances, such as pectin, were not completely broken down or degraded in the short period of time under the water retting condition. By contrast, the fibers were relatively clean under retting schedules R2 (Figure 4c,d) and R3 (Figure 4e,f). Both schedules included added pectinase, which degrades pectin around the fiber bundles;

however, the gummy substances were still visible on parts of the fiber bundle surfaces. After five days of retting, bacteria accumulated on the surfaces of the fiber bundles. The accumulated bacteria started to degrade the remaining gummy substances. At the beginning of retting schedule R4, the fiber bundles were still surrounded by gummy substances because pectinase was not added. Bacterial proliferation on the fiber surface was not observed under ESEM.

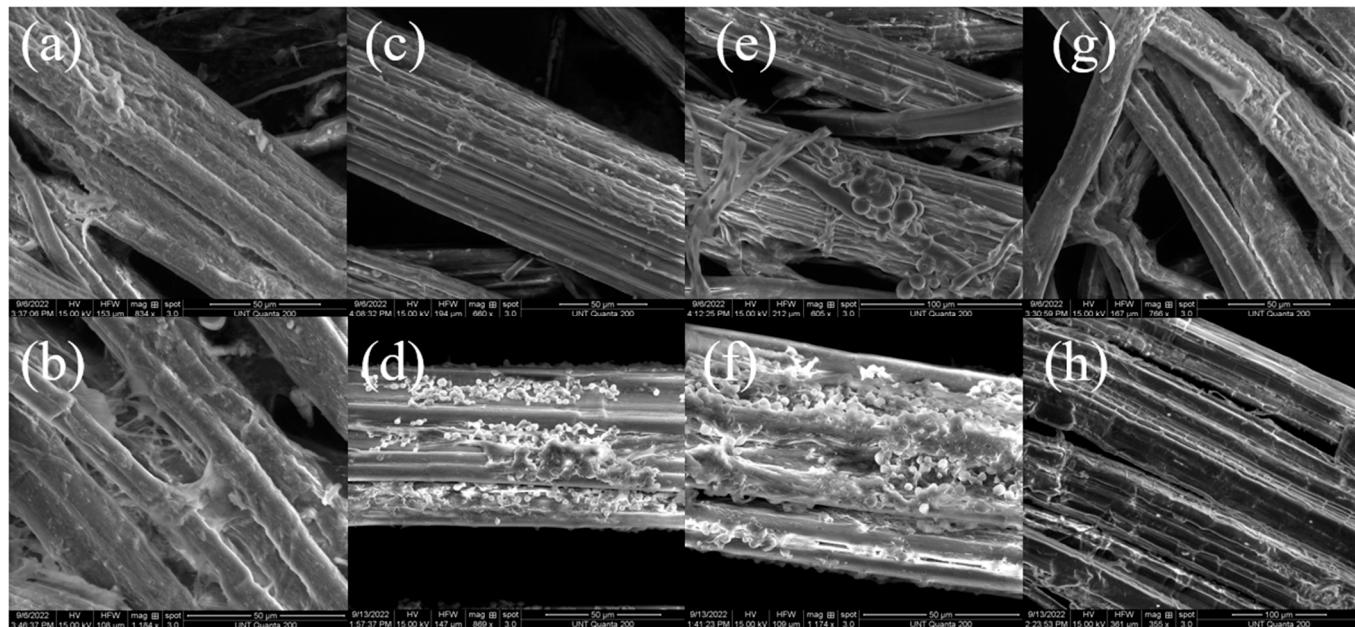


Figure 4. SEM micrographs of hemp fiber sampled from day 1 (**a,c,e,g**) and day 5 (**b,d,f,h**) of four retting schedules (R1–R4), where a and b are from R1, c and d are from R2, e and f are from R3, and g and h are from R4. Fibers were wrapped by gummy substance (**a**), and retting schedule R1 did not remove the gummy substance (**b**). The gummy substance was removed partially (**c,e**) at the beginning of schedules R2 and R3. Bacteria colonized and adhered to the fiber surfaces after 5 days of retting for both R2 and R3 (**d,f**). Bacteria were not enriched on fiber for retting schedule R4 (**g,h**).

The microbial community structure change during hemp retting was explored by principal coordinate analysis (PCoA). The unweighted PCoA results indicated that the hemp samples before retting had similar microbial community compositions (Figure 5a). Microbial communities from the samples retted with pectinase clustered together and those from the samples without pectinase separated, suggesting that pectinase affected the species occurrence in the samples (Figure 5a). Bacterial species in the retting schedule with the pectinase addition on the third and fifth days clustered on the weighted PCoA, indicating that the use of pectinase markedly altered the abundance of the microbiota on the third and fifth days of retting (Figure 5b). The relationship between the retting time and pH showed that pectinase reduced the pH of the retting environment. Neither the water retting (schedule R1) nor the core residue retting (schedule R4) changed the composition of the bacterial community structure during the process.

A total of 2,010,228 sequencing reads were generated ($125,639 \pm 6270$ average per sample). Bacterial relative abundance changes were observed at the phylum level (Figure 6a). A total of 17 bacterial phyla were detected. Five bacterial phyla (Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, and Spirochaetes) were able to be classified to the family level. Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes were the top four bacterial phyla in all the retting conditions, among which Proteobacteria and Firmicutes accounted for $93.13\% \pm 0.093\%$ of the bacteria. Actinobacteria were present in the hemp and at all the initial stages of the hemp retting methods; however, their abundance diminished during the retting process. Pectinase altered the succession of bacterial communities. In

the pectinase-treated retting process, the relative abundance of Firmicutes in schedule R2 increased at the later stages of retting (days 3 and 5) from 2.5% to 89.8% and 93.1%. The difference between the pectinase-treated retting process and non-pectinase-treated retting process was more obvious at the family level, where Rhodospirillaceae (23.7%), Clostridiaceae (14.7%), Sphingobacteriaceae (12.5%), and Ruminococcaceae (9.6%) were the most abundant families present at the late stage of the non-pectinase-treated retting (R1d5), and Bacillaceae (91.4%) was the most abundant family that appeared at the late stage of the pectinase-treated retting (R2d5).

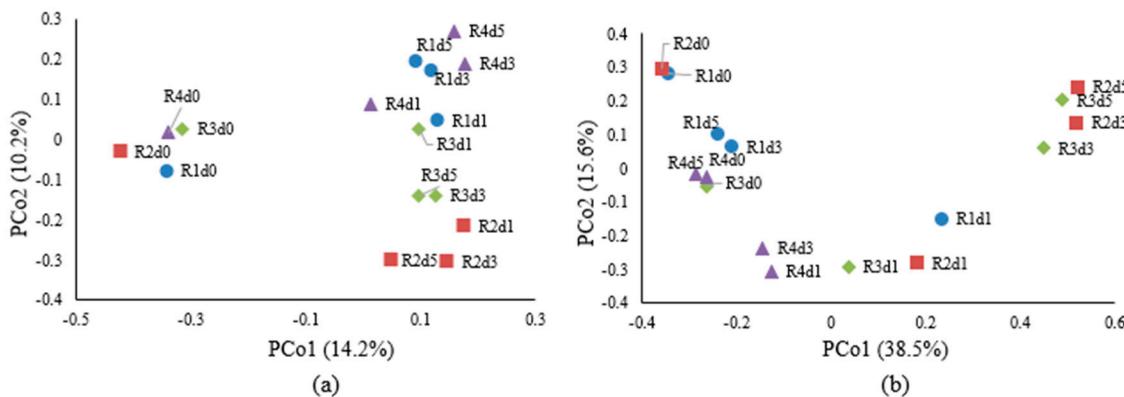


Figure 5. Principal coordinate analysis (PCoA) based on unweighted and weighted UniFrac distances. Unweighted PCoA (a) showed the clustering of bacterial communities at the beginning of retting. The microbial communities showed clustering in the presence and absence of pectinase. Weighted PCoA (b) indicated that the bacterial communities in the presence of pectinase were similar after 3 days of hemp retting. Each point represents a sample.

Ribeiro et al. previously studied the microbial diversity changes during hemp field retting [47]. It was observed that Proteobacteria, Bacteroidetes, Actinobacteria, and Firmicutes were abundant at the phylum level, where Proteobacteria were the most abundant phylum. Proteobacteria, Bacteroidetes, Actinobacteria, and Firmicutes also appeared at the phylum level of the dataset during the simulated six weeks of field hemp retting in the research completed by Law et al. [48]. Results of their research also confirmed that microorganisms were already “on-site” within or on the hemp prior to retting and continued to be present throughout the field retting process, which did not show large shifts in the population over the retting time. Our study also identified the “on-site” bacterial phyla on hemp before retting. However, the bacterial communities were altered markedly in the presence of pectinase. *Proteobacteria* were present in a relatively high abundance (greater than 50%) at the beginning of the retting, but their relative abundance tended to decrease during the R2, R3, and R4 retting processes. This was not the case for the water retting (R1). The relative abundance of Firmicutes increased with the retting time in the R2 and R3 retting schedules with added pectinase. The Bacteroidetes phylum was present at the beginning of each retting method. This bacterial phylum tended to proliferate from the third day of water retting (R1d3) and had a significant abundance on the fifth day of retting schedule R4 (R4d5). Bacteroidetes are known to be anaerobic [49], and anaerobic bacteria are associated with a foul odor during water retting [50]. A significant abundance (greater than 5%) of Actinobacteria in the hemp and at the initial stages of all the retting processes (R1d0, R2d0, R3d0, R4d0) was also detected. However, their relative abundance dropped to less than 0.2% during the rest of the retting process, possibly due to the shift towards anoxic conditions. An evaluation of the hemp microbiome performed by Barnett et al. confirmed that Actinobacteria were in higher relative abundance in the soil, rhizosphere, and root tissue of hemp [51]. These bacterial phyla (Proteobacteria, Bacteroidetes, Actinobacteria, and Firmicutes) have similarly been found in a variety of natural fiber retting conditions, including flax retting [52,53], kenaf retting [15], and jute retting [54,55].

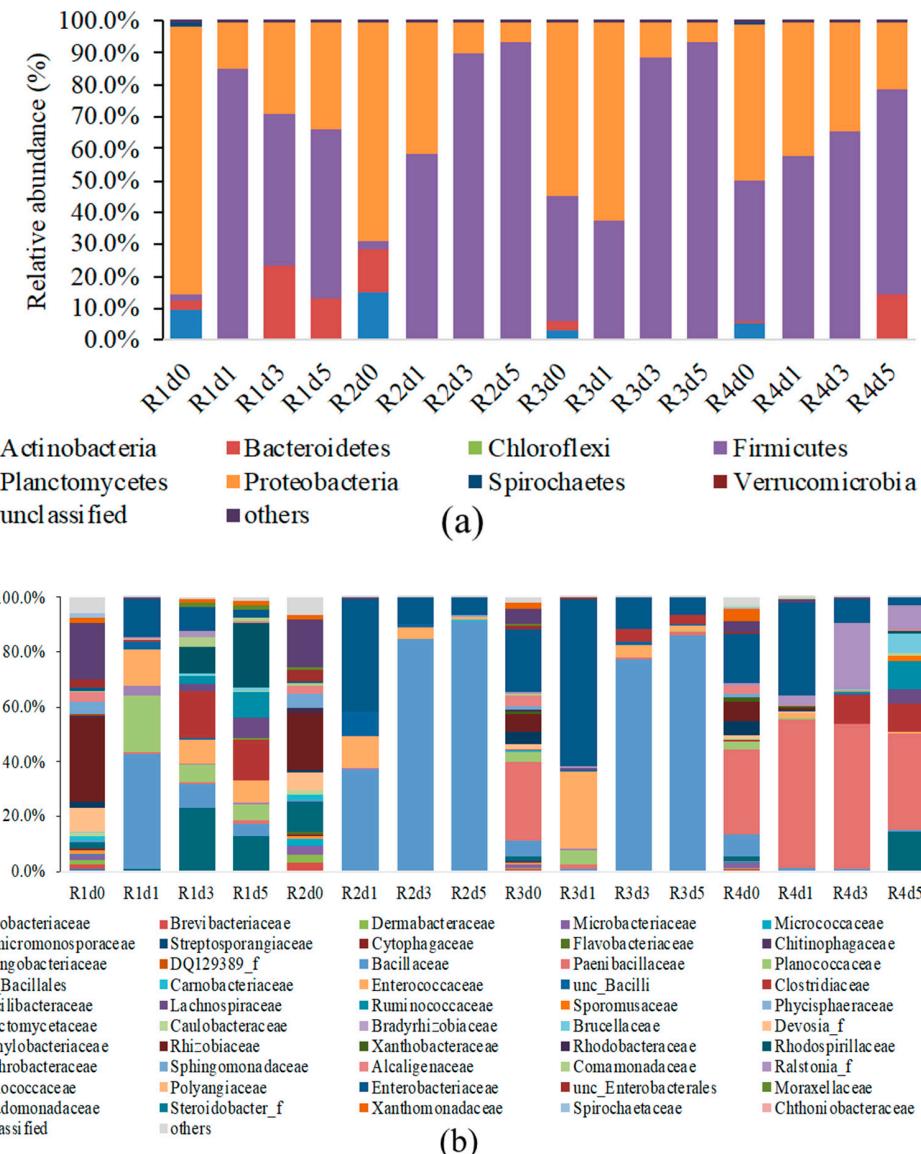


Figure 6. Relative abundances of the phylum-level (a) and family-level (b) bacterial community compositions during retting. Only bacterial phyla with relative abundances >1% and bacterial families with relative abundances >5% are shown.

The relative abundances of the family-level bacterial communities during retting (Figure 6b) revealed more detailed bacterial compositions. It was noted that the relative abundance of Bacillaceae during retting with pectinase (schedule R2) increased with the duration of retting, from 0.3% at the beginning of the retting process to 84.9% and 91.4% on the third and fifth days, respectively. Bacillaceae were not able to be further classified to the genus level. The value of members of the Bacillaceae family in the field of ecological function include involvement in the degradation of soil organic matter, the nitrogen cycle, and the phosphate cycle [56]. Research completed by Zhao et al. showed that flax retting with the addition of the Bacillaceae member *Bacillus cereus* HDYM-02 as the inoculum altered the bacterial succession significantly and effectively accelerated the retting process [16]. They also speculated that the presence of Paenibacillaceae in flax retting inoculated with *B. cereus* HDYM-02 further contributed to the degradation of the gummy pectic substances during the retting process. In our study, Paenibacillaceae were the dominant microorganisms in the retting residues of the hemp cores. The relative abundance of Paenibacillaceae in the retted hemp core was 68.9%, possibly explaining why Paenibacillaceae dominated the

bacterial populations in the retting schedule R4, which contained the cultured bacteria from the retted hemp core. Paenibacillaceae can be classified into the genera *Brevibacillus*, *Paenibacillus*, and *Cohnella*. *Brevibacillus* was identified as the dominant genus in R4. Members of *Brevibacillus* have been shown to produce pectin lyase [57], which suggests that these bacteria may be contributing pectinase enzymes to the retting system.

Retting typically begins as an aerobic process. Aerobes or facultative anaerobes such as *Bacillus* and *Paenibacillus* predominate the initial bacterial communities [58]. Firmicutes, particularly members of the genus *Clostridium*, were reported to be the dominant phylum during the water retting of flax fibers, while the aerobic environment of the dew retting of flax fibers was low in *Clostridium* [52]. The more anaerobic environment of water retting than dew retting was thought to be the primary reason for the difference in the *Clostridium* abundance [14,58], as *Clostridium* species are obligately anaerobic [59]. In this study, Clostridiaceae (including the genus *Clostridium*) were also found in the water retting (Figure 6b, R1d3 and R1d5). On the third and fifth days of retting schedules R2 and R3, the most abundant bacterial communities belonged to the family Bacillaceae. Bacillaceae include facultative anaerobes that can produce acetate from organic matter through anaerobic digestion [60].

The results from the reducing sugar analysis during the bacteria enrichment are shown in Figure 7. The small amount of pectinase (1% w/v) was able to degrade pectin within 24 h. The concentration of reducing sugar within 24 h was slightly higher than that from the water retting. As the Bacillaceae accumulated, the reducing sugar concentration was increased. On the third day, the relative abundance of Bacillaceae exceeded 80%, and the reducing sugar concentration reached its peak. It is worth noting that Bacillaceae were present on the hemp fibers prior to the retting. The enriched bacteria were carried by the fiber. These observations suggest that the addition of pectinase provides a suitable trigger for the enrichment of Bacillaceae. On the third day, the concentration of reducing sugars was the highest and Bacillaceae also dominated the population.

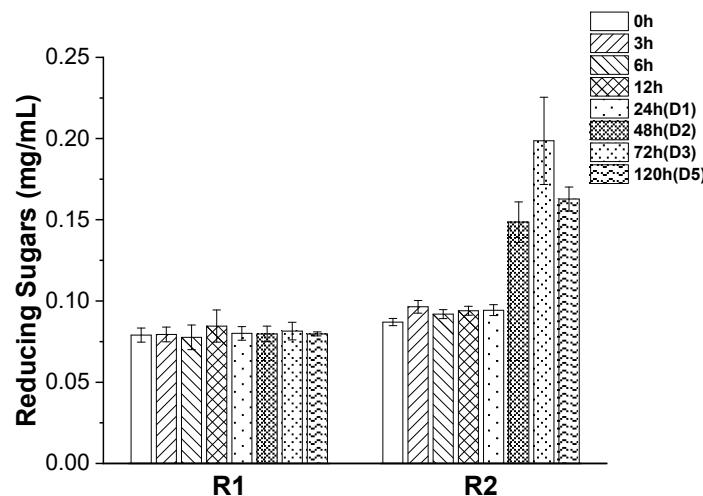


Figure 7. Reducing sugar analysis with DNS method indicated that a small amount of pectinase (1% w/v) could degrade the pectin at the beginning of retting. The reducing sugar concentration within 24 h was slightly higher than that from the water retting. As the Bacillaceae accumulated for 72 h (Day 3), the reducing sugar concentration reached its peak.

4. Conclusions

This study explored the succession of bacteria in the presence of a small amount of pectinase during the retting process of hemp bast fibers. By conducting microbial community analysis and bioinformatics analysis, it was found that using a small amount of pectinase (1% w/v) has a significant effect on the bacterial community succession. The addition of pectinase resulted in an acidic retting environment that was likely enriched for

Bacillaceae during the retting process within three days. On the third day, the reducing sugar concentration reached the highest. After certain pectinase-producing bacteria dominate the communities in the retting system, it may be possible to recycle the Bacillaceae-dominated bacterial retting solution to process the following batches of bacterial fiber retting. Future work will focus on evaluating the removal of pectin and galacturonic acid and analyzing the fungal communities to understand the role of pectinases in microbial aggregation during retting. The effect of bacteria acquired through this aggregation process on retting will be evaluated.

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References

- United States Department of Agriculture. National Hemp Report. 2022. Available online: <https://downloads.usda.library.cornell.edu/usda-esmis/files/gf06h2430/xd07hw825/v692v917t/hempan22.pdf> (accessed on 17 February 2022).
- Liang, K.; Shi, S.Q.; Wang, G. Effect of impregnated inorganic nanoparticles on the properties of the kenaf bast fibers. *Fibers* **2014**, *2*, 242–254. [[CrossRef](#)]
- Xia, C.; Zhang, S.; Shi, S.Q.; Cai, L.; Huang, J. Property enhancement of kenaf fiber reinforced composites by in situ aluminum hydroxide impregnation. *Ind. Crops Prod.* **2016**, *79*, 131–136. [[CrossRef](#)]
- Tulaphol, S.; Sun, Z.; Sathitsuksanoh, N. Chapter six—Biofuels and bioproducts from industrial hemp. *Adv. Bioenergy* **2021**, *6*, 301–338. [[CrossRef](#)]
- Paridah, M.T.; Basher, A.B.; SaifulAzry, S.; Ahmed, Z. Retting process of some bast plant fibres and its effect on fibre quality: A review. *BioResources* **2011**, *6*, 5260–5281.
- Zimniewska, M. Hemp fibre properties and processing target textile: A review. *Materials* **2022**, *15*, 1901. [[CrossRef](#)] [[PubMed](#)]
- Hurren, C.J.; Wang, X.; Dennis, H.G.; Clarke, A.F.K. Evaluation of bast fibre retting systems on hemp. In Proceedings of the 82nd Textile Institute World Conference, Cairo, Egypt, 2002. Available online: <https://hdl.handle.net/10536/DRO/DU:30013875> (accessed on 23 March 2002).
- Hoondal, G.; Tiwari, R.; Tewari, R.; Dahiya, N.; Beg, Q. Microbial alkaline pectinases and their industrial applications: A review. *Appl. Microbiol. Biotechnol.* **2002**, *59*, 409–418. [[CrossRef](#)]
- Foulk, J.A.; Rho, D.; Alcock, M.M.; Ulven, C.A.; Huo, S. Modifications caused by enzyme-retting and their effect on composite performance. *Adv. Mater. Sci. Eng.* **2011**, *2011*, 179023. [[CrossRef](#)]
- Di Candilo, M.; Ranalli, P.; Bozzi, C.; Focher, B.; Mastromei, G. Preliminary results of tests facing with the controlled retting of hemp. *Ind. Crops Prod.* **2000**, *11*, 197–203. [[CrossRef](#)]
- Hossain, M.M.; Siddiquee, S.; Kumar, V. Critical factors for optimum biodegradation of bast fiber's gums in bacterial retting. *Fibers* **2021**, *9*, 52. [[CrossRef](#)]
- Di Candilo, M.; Bonatti, P.M.; Guidetti, C.; Focher, B.; Grippo, C.; Tamburini, E.; Mastromei, G. Effects of selected pectinolytic bacterial strains on water-retting of hemp and fibre properties. *J. Appl. Microbiol.* **2010**, *108*, 194–203. [[CrossRef](#)]
- Tamburini, E.; León, A.G.; Perito, B.; Di Candilo, M.; Mastromei, G. Exploitation of bacterial pectinolytic strains for improvement of hemp water retting. *Euphytica* **2004**, *140*, 47–54. [[CrossRef](#)]
- Donaghy, J.A.; Levett, P.N.; Haylock, R.W. Changes in microbial populations during anaerobic flax retting. *J. Appl. Bacteriol.* **1990**, *69*, 634–641. [[CrossRef](#)]
- Visi, D.K.; D’Souza, N.; Ayre, B.G.; Webber III, C.L.; Allen, M.S. Investigation of the bacterial retting community of kenaf (*Hibiscus cannabinus*) under different conditions using next-generation semiconductor sequencing. *J. Ind. Microbiol. Biotechnol.* **2013**, *40*, 465–475. [[CrossRef](#)]
- Zhao, D.; Liu, P.; Pan, C.; Du, R.; Ping, W.; Ge, J. Bacterial succession and metabolite changes during flax (*Linum usitatissimum* L.) retting with *bacillus cereus* HDYM-02. *Sci. Rep.* **2016**, *6*, 31812. [[CrossRef](#)]
- De Prez, J.; Van Vuure, A.W.; Ivens, J.; Aerts, G.; de Van Voorde, I. Enzymatic treatment of flax for use in composites. *Biotechnol. Rep.* **2018**, *20*, e00294. [[CrossRef](#)] [[PubMed](#)]
- Akin, D.E.; Dodd, R.B.; Perkins, W.; Henriksson, G.; Eriksson, K.L. Spray enzymatic retting: A new method for processing flax fibers. *Text. Res. J.* **2000**, *70*, 486–494. [[CrossRef](#)]

19. Akin, D.E.; Foulk, J.A.; Dodd, R.B.; McAlister, D.D., III. Enzyme-retting of flax and characterization of processed fibers. *J. Biotechnol.* **2001**, *89*, 193–203. [CrossRef]
20. Bernava, A.; Reihmane, S.; Strazds, G. Influence of pectinase enzyme beisol PRO on hemp fibres retting. *Pap. Present. Proc. Est. Acad. Sci.* **2015**, *64*, 77–81. [CrossRef]
21. Miller, G.L. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* **1959**, *31*, 426–428. [CrossRef]
22. Sandri, I.G.; Silveira, M.M.d. Production and application of pectinases from *Aspergillus niger* obtained in solid state cultivation. *Beverages* **2018**, *4*, 48. [CrossRef]
23. Ajayi, A.A.; Lawal, B.; Salubi, A.E.; Onibokun, A.E.; Oniha, M.I.; Ajayi, O.M. Pectinase production by *Aspergillus niger* using pineapple peel pectin and its application in coconut oil extraction. *Pap. Present. IOP Conf. Ser. Earth Environ. Sci.* **2021**, *655*, 012014. [CrossRef]
24. Jalil, M.T.M.; Ibrahim, D. Partial purification and characterization of pectinase produced by *Aspergillus niger* LFP-1 grown on pomelo peels as a substrate. *Trop. Life Sci. Res.* **2021**, *32*, 1. [CrossRef] [PubMed]
25. Apprill, A.; McNally, S.; Parsons, R.; Weber, L. Minor revision to V4 region SSU rRNA 806R gene primer greatly increases detection of SAR11 bacterioplankton. *Aquat. Microb. Ecol.* **2015**, *75*, 129–137. [CrossRef]
26. Parada, A.E.; Needham, D.M.; Fuhrman, J.A. Every base matters: Assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples. *Environ. Microbiol.* **2016**, *18*, 1403–1414. [CrossRef] [PubMed]
27. Schloss, P.D.; Westcott, S.L.; Ryabin, T.; Hall, J.R.; Hartmann, M.; Hollister, E.B.; Robinson, C.J. Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* **2009**, *75*, 7537–7541. [CrossRef] [PubMed]
28. Glöckner, F.O.; Yilmaz, P.; Quast, C.; Gerken, J.; Beccati, A.; Ciuprina, A.; Westram, R. 25 years of serving the community with ribosomal RNA gene reference databases and tools. *J. Biotechnol.* **2017**, *261*, 169–176. [CrossRef] [PubMed]
29. Edgar, R.C.; Haas, B.J.; Clemente, J.C.; Quince, C.; Knight, R. UCHIME improves sensitivity and speed of chimer detection. *Bioinformatics* **2011**, *27*, 2194. [CrossRef] [PubMed]
30. Wang, Q.; Garrity, G.M.; Tiedje, J.M.; Cole, J.R. Naive bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* **2007**, *73*, 5261–5267. [CrossRef] [PubMed]
31. Yoon, S.; Ha, S.; Kwon, S.; Lim, J.; Kim, Y.; Seo, H.; Chun, J. Introducing EzBioCloud: A taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. *Int. J. Syst. Evol. Microbiol.* **2017**, *67*, 1613. [CrossRef]
32. Lozupone, C.; Knight, R. UniFrac: A new phylogenetic method for comparing microbial communities. *Appl. Environ. Microbiol.* **2005**, *71*, 8228–8235. [CrossRef]
33. Lee, C.H.; Khalina, A.; Lee, S.; Liu, M. A comprehensive review on bast fibre retting process for optimal performance in fibre-reinforced polymer composites. *Adv. Mater. Sci. Eng.* **2020**, *2020*, 6074063. [CrossRef]
34. Müller, M.J.; Stachurski, S.; Stoffels, P.; Schipper, K.; Feldbrügge, M.; Büchs, J. Online evaluation of the metabolic activity of *Ustilago maydis* on (poly) galacturonic acid. *J. Biol. Eng.* **2018**, *12*, 34. [CrossRef]
35. Sharma, S.; Mandhan, R.P.; Sharma, J. *Pseudozyma* sp. SPJ: An economic and eco-friendly approach for degumming of flax fibers. *World J. Microbiol. Biotechnol.* **2011**, *27*, 2697–2701. [CrossRef]
36. Zhao, D.; Pan, C.; Ping, W.; Ge, J. Degumming crude enzyme produced by *Bacillus cereus* HDYM-02 and its application in flax retting. *BioResources* **2018**, *13*, 5213–5224. [CrossRef]
37. Garg, G.; Singh, A.; Kaur, A.; Singh, R.; Kaur, J.; Mahajan, R. Microbial pectinases: An ecofriendly tool of nature for industries. *3 Biotech* **2016**, *6*, 47. [CrossRef]
38. Zhang, G.; Li, S.; Xu, Y.; Wang, J.; Wang, F.; Xin, Y.; Liu, H. Production of alkaline pectinase: A case study investigating the use of tobacco stalk with the newly isolated strain *Bacillus tequilensis* CAS-MEI-2-33. *BMC Biotechnol.* **2019**, *19*, 45. [CrossRef]
39. Alqahtani, Y.S.; More, S.S.; Shaikh, I.A.; KJ, A.; More, V.S.; Niyonzima, F.N.; Khan, A.A. Production and purification of pectinase from *Bacillus subtilis* 15a-b92 and its biotechnological applications. *Molecules* **2022**, *27*, 4195. [CrossRef] [PubMed]
40. Arotupin, D.J.; Akinyosoye, F.A.; Onifade, A.K. Purification and characterization of pectinmethyl esterase from *Aspergillus repens* isolated from cultivated soil. *Afr. J. Biotechnol.* **2008**, *7*, 1991–1998. [CrossRef]
41. Fahmy, A.S.; El-Beih, F.M.; Mohamed, S.A.; Abdel-Gany, S.S.; Abd-Elbaky, E.A. Characterization of an exopolygalacturonase from *Aspergillus niger*. *Appl. Biochem. Biotechnol.* **2008**, *149*, 205–217. [CrossRef]
42. Kumar, A.; Sharma, R. Production of alkaline pectinase by bacteria (cocci spp.) isolated from decomposing fruit materials. *J. Phytol.* **2012**, *4*, 1–5.
43. Ahlawat, S.; Mandhan, R.P.; Dhiman, S.S.; Kumar, R.; Sharma, J. Potential application of alkaline pectinase from *Bacillus subtilis* SS in pulp and paper industry. *Appl. Biochem. Biotechnol.* **2008**, *149*, 287–293. [CrossRef] [PubMed]
44. Pakarinen, A.; Zhang, J.; Brock, T.; Maijala, P.; Viikari, L. Enzymatic accessibility of fiber hemp is enhanced by enzymatic or chemical removal of pectin. *Bioresour. Technol.* **2012**, *107*, 275–281. [CrossRef] [PubMed]
45. Macfarlane, S.; Macfarlane, G.T. Regulation of short-chain fatty acid production. *Proc. Nutr. Soc.* **2003**, *62*, 67–72. [CrossRef] [PubMed]
46. Banerjee, S.; Sar, A.; Misra, A.; Pal, S.; Chakraborty, A.; Dam, B. Increased productivity in poultry birds by sub-lethal dose of antibiotics is arbitraged by selective enrichment of gut microbiota, particularly short-chain fatty acid producers. *Microbiology* **2018**, *164*, 142–153. [CrossRef] [PubMed]

47. Ribeiro, A.; Pochart, P.; Day, A.; Mennuni, S.; Bono, P.; Baret, J.; Mangin, I. Microbial diversity observed during hemp retting. *Appl. Microbiol. Biotechnol.* **2015**, *99*, 4471–4484. [[CrossRef](#)] [[PubMed](#)]
48. Law, A.D.; McNees, C.R.; Moe, L.A. The microbiology of hemp retting in a controlled environment: Steering the hemp microbiome towards more consistent fiber production. *Agronomy* **2020**, *10*, 492. [[CrossRef](#)]
49. Wexler, H.M. *Bacteroides*: The good, the bad, and the nitty-gritty. *Clin. Microbiol. Rev.* **2007**, *20*, 593–621. [[CrossRef](#)] [[PubMed](#)]
50. Bajpai, P. Chapter 8—Industrial applications of xylanases. In *Xylanolytic enzymes*; Bajpai, P., Ed.; Academic Press: Amsterdam, Netherlands, 2014; pp. 69–104. [[CrossRef](#)]
51. Barnett, S.E.; Cala, A.R.; Hansen, J.L.; Crawford, J.; Viands, D.R.; Smart, L.B.; Buckley, D.H. Evaluating the microbiome of hemp. *Phytobiomes J.* **2020**, *4*, 351–363. [[CrossRef](#)]
52. Djemiel, C.; Grec, S.; Hawkins, S. Characterization of bacterial and fungal community dynamics by high-throughput sequencing (HTS) metabarcoding during flax dew-retting. *Front. Microbiol.* **2017**, *8*, 2052. [[CrossRef](#)]
53. Djemiel, C.; Goulas, E.; Badalato, N.; Chabbert, B.; Hawkins, S.; Grec, S. Targeted metagenomics of retting in flax: The beginning of the quest to harness the secret powers of the microbiota. *Front. Genet.* **2020**, *11*, 581664. [[CrossRef](#)]
54. Munshi, T.K.; Chattoo, B.B. Bacterial population structure of the jute-retting environment. *Microb. Ecol.* **2008**, *56*, 270–282. [[CrossRef](#)]
55. Das, B.; Chakrabarti, K.; Tripathi, S.; Chakraborty, A. Review of some factors influencing jute fiber quality. *J. Nat. Fibers* **2014**, *11*, 268–281. [[CrossRef](#)]
56. Mandic-Mulec, I.; Stefanic, P.; van Elsas, J.D. Ecology of bacillaceae. In *The Bacterial Spore: From Molecules to Systems*; John Wiley & Sons: Hoboken, NJ, USA, 2016; pp. 59–85. [[CrossRef](#)]
57. Demir, N.; Nadaroglu, H.; Demir, Y.; Isik, C.; Taskin, E.; Adiguzel, A.; Gulluce, M. Purification and characterization of an alkaline pectin lyase produced by a newly isolated *Brevibacillus borstelensis* (P35) and its applications in fruit juice and oil extraction. *Eur. Food Res. Technol.* **2014**, *239*, 127–135. [[CrossRef](#)]
58. Tamburini, E.; León, A.G.; Perito, B.; Mastromei, G. Characterization of bacterial pectinolytic strains involved in the water retting process. *Environ. Microbiol.* **2003**, *5*, 730–736. [[CrossRef](#)]
59. Bowman, J.P. 14—Protein-based analysis and other new and emerging non-nucleic acid based methods for tracing and investigating foodborne pathogens. In *Tracing Pathogens in the Food Chain*; Brul, S., Fratamico, P.M., McMeekin, T.A., Eds.; Woodhead Publishing: Cambridge, UK, 2011; pp. 292–341. [[CrossRef](#)]
60. Park, G.W.; Seo, C.; Jung, K.; Chang, H.N.; Kim, W.; Kim, Y. A comprehensive study on volatile fatty acids production from rice straw coupled with microbial community analysis. *Bioprocess. Biosyst. Eng.* **2015**, *38*, 1157–1166. [[CrossRef](#)] [[PubMed](#)]

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