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Improving Pulping Performance as Well as Reducing Consumption and Increasing Efficiency via Microbial Consortium Pretreating Bamboo

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Abstract: The bio-refining process of bamboo is more challenging compared to wood due to its dense and stabilized complex polymer structure, as well as its abundance of degradation-resistant components. Consequently, the bio-refining of bamboo requires more energy and time consumption compared to the bio-refining of wood. In this study, co-cultured microorganisms were utilized for the pretreatment of bamboo to improve pulping performance, reduce consumption, and increase efficiency. These microorganisms were constructed by combining environmental microorganisms found in bamboo pulp with *Bacillus* sp. that were self-screened. The results of 16S rRNA analysis showed that the genera *Proteobacteria*, *Firmicutes*, *Bacteroidota*, and *Actinobacteriota* gradually became dominant during the treatment process. Additionally, the PICRUSt results indicated that the co-culture microbial consortium C strategy strengthened key enzyme activities related to the degradation of bamboo lignocelluloses. The microbial consortium pretreatment resulted in removing lignin and hemicellulose at rates of 21.96% and 26.21%, respectively. This process also caused a decrease in the crystalline index, indicating the presence of disordered crystalline regions. This change was beneficial for the subsequent Kraft pulping process. Compared to the conventional bamboo pulp, the yield of pretreated bamboo pulp increased slightly, while the cellulose purity and paper properties were significantly superior. The obtained Kraft pulp, which underwent microbiological pretreatment, met the requirements for superior Kraft pulp products despite a 65 min decrease in cooking time and a 10 °C decline in maximum cooking temperature. This study proves that co-cultured microbial consortium used for pretreating bamboo are beneficial for bamboo Kraft pulping. This approach can be considered environmentally friendly and leads to energy saving and cost reduction in bamboo bio-refining processes.

Keywords: bamboo; microbial consortium; pretreatment; pulping performance

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1. Introduction

As the most abundant biomass resource on earth, lignocellulose is the ideal raw material for producing energy, bio-based chemicals, polymer materials, etc. Chemical pulping is an established business model in the pulp and paper industry, which uses chemicals to remove lignin from complex organic substrates to produce cellulose-rich pulps. However, lignocellulosic biomass, especially bamboo biomass, has a compact and rigid structure known as biomass recalcitrance [1,2]. Biomass recalcitrance is closely related to the chemical and physical features of the plant cell wall. The presence of lignin, hemicelluloses, pectin, ash, etc., and their spatial interlinks have constructed physical barriers to protect cellulose from degradation because of biomass recalcitrance. Besides the large amount of energy and alkali required in the chemical pulping process, there was a

lower utilization rate of raw materials and a large amount of black liquor produced due to the barriers.

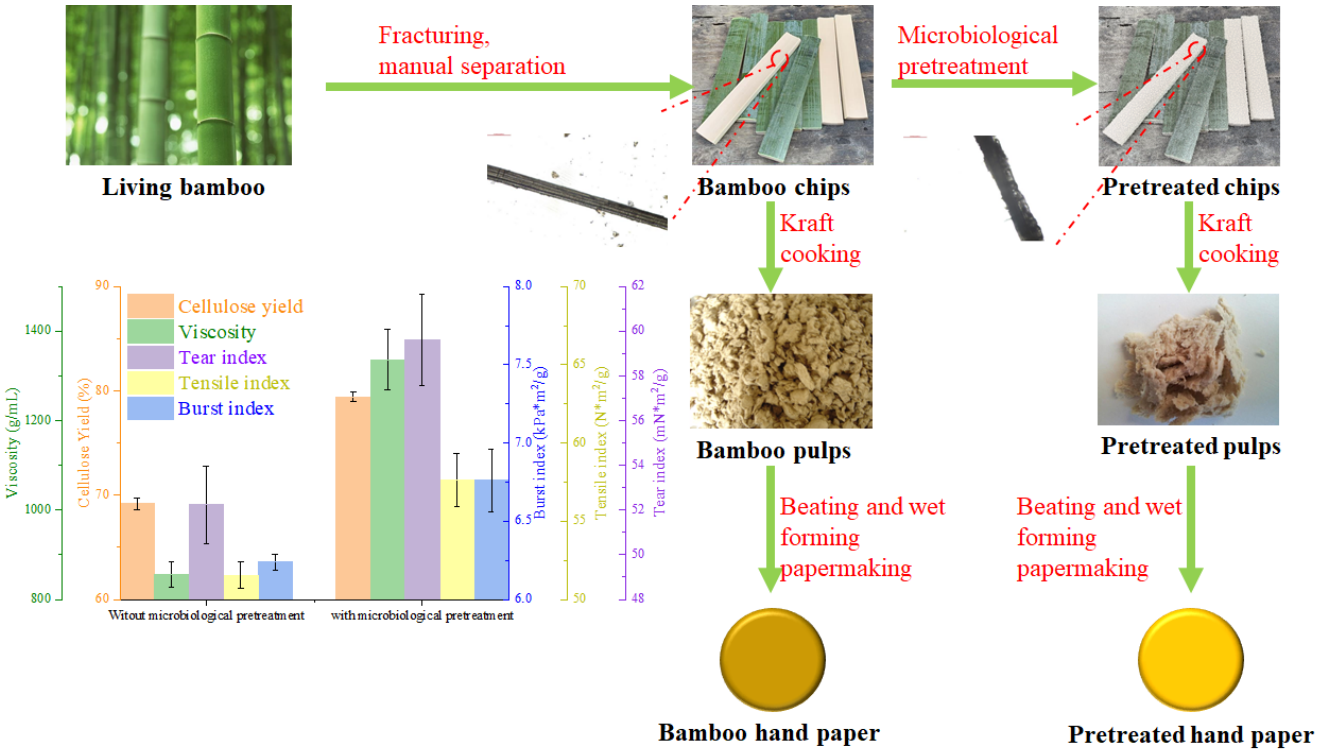
Lignocellulosic biomass pretreatment is crucial in improving rove downstream utilization. Previous research has focused on altering the recalcitrant structure of biomass through physical, chemical, biological, and hybrid pretreatments to facilitate further utilization of biomass. In pulp and papermaking, the pretreatment of raw material with microorganisms before pulping is called bio-pulping, an environmentally friendly alternative to traditional pulping methods. Bio-pulping aims to remove or loosen lignin in raw material without the decomposition of cellulose. In bio-pulping, lignin and hemicellulose can be effectively removed through the action of different enzymes produced by fungi [3,4]. It was found that white rot fungi, such as Ceriporiopsis subvermispora, Corioulus versicolor, Ganoderma colossum, Phanerochates chrysosporium, Phlebiopsis gigantean, Physisporinus rivulosus, *Trametes versicolor*, *Schizophyllum commune*, etc., are most the suitable for the biological pulping process [3–10]. Compared with other traditional pulping processes, bio-pulping improves pulp quality and paper properties and reduces energy costs and environmental impacts [4,11,12].

Fungi have been found to have a high lignin-degrading ability; however, they are sometimes not stable in practical treatment under extreme environmental and substrate conditions [13]. In recent years, because of the tremendous ecological adaptability and biochemical versatility of bacteria, more and more studies have been conducted on bacterial lignin degradation. Lignin-degrading bacteria are mainly *Acinetobacter*, *Neosphingobacter*, *Aeromonas*, *Pseudomonas*, *Bacillus*, *Tuptomonas*, etc. [14], which can also produce lignin peroxidase, lignin manganese peroxidase and/or laccase, while the organic acids produced by bacteria have been found to promote lignin degradation [15]. In addition, a growing body of research has found that bacterial communities were more effective in lignocellulose degradation than single bacterial/fungal or enzyme treatments [16]. After bacterial consortium pretreatment, the degradation rate of lignocellulosic biomass [17] biogas production [18] and/or compost quality [19] during downstream biotransformation were enhanced. These previous studies illustrate that bacteria also contribute to lignin degradation as well as fungus. It was feasible to pulp pretreated plant fiber raw materials with bacteria/flora. Wang et al. [20] found that the pretreatment of lignin-degrading bacteria LDC screened from reed wetland sludge harvested an improved physical performance of reed mechanical pulp and reduced energy consumption. Han [21] realized the chemical pulping of bamboo fermented by engineering bacteria of *Bacillus licheniformis* with a weakened cellulase gene. However, there needs to be more information on the influence of bacterial communities on pulping, especially bamboo pulping.

Due to the wood pulp shortage in China, wood pulp was employed in many areas. Bamboo, widely found in Sichuan, Guizhou, Chongqing, Guangxi, Fujian, and Yunnan provinces, is a crucial raw material for pulp- and papermaking. Currently, the production capacity of bamboo pulp is approximately 2.42 million tons/a, and there are 23 enterprises above the designated size, with the output value reaching 13.2 billion [22]. Thus, it is worth exploring the potential of bamboo pulping with low pollution.

Our previous study found a bacterial consortium with the ability of bamboo lignocellulose degrading contained in bamboo pulp from Luzhou (China). In combination with *Bacillus.* sp. screened from a sequencing batch-activated sludge system operated with bamboo black liquor, a bacterial consortium with the capability of lignocellulose degradation was constructed, named C. Hemicellulose, and Lignin in bamboo was decomposed efficiently by C, while cellulose had little effect. Therefore, this study aimed to (1) construct a microbial consortium for selectively degrading lignin and hemicelluloses of bamboo and (2) develop a method to pretreat complex biomass and improve the pulping performance of bamboo. This study focused on microbial consortium construction, bamboo composition, and structure bioconversion behaviors. Moreover, the pulp properties, physical characteristics of hand paper, and pulping parameters with pretreated or not pretreated were also compared. This research paper also evaluated the potential performance of

bamboo bio-pulping to reduce consumption and increase efficiency by bacterial consortium C pretreatment. Graphical abstract of this research was presented in Scheme 1.



Scheme 1. The treatment processes and effectiveness of microbiological pretreatment for Kraft cooking from bamboo.

2. Materials and Methods

2.1. Materials

The bamboo chips, with a width of 0.5 cm and a length of 3–5 cm, were collected from living bamboo in Luzhou, Sichuan Province, China, via fracturing and manual separation. The main components of these bamboo chips are cellulose (46.25% of content), lignin (35.63% of content), and hemicellulose (16.37% of content).

Three kinds of microorganisms were used in the pretreatment of bamboo chips prior to Kraft pulping. The first kind was a pure microbial bacterium screened from the sequencing batch-activated sludge system operated with stably diluted bamboo pulping black liquid and identified as *Bacillus* sp. in a previous work, and named as A. A was inoculated when the cell density was approximately 1.0 in order to ensure excellent microbial activity. The second one, marked as B, was derived from the fine fibers (less than 200 mesh) of unbleached bamboo Kraft pulp from Fengsheng Pulp and Paper Co. Ltd., Qianwei, Sichuan Province, China. The dominant genera of B are *Alcaligenes* (22.42%), *Ochrobactrum* (9.24%), *Proteus* (6.79%), *Enterococcus* (6.08%), and *Escherichia-Shigella* (5.69%) by 16S rRNA technology. B was inoculated into bamboo chips through the incorporation of corresponding unbleached Kraft pulp. The last one, marked as C, was defined as A and B co-culture microbial consortium with a halved inoculated dose.

All the other chemical reagents were of analytical grade and used as received from Sinopharm Chemical Reagent Co., Ltd. without further purification.

2.2. Experimental Process

2.2.1. Microbiological Pretreatment of Bamboo Chips

The microbiological pretreatment of bamboo chips proceeded in an aerobic fermenter with a solid–liquid ratio of 1:3.6, an initial pH of 7, and a temperature of 37 °C. Ca. 300 g

bamboo chips (based on oven-dried weight) were inoculated with A (750 mL of inoculating liquid), B (5.00 g of fines fibers from unbleached bamboo Kraft pulp), and C (375 mL of inoculating liquid and 2.50 g of fines fibers), respectively. The solid–liquid ratio was adjusted by Herr's buffer. Following microbiological treatment, the separation of solid/liquid, washing with deionized water, and air-dried were carried out to obtain microbiologically treated bamboo chips. In order to evaluate the effect of indigenous microorganisms in bamboo, the specimen, treated without exogenous microorganisms under the same conditions, was carried out and marked as Control. The specimen missing microbiological treatment was marked as Origin. Every treatment group was repeated twice. The yield of specimens was calculated according to Formula (1).

$$Y = \frac{m_2}{m_1} \times 100\% \quad (1)$$

where m_1 is the initial oven-dried weight of bamboo chips and m_2 is the oven-dried weight of bamboo chips via microorganisms' treatment.

In order to obtain accurate information on microorganisms during microbiological treatment, the homogeneous mixture of bamboo chips, degradation products of bamboo, and fermentation liquor in a fermentation cylinder was sampled from 4 evenly distributed points at 0, 7, and 10 d to obtain sample 0, sample 1, and sample 2, respectively. The obtained sample was randomly mixed with one of the other 3 corresponding samples at a liquor ratio of 1:1. Finally, the obtained specimens were marked as A0, A1, A2, B0, B1, B2, C0, C1, and C2, according to the inoculant and sampling time. The filtrate of specimens sampled at 7 d and 10 d were solid–liquid separated by 60-mesh polyester bags. The obtained filtrates were centrifuged at 5000 rpm with a temperature of 4 °C for 5 min. The obtained sediments from centrifugation were immediately frozen at −80 °C and used to determine bacterial diversity.

2.2.2. Conventional Kraft Cooking, Hand Paper-Making

Kraft cooking was performed in an intermittent rotary electric cooking pot (VRD-42SD-A, CNPPRI, Beijing, China) with a liquor ratio of 1:3, an active alkali loading of 15% (based on the weight of Na_2O), and a sulfidity of 15% (based on the weight of Na_2O). The Kraft cooking process is shown in Figure S1. The samples for Kraft cooking above were all pretreatment groups for 10 d because of the obvious erosion of bamboo. Unpretreated bamboo was also prepared for comparison. The 15% active alkali loading and 15% sulfidity, which are the critical pulping conditions for raw bamboo, were determined via a preliminary experiment (Figure S2). After solid–liquid separation by extrusion, washing, screening, and dewatering, the unbleached biochemical pulp was prepared for the following hand papermaking process.

According to GB/T24324, a proper amount of biochemical pulp was beaten by PFI with a refining consistency of 10% until the degree of 45°SR, and then the hand paper (60 g/m^2) was prepared. An equal amount of the above pulp dispersed thoroughly was used to prepare wet paper sheets with a rapid casser paper sheet shaper (ASN-32N2F, CNPPRI, Beijing, China). The wet sheets were then dried with a drum dryer (RD-01, CNPPRI, Beijing, China) after being pressed and dehydrated to obtain the hand papers for the properties test.

2.2.3. Modified Kraft Cooking

In this section, declined cooking time and cooking temperature were carried out to reduce energy consumption and increase efficiency during Kraft cooking. Meanwhile, the obtained hand paper should meet the requirements of a superior product in Kraft bamboo pulp. Cooking time declined from 185 min to 150 min, and finally to 120 min. Maximum temperature declined from 165 °C to 155 °C. The modified Kraft cooking process is shown in Figure S1.

2.3. Chemical Components Determination of Pulp

The total yield of pulp specimens was calculated according to Formula (2).

$$\text{Total yield}(\%) = \frac{M_2}{M_1} \times 100\% \quad (2)$$

where M_1 is the initial oven-dried weight of bamboo chips and M_2 is the oven-dried weight of the pulp.

The main chemical components, such as the contents of cellulose, lignin, and hemicellulose, were defeminated according to NREL/TP-510-42618 and NREL/TP-510-42619. The holocellulose content is defined as the sum value of cellulose and hemicellulose content in this study.

The degradation rate and degradation selectivity of hemicellulose/lignin were calculated according to Formulas (3) and (4) [23], respectively.

$$\text{Degradation rate } (\%) = \frac{C_1 - C_f}{C_i} \times 100\% \quad (3)$$

$$\text{Degradation selectivity } (\%) = \frac{C_i - C_f}{\text{Yield loss}} \times 100\% \quad (4)$$

where C_i is the initial content of lignin/hemicellulose and C_f is the lignin/hemicellulose content of specimens following treatment.

The relative yield of cellulose in pulp was calculated according to Formula (5).

$$\text{Cellulose yield } (\%) = \frac{C_f}{C_i} \times 100\% \quad (5)$$

where C_i is the initial cellulose content of bamboo chips prior to microbiological treatment and C_f is the cellulose content of Kraft pulp.

According to GB-T 1548-2016, the intrinsic viscosity of the pulp specimen was measured by the method of cupri-ethylene-diamine and calculated according to Formula (6).

$$\eta_r = h_n \times t_n \quad (6)$$

where η_r is the relative viscosity of cellulose; h_n is the constant of viscometer (1.1054, s^{-1}); t_n is the recorded time (s); and [η] is the intrinsic viscosity of cellulose, which can be obtained via table lookup according to the calculated η_r.

The content of mainly chemical components, degradation rate, and degradation selectivity of hemicellulose/lignin of bamboo chips with/without pretreatment were determined according to methods similar to the pulp.

2.4. Fiber Morphology and Properties of Hand Papermaking

Length, width, and fine fiber content of pulp fibers were measured by a fiber quality analyzer (12440585, ABB AB/Lorentzen & Wettre, Sweden) with a concentration of 0.05 wt%. Properties of hand paper, such as the tensile index, tear index, and burst index, were measured according to GB/T 22898-2008, GB/T 455-2002, and GB/T 454-2020, respectively.

2.5. XRD Characterization

The XRD pattern of specimens was recorded by an X-ray diffractometer (D2 Phaser, Bruker, Germany) equipped with Cu-Ka radiation (k = 1.54 Å) as an X-ray resource operating (30 kV, 10 mA) at a scan rate of 0.2° per second in a 2θ range of 10–60°. The crystalline index (CrI) of cellulose was calculated according to Formula (7) [24,25].

$$\text{CrI} = (I_{Cr} - I_{am})/I_{Cr} \quad (7)$$

where the subscript Cr and am are related to the crystalline regions and amorphous regions, respectively, and I is the diffraction intensity. I_{Cr} and I_{am} are the diffraction intensities at $2\theta = 22.6^{\circ}$ and $2\theta = 18.0^{\circ}$, respectively.

2.6. 16S rRNA Gene Sequencing and Functional Prediction

2.6.1. 16S rRNA Gene Sequencing

The FastDNA ® RSPIN kit for Soil (Mpbio, Santa Ana, CA, USA) kit was used to extract genomic DNA. PCR was performed using 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGT WTCTAAT-3′) primers [26], and the amplified products were subjected to 2% agarose gel electrophoresis. The library construction was performed using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA). The constructed library was quantified by QuantiFluor™-ST (Promega, Madison, WI, USA). The qualified libraries were sequenced using the MiSeq PE300 platform (Illumina, San Diego, CA, USA).

2.6.2. Enzyme Functional Prediction

The OUT abundance table obtained on the basis of 16S rRNA data was compared with the Greengene database using PICRUSt technology to obtain the corresponding COG and KEGG functional information and their abundance (mainly focusing on functional enzyme genes). Before functional prediction, OTUs of 16S rRNA sequences were normalized by PICRUSt [17].

2.6.3. Bioinformatics Analysis

The effective sequences of original data filtered and spliced were clustered into OTUs with 97% similarity, and the representative sequences were annotated using the Silva database and RDP classifier algorithm [27]. The OTU abundance and diversity index, as well as the consortium structure at phylum and genus, were then statistically analyzed based on the results of the taxonomic analysis. Differences among samples were analyzed using PCoA analysis and Anosim analysis based on Bray–Curtis [28]. One-way ANOVA, with fdr for *p*-value multiple tests, was used to evaluate the significance level of species differences and species differences among various groups [29]. Statistical differences in physical and chemical indexes were tested by one-way analysis and the t-test. Plots were drawn using Origin Pro 8.0.2 software. All reported values were the average of triplicate results (mean ± SD). Results with $p < 0.05$ between groups were declared statistically significant for all analyses.

3. Results and Discussion

3.1. Chemical Components and Physical Structure of Bamboo via Microbiological Pretreatment

The components and yield of bamboo with/without microbiological pretreatments are shown in Figure 1a. All the specimens exhibited yield loss via microbiological pretreatment. The inoculation of exogenous microorganisms leads to a decrease in yield (88.08% for A, 91.88% for B, and 87.52% for C) compared to specimens pretreated by indigenous microorganisms (93.67%). Fortunately, the holocellulose content of specimens increases (A and B), and the lignin content of specimens decreases (A, B, and C), revealing the fact that the inoculation of A, B, or C is beneficial for the removal of lignin during microbiological pretreatment.

In order to understand the variation of carbohydrates during microbiological treatment, the cellulose and hemicellulose content were detected, as shown in Figure 1b. With the proceeding of microbiological pretreatment, both Origin and A, B, C, the cellulose content for specimen/holocellulose increased, and the hemicellulose content for specimen/holocellulose decreased via microbiological treatment, indicating that hemicellulose rather than cellulose can be removed selectively during both endogenous and exogenous microbiological pretreatment. Interestingly, the single addition of A or B was incapable of increasing the cellulose content for holocellulose and the decrease of hemicellulose content

for holocellulose when compared with single endogenous microorganism treatment. The cellulose for holocellulose of samples increased significantly from 76.67% in Origin to 79.66% in C. Hemicellulose for holocellulose decreased significantly from 15.53% in Control to 12.08% in C because of the addition of C, which indicated that the synergistic effect of A and B may be more beneficial for increasing the cellulose content of holocellulose.

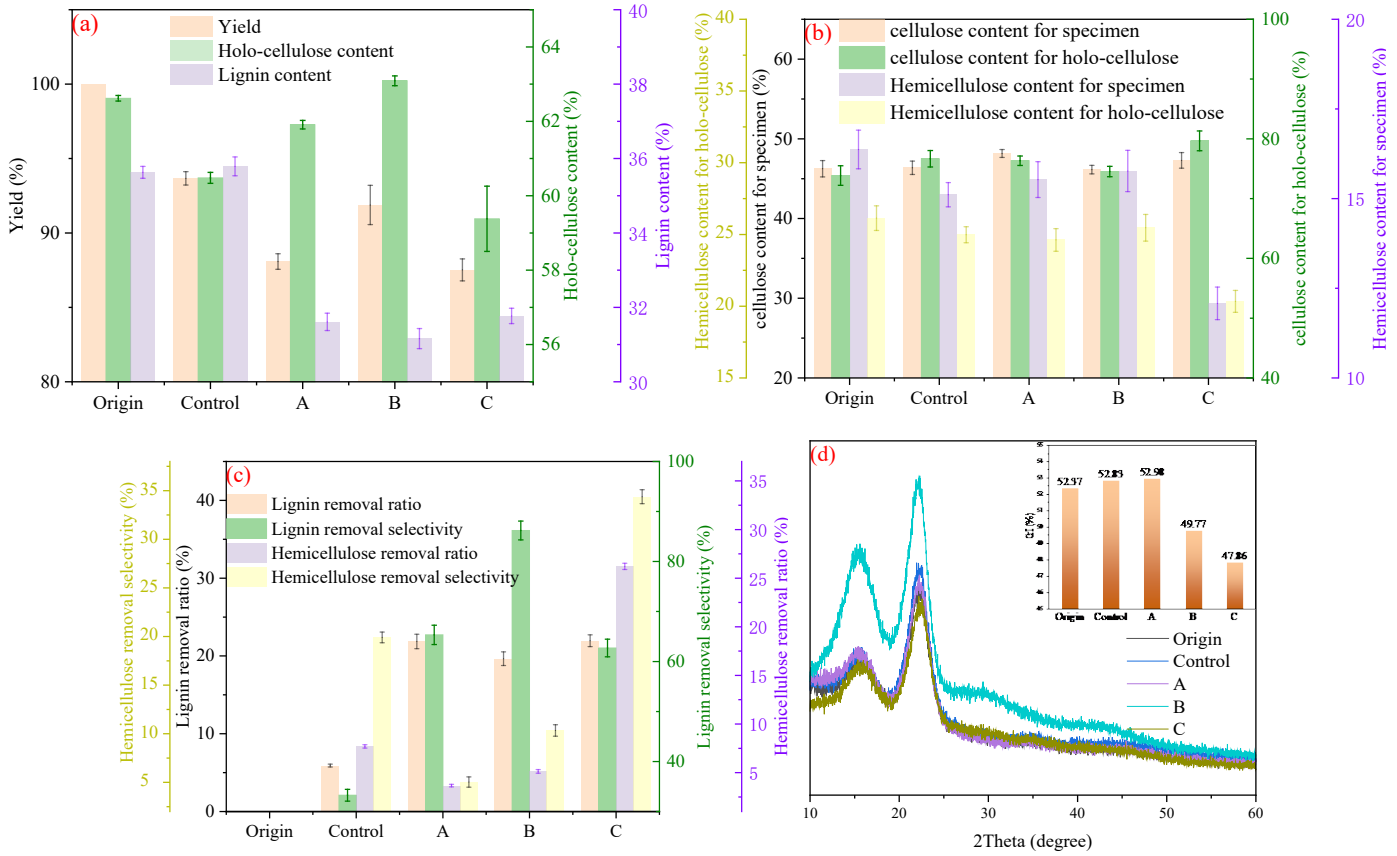


Figure 1. (**a**) Yield, holocellulose, and lignin content; (**b**) cellulose and hemicellulose content; (**c**) removal ratio and removal selectivity of lignin and hemicellulose; (**d**) crystal index of specimens via microbiological pretreatment. A: bamboo chips treated by microorganisms A, B: bamboo chips treated by microorganisms B, C: bamboo chips treated by microbial consortium C.

To further demonstrate the effect of various microbiological pretreatment on the degradation of hemicellulose and lignin, the degradation rates and degradation selectivity of hemicellulose/lignin were also calculated and are shown in Figure 1c. The inoculation of exogenous microorganisms significantly increased the lignin degradation rate and lignin degradation selectivity of specimens compared to the indigenous microorganism. However, the hemicellulose degradation rate and hemicellulose degradation selectivity declined when A or B was inoculated separately, declaring that it was not conducive to remove hemicellulose in bamboo. C pretreatment showed the highest degradation rate and hemicellulose degradation selectivity. The two indexes increased by 106.27% and 72.68%, respectively, compared with the control group, which further indicated the synergistic effect of mixed A and B, conducing to the removal of hemicellulose. The structure of lignin played an essential role in the degree of inhibition of bio-refining. After removing part of the lignin, the specific surface area increased, and the accessibility of the base material to the enzyme/chemical solution also increased [30]. In addition, the impregnation level of the enzyme/chemical solution of the base material will also be improved due to the removal of hemicellulose, redistribution of lignin, and structural changes [31]. Therefore, the significant reduction of hemicellulose in this study may be another critical way to improve the performance of biomass substrates cooking.

The XRD pattern of specimens was recorded and is shown in Figure 1d, and the crystal index (CrI) of specimens was also calculated and is shown in Figure 1d. All the XRD pattern of specimens with/without microbiological treatment exhibit typical XRD diffraction peaks of cellulose I_β. Among them, the diffraction peaks at $2\theta = 15.1^\circ$, 16.4°, 22.6°, and 34.5° are attributed to 1–10, 110, 200, and 004 planes of cellulose I_β, respectively [24], indicating the scare variation in cellulose crystalline structure during these microbiological pretreatments. However, the CrI of bamboo specimens was obviously varied. Compared with the original specimen (with a CrI of 52.37%), specimens pretreated via microorganism exhibited a lower CrI, attributed to the effect of microorganisms on crystalline cellulose and arousing disorder in the region of ordered crystals, which could not be removed during microbiological pretreatment. The variation of CrI of specimens demonstrates the variation in the relative number of crystalline regions and amorphous regions in specimens. In addition to chemical components, the number of crystalline regions is another critical factor affecting the lignocellulosic degradation of bamboo. The decreased CrI indicates that the ordered crystalline regions are trending towards disordered regions, which may be more well organized than the original amorphous regions of cellulose in specimens, resulting in cellulose fibers with high accessibility. Bamboo following microbiological treatment may be more suitable for cooking or bio-refining due to the decreased CrI, which also indicates the destructive hydrogen bonding network in cellulose. Similar results are demonstrated in the literature [32].

The aforementioned variations confirm the reconstructed chemical components and crystalline structure of bamboo following microbiological treatment. These variations may favor the followed bio-refine or pulping of bamboo. Furthermore, the incorporated indigenous microorganism and various exogenous microorganisms exhibit distinct discrepancy effects on chemical components and CrI of specimens. The discrepancy of incorporated exogenous microorganisms and their different evolution and functionalization routes may be responsible for the above variation.

3.2. 16S rRNA Sequencing and Analysis

3.2.1. Differences in Microbial Consortium Composition and Species Distribution

The stacked bar chart could visually display the composition of dominant species at a taxonomic level and the overall distribution of dominant species. A total of 302 OTUs were obtained by sequencing 12 samples from the 3 treatment groups that belonged to 5 phyla, 13 classes, 49 orders, 86 families, and 157 genera. The composition of bacterial communities at the phylum level (relative abundance greater than 1%) in treatments A, B, and C is shown in Figure 2a. Similar to previous studies on lignocellulose decomposition by pyrosequencing [33] and Illumina MiSeq sequencing [34], *Proteobacteria*, *Firmicutes*, *Bacteroidota*, and *Actinobacteriota* were dominant in all samples. The abundance of the four phyla accounted for 44.06~74.82%, 26.85~48.02%, 0.82~6.13%, and 0.01~8.19% of the whole sequence, respectively. *Proteobacteria* and *Firmicurium* remained overwhelmingly dominant in all microbial treatment groups, being the main phylum of lignin-degrading bacteria [35]. The proportion of *Bacteroides* decreased, possibly involving the degradation of hemicellulose and amorphous regions of cellulose [36], but the abundance in sample C was consistently higher than that in sample A or B. In addition, *actinomyces* significantly increased in all treatment groups, and *Actinobacter* abundance was higher in the C treatment group (0~0.56% for A, 2.92~7.34% for B, and 0.15~8.19% for C). *Actinobacter* has been reported to be involved in the decomposition of cellulose, hemicellulose, and lignin [37].

The above results showed that the addition of C composed of A and B could accelerate the decomposition of bamboo, and the change in bamboo yield also supports this conclusion (Figure 1a). The succession of the bacterial consortium can also affect the performance of bamboo component structure redistribution, which is supported by the changes in lignin and hemicellulose content in bamboo (Figure 1a).

For more details, microbial communities were further compared at the genus level, as shown in Figure 2b. A total of 31 genera (relative abundance > 1%) were identified

in all 12 samples from the 3 treatment groups. Although the same genus species were detected in the samples from the 3 treatment groups, the abundance of the dominant genus was varied. They all contained some bacterial genera with similar relative abundances and high abundance levels, such as *Escherichia Shigella*, *Enterobacter*, *Acinetabacter*, *Proteus*, *Proteus*, *Escherichia coli*, *Enterobacter*, *Acinetobacter*, and *Enterococcus*. Among the top 20 bacterial genera, the dominant genera in sample A also included *Alcaligenes* (6.69~20.76%), *Ochrobactrum* (4.34~7.57%), *Bacillus* (4.12~7.06%), *Alkaliphilus* (4.01~6.61%), *Pseudomonas* (2.43~6.16%), *Tissierella* (3.49~4.23%), and *Pusillimonas* (0.41~4.69%). The dominant genera of sample B also included *Lactobacillus* (4.36~5.99%), *Lysinibacillus* (5.69~7.13%), *Corynebacterium* (2.72~3.90%), *Corynebacterium* (2.72~3.90%), *Kurthia* (≤8.71%), and *unclassified_f__Lachnospiraceae* (1.67~2.21%). In addition, *Weissella*, *Ochrobactrum*, *Alkaliphilus*, *Sphingobacterium*, and *Stenotrophomonas* were also found in certain amounts. The bacterial genus composition of sample C was similar to that of B. Compared with sample B, the relative abundance of *Proteus*, *Lactobacillus*, *Weissella*, *Ochrobactrum*, *Bacillus*, *Sphingobacterium*, and *Stenotrophomonas* was upregulated in sample C due to the addition of A. The genera with decreased relative abundance were *Enterococcus*, *Lysinibacillus*, *Alkaliphilus*, *Tissierella*, and *Kurthia*. In addition, the relative abundance of *Corynebacterium* and *unclassified_f__Lachnospiraceae* decreased significantly in the early stage of microbial interaction and then increased in the later stage. This may be the result of the interaction between bacteria genus A and exogenous microbial lineage B on the basis of maintaining the endogenous microorganisms in bamboo wood, and this succession is also closely related to the spatiotemporal gradient of nutrients [38].

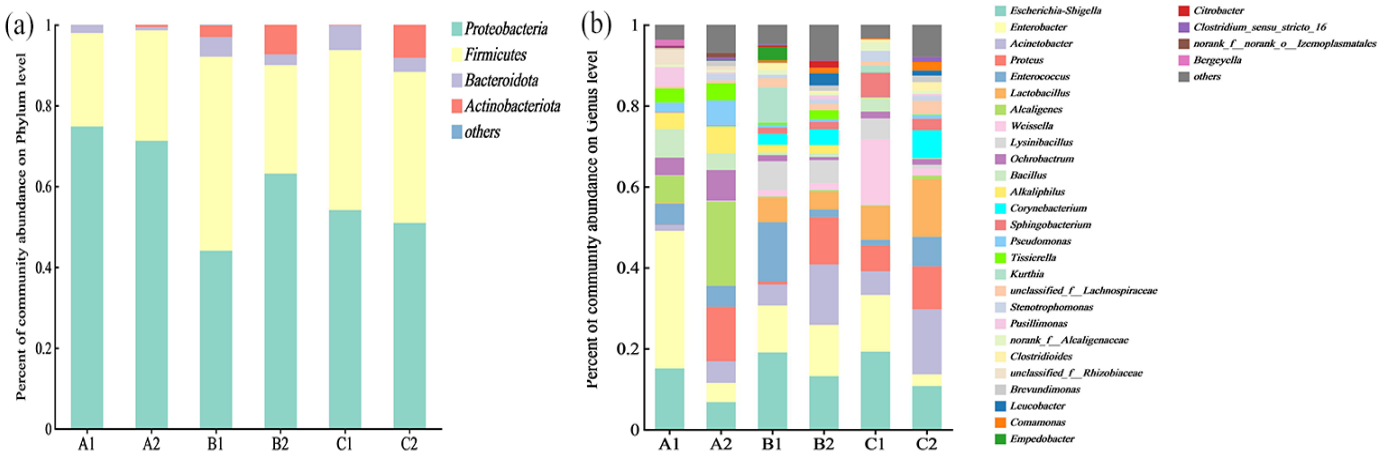


Figure 2. Composition and succession of bacteria communities during microbiological pretreatment at the (**a**) phylum level; (**b**) genus level.

Figure 3a shows the UPGMA clustering tree based on the OTU level. It can be seen that sample A forms a cluster of its own. However, samples B and C in the same period were clustered into A cluster, indicating that they were closely related to each other but distantly related to sample A. PCoA analysis based on the Bray–Curtis distance algorithm, and Anosim analysis, was used to further investigate the differences in bacterial consortium composition structure among different microbial treatments (Figure 3b). The results of PCoA analysis showed that the samples of the 3 treatment groups were far apart, indicating that there were differences in species composition among the groups. The results of Anosim ($R = 0.6007 > 0$, $p = 0.002 < 0.05$) further confirmed that there were significant differences in bacterial consortium composition among the 3 treatment groups.

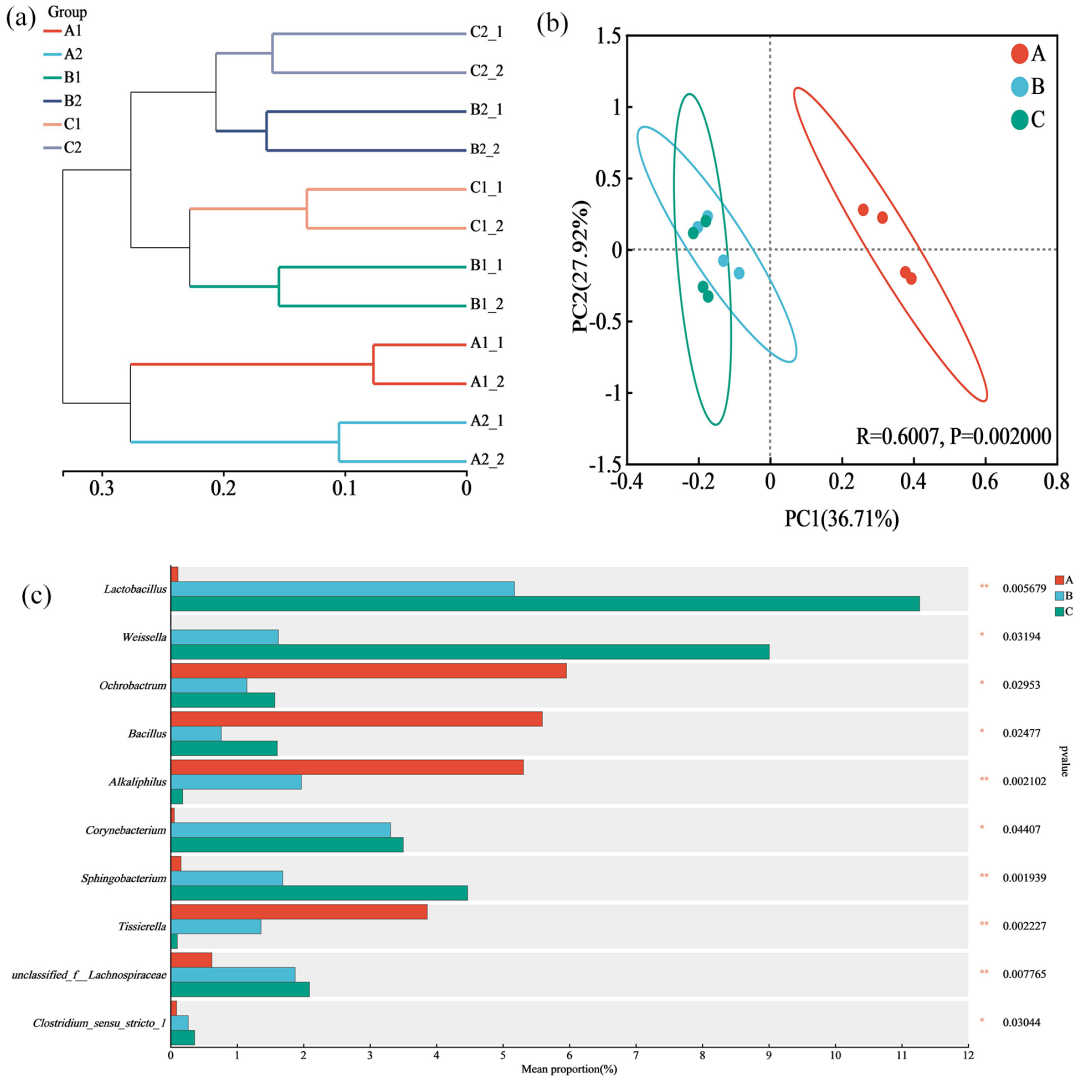


Figure 3. (**a**) Hierarchical clustering analysis of bacterial samples; (**b**) PCoA analysis of the three bacterial flora samples; (**c**) comparison of the top 10 genera for multi−group. The statistical analysis was performed using one−way ANOVA, with fdr for p−value multiple tests. * $0.01 < p < 0.05$, ** $0.001 < p < 0.001$. A, B and C denoted all samples collected on day 7d and 10d, in groups treated by microorganisms A, B and C.

The results of bacterial genera with significant distribution differences between samples from each treatment group are shown in Figure 3c. The results showed that the top 10 genera in abundance were *Lactobacillus*, *Weissella*, *Ochrobactrum*, *Bacillus*, *Alkaliphilus*, *Corynebacterium*, *Sphingobacterium*, *Lactobacillus*, *Tissierella*, *unclassified_f__Lachnospiraceae*, and *Clostridium_sensu_stricto_1*. Four bacterial genera (*Ochrobactrum*, *Bacillus*, *Alkaliphilus*, *Tissierella*) were significantly abundant in A, but none in B. The relative abundance of the six genera *Lactobacillus*, *Weissella*, *Sphingobacterium*, *Corynebacterium*, *unclassified_f__Lachnospiraceae*, and *Clostridium_sensu_stricto_1* in sample C was significantly higher than that in A or B cultured alone. For the function of significantly different genera, it was found that *W. confusa* XU1 had the highest biomass yield when xylo-oligosaccharide was used as a single carbon source, and XU1 preferred xylo-oligosaccharide to glucose and xylose [39]. *Sphingobacterium* isolated from Athyrium wallichianum Ching has the ability to degrade cellulose and xylan [40]. *Corynebacterium* has better "talent" than ordinary industrial strains in degradation and tolerance of phenolic aromatic compounds in lignocellulosic saccharification solution [41], while there are not many studies on *Lactobacillus* about lignocellulose degradation. In addition, the advantage of *Ochrobactrum* (relative abundance C > B) is

more reflected in the aspects related to lignin degradation. Some members of the same family of *Ochrobactrum* can effectively degrade lignin or phenolic substances, and some also show the ability to utilize lignin and hemicellulose without cellulose reduction [42]. Thus, the degradation differences of chemical components in different treatment groups may be affected by the distribution differences of functional bacteria to some extent. On the contrary, the variation of substrate component structure, fermentation environment, bacterial metabolism, and activity will also affect the difference in bacteria.

3.2.2. Enzyme Annotation Analysis Related to Lignocellulose Degradation

Enzymes secreted by microorganisms play an irreplaceable role in the degradation of lignocellulose and the substrates related to lignocellulose degradation. As shown in Figure 4a, the abundance of enzymes with debranching activity for hemicellulose degradation L-arabinoside (EC: 3.2.1.55) and β-glucosidase (EC: 3.2.1.21) in sample C did not change significantly compared with that in sample B. However, endo-β-1, 4-xylanase (EC: 3.2.1.8) and β-xylosidase (EC: 3.2.1.37) were significantly enriched in sample C compared with samples A or B. Endo-β-1, 4-xylanase can cleave xylan randomly inside hemicellulose to produce xylose oligosaccharides mixtures. β-xylose glycosidase releases xylose by removing monosaccharides from the nonreducing terminal groups of short oligosaccharides. They are the most critical enzymes for bamboo hemicellulose degradation [43]. Therefore, the higher abundance of hemicellulose in the C treatment could more easily break down the hemicellulose fraction of the bamboo, resulting in a significantly higher hemicellulose degradation rate (26.21% for C compared with 3.67% for A and 5.13% for B) and hemicellulose selectivity (34.38% for C, 5.03% for A, and 10.34% for B) in the 3 treatment groups (Figure 1c).

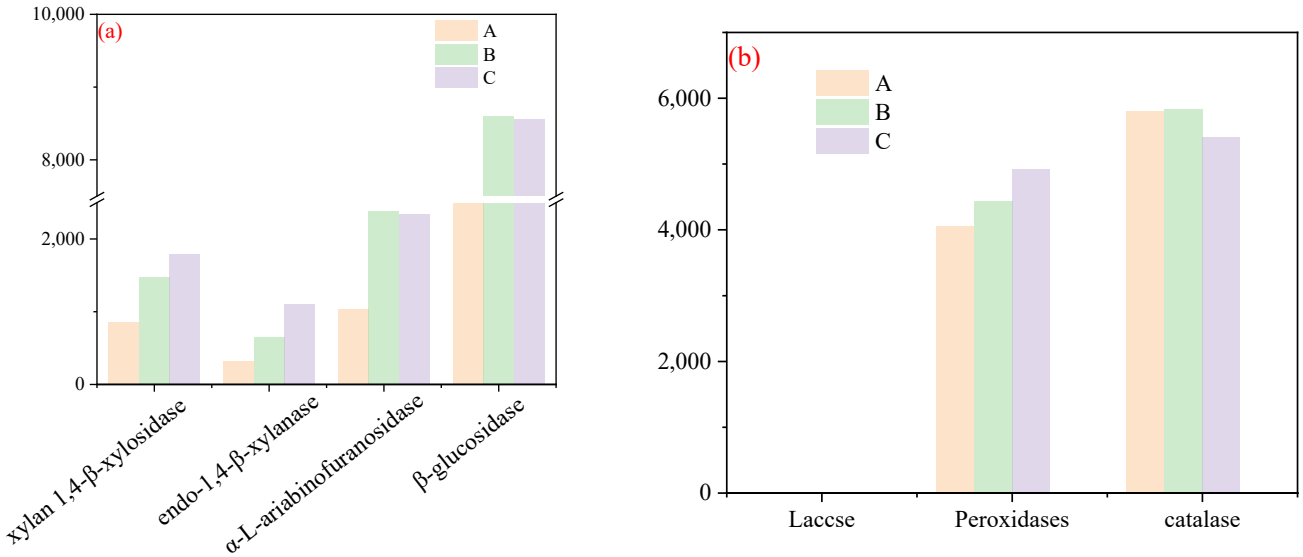


Figure 4. The abundance of associated enzymes predicted by PICRUSt. (**a**) Abundance of enzymes associated with hemicellulose degradation; (**b**) abundance of enzymes associated with lignin degradation. A, B and C denoted all samples collected on day 7d and 10d, in groups treated by microorganisms A, B and C.

In all samples, laccase was not predicted (EC:1.10.3.2), probably due to spatiotemporal variation in sampling, but the abundant and comparable abundance of peroxidase (EC: 1.11.1.-) and peroxidase (EC: 1.11.1.6) were predicted (Figure 4b), which was confirmed by the previously detected lignin removal rate (ca. 20%).

3.3. Effect of Microbial Pretreatment on Bamboo Bio-Refining

3.3.1. Effect from the Perspective of Pulping Product Characteristics

For the bio-refining process of lignocellulose, prior to utilizing cellulose or hemicellulose, lignin should be removed first, which is similar to the process of pulping. However, the former needed the increased accessibility of cellulose to enhance the enzymatic hydrolysis efficiency. The latter needed to be improved by increased delignify efficiency and decreased cellulose degradation. Even though the final purpose of bio-refining and pulping are discrepant, for both of them, the delignification process must be involved. It is obvious that the microbial consortium and related technology can be carried out as a pretreatment to reduce the treatment conditions of the following delignification during pulping via the enhanced impregnation effect from chemical reagents to bamboo chips. Meanwhile, the degradation of cellulose can be reduced.

In order to explore the influence of different microbiological pretreatments on the process of pulping, Kraft cooking under the same conditions was carried out, with the corresponding results shown in Figure 5. Following Kraft cooking, specimens pretreated by indigenous microorganisms and exogenous microorganisms exhibit slightly increased yield loss compared with the original specimen (Figure 5a). It is worth noting that specimen C, following Kraft cooking, exhibits the highest total yield. Chemical cooking is mainly used to remove most of the lignin and hemicellulose of bamboo and retain cellulose. Compared with the original specimen, the weight of bamboo chips decreases following microbiological pretreatment. Fortunately, the loss of cellulose is scarcely detected during pretreatment. Hence, the pretreated chips with higher cellulose content were applied for the Kraft cooking that followed. This will lead to an increase in the yield of pulp. Therefore, the yield loss during pretreatment will not observably affect the yield of pulp due to chips with higher cellulose content being used for Kraft cooking.

All pulp specimens, whether subject to microbiological pretreatment or not, exhibit a lignin content less than 2.00%, which is attributed to the strong delignification during Kraft cooking. For the retaining of hemicelluloses and cellulose, different microbiological treatments lead to a different pattern. The holocellulose content of bamboo pulp, which is pretreated by microorganisms, is higher than that of bamboo pulp that is missing microorganism pretreatment, by up to 97.72% (C), as shown in Figure 5a. The cellulose content of pulp (89.67%), which is pretreated by C, is higher than that of pulp, which is missing pretreatment (81.83%), pretreated by indigenous microorganism (82.78%), and exogenous microorganisms A (81.45%) or B (81.72%) (Figure 5b). Furthermore, cellulose is the main component of cellulose paper and plays a crucial role in the physical properties of cellulose paper. The cellulose content for holocellulose of pulp pretreated by microorganisms, in addition to C, is lower than that of pulp pretreated by indigenous microorganisms. The opposite is true for the hemicellulose content for holocellulose or specimen of pulp.

It is evident that the discrepancy in the components of pulp is owing to the pretreatment via various microorganisms due to the Kraft cooking which proceeds under the same conditions. Obviously, bamboo pretreated by the indigenous microorganism and exogenous microorganisms (A or B) exhibits a similar retention pattern of cellulose and hemicellulose in the cooking process, i.e., the holocellulose content of these pulps increases, the cellulose content for holocellulose of these pulps decreases, and the hemicellulose content for both holocellulose and specimen increases, compared with those indexes for pulp missing microorganism pretreatment. Fortunately, the holocellulose of pulp pretreated by C, i.e., the blended A and B, can be reserved commendably (Figure 5a). In addition, the cellulose content and hemicellulose content for holocellulose of pulp pretreated by C is higher and lower than that of other specimens, respectively, which is similar to the components of bamboo following microorganism pretreatment, which can be further confirmed by the result of the degradation rate/degradation selectivity of lignin and hemicelluloses (Figure 5c). Only pulp pretreated by C exhibits an improved cellulose yield, from 69.18% for pulp missing pretreatment (pretreated by an indigenous microorganism) to 79.40%. Further, the viscosity of pulp that suffers pretreatment improves significantly from 856 mL/g to

1259–1367 mL/g (Figure 5d), indicating that microorganism pretreatments are beneficial to retain carbohydrates (holocellulose) during the cooking process.

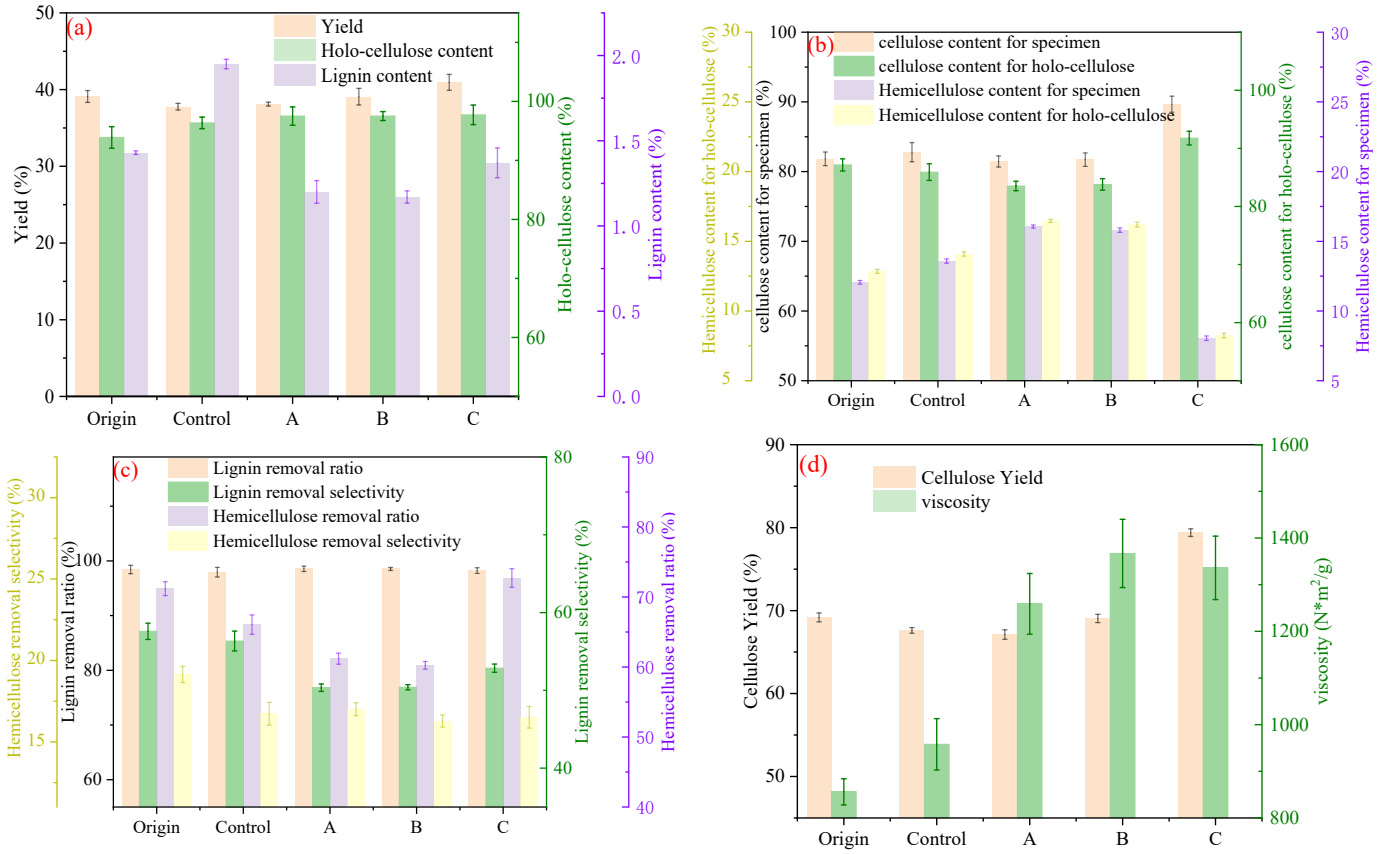


Figure 5. (**a**) Yield, holocellulose, and lignin content; (**b**) α-cellulose and hemicellulose content; (**c**) removal ratio and removal selectivity of lignin and hemicellulose; (**d**) cellulose yield and viscosity of specimens via Kraft cooking. A: pulp obtained from bamboo chips pretreated by microorganisms A, B: pulp obtained from bamboo chips pretreated by microorganisms B, C: pulp obtained from bamboo chips pretreated by microbial consortium C.

The physical properties of the hand paper are shown in Figure 6b. The key physical strengths of the samples, such as tensile index, tear index, and burst index, were significantly improved due to pretreatment by three exogenous microorganisms before cooking, with the highest increases of 16.3%, 33.97%, and 11.38%, respectively, compared with Origin. However, the tearing index of the C group was slightly lower than the other two groups of exogenous microorganisms, A and B. This may be mainly related to the differences in the content of fine fibers in the samples [44]. The fine fiber contents for A, B, and C are 14.8%, 13.2%, and 18.8%, respectively (Figure 6a). The specimens involved in microbiological pretreatment by C exhibit significantly high lignin/hemicellulose degradation selectivity due to the regulation of enzyme activities of lignin and hemicellulose degradation of bamboo chips by inoculated microorganisms/strains, resulting in an improved pulp quality that was confirmed by the increased cellulose content. In addition, the indexes, which affect the strength properties of hand paper, such as viscosity, fiber characteristics, etc., do not significantly reduce. Fortunately, the physical properties of hand paper, whether subject to microorganism pretreatment or not, can meet the requirement of the superior product of Kraft bamboo pulp without bleach (Q/79397065-5.2-2020).

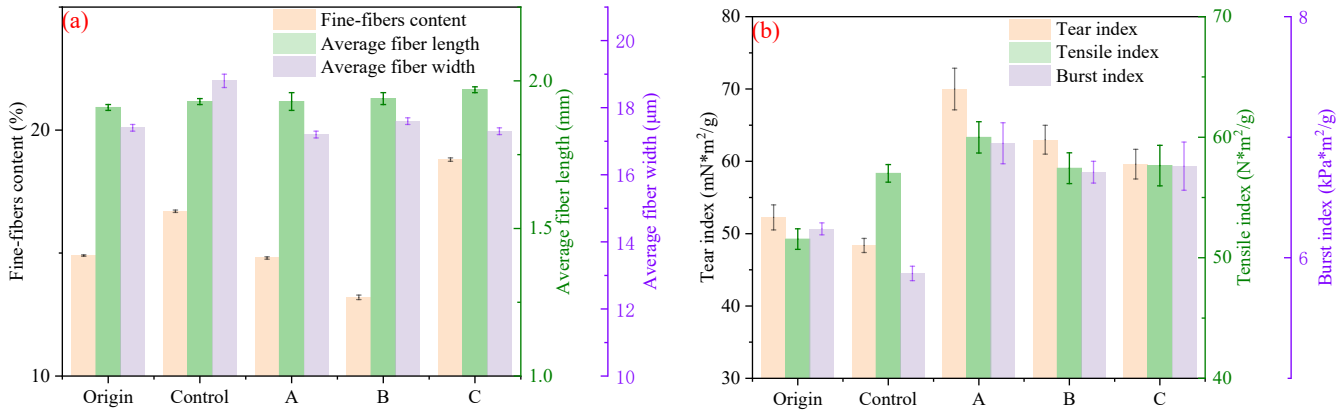


Figure 6. Fiber morphology and properties of hand papermaking of bamboo sulfate pulp (45°SR): (**a**) fiber morphology; (**b**) physical properties. A: hand-paper obtained from bamboo pulp A, B: hand-paper obtained from bamboo pulp B, C: hand-paper obtained from bamboo pulp C.

3.3.2. Effect of Pulping Process on the Potential for Reducing Consumption and Increasing Efficiency

In order to investigate the effect of microbiological pretreatment on the energy-saving and cost-reducing Kraft cooking for bamboo, the decreased Kraft cooking conditions were introduced. With the decrease in cooking times (from 185 min to 150 min and 120 min) and the decrease in cooking temperature (from 165 °C to 155 °C), Kraft pulp involved in C pretreatment still exhibits a stabilized total yield (ca. 40%), while the declining viscosity properties of this pulp and the efficiency on delignification are also observed. The physical properties of this hand paper, although inferior to the hand paper involved in C pretreatment in Section 3.3.1, still meet the requirement of superior products of Kraft bamboo pulp without bleach (Q/79397065-5.2-2020, Table S1). Under the final declined cooking conditions (time and temperature), Kraft pulp and hand paper involved raw bamboo, with a total yield of 34.33%, a tensile index of 38.6 N × m/g, a tear index of 29.46 mN × m^2/g, and a burst index of 5.93 kPa × m^2/g obtained (Table S1). In addition, the above physical properties cannot simultaneously meet the requirement of superior products of Kraft bamboo pulp without bleach (Q/79397065-5.2-2020). Fortunately, the potential effect of microbiological treatment on energy-saving and cost-reducing Kraft cooking can be confirmed. This microbiological treatment can reduce the cooking time (ca. 65 min) and the cooking temperature (10 °C) subject to meeting the requirements of the final products. Moreover, this microbiological treatment is beneficial for the beating of pulp. Under the decreased cooking conditions, the obtained pulp involved C pretreatment beating to 45°SR, requiring ca. 5600 circles by PFI, which is lower than the missing pretreatment pulp that required circles beating to 45°SR (ca. 6400 circles). The lower requirement in beating results in further energy saving and cost reducing for the bamboo pulping and papermaking industry.

4. Conclusions

In this study, microbiological pretreatment was used to improve Kraft cooking from bamboo chips (*Neosinocalamus affinis*). Bamboo, via the co-cultured A and B microbiological pretreatment and Kraft cooking, can obtain unbleached Kraft pulp with the highest total yield (greater than 40%) and the highest cellulose yield (ca. 80%). Compared with hand paper without microbiological treatment, the tensile index, tear index, and burst index of the corresponding hand paper increased by 16.3%, 33.97%, and 11.38%, respectively. The results of gene sequencing via 16S rRNA, the co-cultured microbial flora is mainly constituted of *Proteobacteria*, *Firmicutes*, *Bacteroidota*, and *Actinobacteriota*. The results of PICRUSt confirmed that the co-culture of A and B can enhance the enzymatic activity of enzymes that are related to the degradation of hemicellulose and lignin. With the

combined effect of microorganisms and related enzymes, the hemicellulose content and lignin content of bamboo chips decrease, indicating that the co-cultured microorganisms exhibit a selective removal effect on lignin and hemicellulose. The selective removal of lignin and hemicelluloses is beneficial for the improved accessibility of bamboo chips and the swelling ability of cooking liquor during Kraft cooking, resulting in an improved degradation selectivity of hemicellulose and lignin. Moreover, the decreased crystal index of pretreated bamboo chips indicates that the crystalline regions of cellulose might be disordered during microbiological pretreatment. The disordered regions of cellulose are also beneficial for Kraft cooking, but they are rarely removed during Kraft cooking. It is evident that the co-cultured microorganism can be used for bamboo pretreatment to reduce the Kraft cooking conditions, especially with the decline of cooking time and maximum cooking temperature. With a 65min reduction in cooking time and a 10 °C decline in maximum cooking temperature, the obtained unbleached Kraft pulp can also meet the requirements of superior products of unbleached Kraft bamboo pulp (Q/79397065-5.2-2020) despite the physical properties of the corresponding hand-paper decrease. Hence, the previously mentioned microbiological treatment can be considered an efficient approach for energy-saving and cost-reducing bamboo bio-refining.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9040400/s1, Figure S1: The cooking curue before/after modifying; Figure S2: Results of yeild of cooked pulp with different active alkali loading and sulfidity; Table S1: The strength properties of hand papers and the requirement in strength properties of unbleached bamboo pulp.

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