fermentation

MDPI

Article

Synergistic Enhancement Effect of Compound Additive of Organic Alcohols and Biosurfactant on Enzymatic Hydrolysis of Lignocellulose

Cuiyi Liang [1], Qifa Feng [1,2], Si Lu [1], Qiong Wang [1], Yunzi Hu [1], Zhongming Wang [1,2], Wen Wang [1,2,*] and Wei Qi [1,2,*]

[1] Guangdong Key Laboratory of New and Renewable Energy Research and Development, Guangzhou Institute of Energy Conversion, Chinese Academy of Sciences, Guangzhou 510640, China
[2] Materials and Chemical Industry, University of Chinese Academy of Sciences, Beijing 100049, China
* Correspondence: wangwen@ms.giec.ac.cn (W.W.); qiwei@ms.giec.ac.cn (W.Q.)

Abstract: The insufficient of lignocellulose degradation enzymes, such as cellulase and hemicellulase, is the major obstacle that hinders the bioconversion of lignocellulosic biomass to monosaccharides, especially during the woody biomass hydrolysis process. The addition of additives has received significant attention due to their enhancement of the enzymatic degradation efficiency of lignocellulose. In the present study, a combination of organic alcohols and a biosurfactant could synergistically enhance the saccharification of the cellulose substrate of Avicel, as well as that of pretreated poplar. Results showed that compound additives can greatly improve the conversion rate of enzymatic hydrolysis. The combination of 0.1% (*v*/*v*) n-decanol and 1% (*v*/*v*) sophorolipid dramatically increased the poplar enzymatic conversion rate from 17.9% to 85%, improving it by 67.1%. Enzyme-rich *Hypocrea* sp. W_{63} was fermented to obtain beta-glucosidase (BGL) and xylanase (XYL), which were used as auxiliary enzymes during enzymatic hydrolysis. It was found that the effects of such a combination of additives improved the filter paper activity, stability, and longevity, helping in the recovery of the cellulase cocktail. The compound additives associated with the commercial cellulase and *Hypocrea* sp. W_{63} enzyme solution formed an excellent formula for improving the stability of BGL and XYL. The results provide insight into compound additives and the use of a cellulase and auxiliary enzyme cocktail to improve enzymatic hydrolysis for lignocellulose conversion into biofuels.

Keywords: woody biomass; organic alcohols; biosurfactant; compound additives; enzymatic hydrolysis

Citation: Liang, C.; Feng, Q.; Lu, S.; Wang, Q.; Hu, Y.; Wang, Z.; Wang, W.; Qi, W. Synergistic Enhancement Effect of Compound Additive of Organic Alcohols and Biosurfactant on Enzymatic Hydrolysis of Lignocellulose. *Fermentation* **2022**, *8*, 725. https://doi.org/10.3390/fermentation8120725

Academic Editors: Xiaoqing Lin and Alexander Rapoport

Received: 18 October 2022
Accepted: 6 December 2022
Published: 11 December 2022

1. Introduction

The transformation of lignocellulosic biomass into clean and renewable energy is one of the most effective ways to solve the problems of fossil resource shortage, energy security, and environmental deterioration [1]. Nevertheless, the bioconversion of lignocellulosic biomass has some technological bottlenecks, one of which is the high cost of the process of lignocellulose degradation to fermentable monosaccharides through the use of cellulolytic enzyme cocktails [2]. Many researchers have focused on improving the conversion rate to reduce the amount of cellulase used [3]. Different strategies have been applied to overcome this challenge and enhance the efficiency of the enzymatic hydrolysis, such as the screening of microorganisms to find auxiliary enzymes with better performance [4], the optimization of the enzymatic hydrolysis process, and the supplementation of additives [5]. However, the performance of additives is strongly influenced by the biomass type, enzyme cocktail used, hydrolysis condition, and additive composition [6].

Woody biomass is a potential feedstock that can be biorefined into high-value products through the bioconversion process [7,8]. Waste poplar, a woody manufacturing residue, can be used as a suitable biorefinery feedstock because poplar is a fast growing woody

species that is widely distributed around the world. It has a matrix composed of three major biopolymeric components: lignin (10–21%), hemicellulose (25–28%), and cellulose (45–51%) [9]. It has a more compactable property than crop straw and grassy biomass, which is attributed to its high heterogeneity and complex structure [10,11]. It is difficult to achieve an ideal enzymatic hydrolysis of woody biomass, as this is only possible after a certain pretreatment [12]. Cellulase is a group of crucial enzymes that can hydrolyze lignocellulosic carbohydrates into fermentable sugars. It is often referred to as the "cellulase complex", which traditionally contains three major enzymes: cellobiohydrolase (CBH; EC 3.2.1.91), endoglucanase (EG; EC 3.2.1.4), and beta-glucosidase (BGL; EC 3.2.1.21). Among them, BGL is a key enzyme in the sugar–enzyme platform of lignocellulose degradation. This was because that CBH and EG act synergistically to degrade native cellulose to cellobiose, which is an inhibitor of both enzymes [13]. In this sences, BGL can hydrolyse the β-1, 4-glycosidic linkage of cellobiose to generate D-glucose. Hence, BGL not only catalyses the final step of cellulose degradation but also can allow the cellulolytic enzymes to function more efficiently by eliminating cellobiose-mediated inhibition [14]. On the other hand, xylanase (XYL) is a vital auxiliary enzyme that disrupts xylan and xylooligomers to enhance the cellulases' access to the biomass substrate [15]. Hence, BGL and XYL are involved in the important biological degradation of lignocellulose and other soluble oligosaccharides into fermentable sugar [16]. However, most commercial cellulases that are used for lignocellulosic hydrolysis are produced by filamentous fungi that have little BGL activity [17]. In recent years, solvent media engineering has received increasing attention due to the optimization of the enzymatic conversion of lignocellulose [18,19]. Our previous study screened out a strain of *Hypocrea* sp. W_{63} that produces a high amount of BGL and XYL [20]. Herein, this research is expected to improve the enzymatic hydrolysis efficiency of woody biomass.

The enzymatic hydrolysis process is known as the most important step throughout the conversion of lignocellulosic biomass into monosaccharides. However, a high lignin content in lignocellulose hinders the enzymatic hydrolysis process due to the nonproductive adsorption of cellulase and hemicellulase, therefor increasing hydrolytic enzyme loading [21]. Some additives can play a strengthening role in the enzymatic hydrolysis process by stopping the nonproductive adsorption of enzymatic proteins. The supplementation of additives (nonionic surfactants, nonhydrolytic disruptive proteins, anionic surfactants, biosurfactants, and lignosulphonates) has received a high amount of attention due to their enhancement of the enzymatic degradation efficiency of lignocellulose [22–24]. Organic alcohols (OAs), as an environmentally friendly, nonionic surfactant, have a good defoaming performance. Recent studies have found that low doses of OAs (0.02–2% v/v) can significantly increase the enzymatic hydrolysis of cellulose films from 65.1 to 77.9%, effectively reducing the viscosity coefficient of the cellulose enzymatic hydrolysis system and improving the cellulose's accessibility to enzymes [23,25]. Additionally, biosurfactants which are easily biodegradable and add no pollution to the environment are considered to be one of the most effective additives for the enzymatic hydrolysis of lignocellulose [26,27]. It was found the sophorolipid (SL), a kind of biosurfactant, had a good promotion effect on the enzymatic hydrolysis of alkali-treated bagasse, and the presence of SLs increased the glucose yield by 17.8% compared with the control [3]. Thus, it was assumed that a combination of additives would be an interesting strategy to further improve the saccharification efficiency as they probably have a synergistic mechanism [28].

Until now, there have been insufficient data for systematically evaluating the mechanism of different combinations of additives in the enzymatic hydrolysis of crystalline cellulose and woody biomass. This study screened different OAs for the improvement of enzymatic hydrolysis and investigated the effect of the combination of OA and SL additives, using a BGL- and XYL-rich crude enzyme from W_{63} as an extra auxiliary enzyme in the enzymatic hydrolysis of Avicel and pretreated poplar. The relative decrease/increase in enzymatic activity and stability of the commercial cellulase or W_{63} enzyme cocktail with

the addition of certain compound additives was also estimated. This research provides a strategy to boost the enzymatic hydrolysis process of lignocellulosic biomass.

2. Materials and Methods

2.1. Materials

Poplar chips were provided by Shandong Longlive Bio-technology Co., Ltd. (Dezhou, China). They were pre-milled and screened for particle sizes between 60 and 80 meshes. The contents of glucan, xylan, and acid-insoluble lignin were 41.96%, 16.90%, and 24.27%, respectively, as analyzed by the NREL's standard for laboratory analytical procedures (LAPs) [29].

A commercial mixture of cellulase LLC02 with an enzyme activity of 225 FPU/mL (filter paper unit) was purchased from Kangdien Biotech Co., Ltd. (Qingdao, China). Avicel crystalline cellulose (PH101) was purchased from Sigma-Aldrich (Shanghai, China). OAs (methanol, ethanol, glycerol, n-butanol, n-pentanol, n-octanol, and n-decanol) were marked as C1-C5, C8, and C10, according to the carbon chain length in the alcohol. Isoamyl alcohol and phenyl ethanol were marked as I5 and P8, respectively, according to the carbon atom. All the above OAs were purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. (Shangai, China). Sophorolipid (SL) were purchased from Zuoke Biotech Co., Ltd. (Guangzhou, China).

2.2. BGL Preparation

Hypocrea sp. W_{63} was stored in our laboratory (patent No. US 9,890,371 B2). The fungal strain was inoculated into a 150 mL Erlenmeyer flask with 50 mL of potato-dextrose liquid medium for the fermentative production of BGL and XYL at 30 °C and 150 rpm for 72 h. The fermentation formula contained 2 g/L of KH_2PO_4, 6 g/L of peptone, 0.3 g/L of $CaCl_2{\bullet}2H_2O$, 0.3 g/L of $MgSO_4{\bullet}7H_2O$, and 1 g/L of NH_4NO_3, as well as 1% (*v*/*v*) of trace elements containing 1 g/L of $CuSO_4$, 1 g/L of $MnSO_4$, 1 g/L of $FeSO_4{\bullet}7H_2O$, and 1 g/L of $CoCl_2$. The cellulose substrate of rice straw was supplied at a concentration of 5% (*w*/*v*). Fermentation was carried in a 250 mL Erlenmeyer flask with a 10% (*v*/*v*) seed culture of W_{63} inoculated into a volume of 100 mL. After fermentation at 30 °C and 150 rpm for 120 h, the fermented broth was centrifuged at 4 °C and 8000 rpm for 10 min. The supernatant was collected as a W_{63} crude enzyme solution that is rich in BGL and XYL, and was stored at 4 °C before use.

2.3. Methods

2.3.1. Pretreatment of Poplar

In total, 5 g of milled poplar was added into a 125 mL pressure bottle containing a 0.5% (*w*/*v*) H_2SO_4 solution to form a solid–liquid ratio of 1:20. The bottle was autoclaved at 121 °C for 1 h and then cooled to room temperature. The acid-treated poplar was washed with distilled water until it was pH neutral, and oven-dried at 60 °C until it reached a constant weight. The alkaline pretreatment of the acid-treated poplar was performed with a 2% (*w*/*v*) NaOH solution under the same conditions as the acid pretreatment. The treated poplar was washed and dried at 60 °C until it reached a constant weight. The content of glucan and acid-insoluble lignin of the two-step-treated poplar was 55.01% and 28.66%, respectively.

2.3.2. Screening of Different OAs

In total, 1 g of Avicel (PH101) was added to a 25 mL Erlenmeyer flask containing the 10 FPU/g LLC02 commercial cellulase substrate and the 40 mg/g W_{63} crude enzyme protein substrate that is rich in BGL and XYL. Then, a 0.1% (*v*/*v*) or 1% (*v*/*v*) additive of OAs (C1–C5, C8, and C10) was loaded according to the carbon chain length. Additionally, a 0.1% (*v*/*v*) or 1% (*v*/*v*) additive of OA isomers I5 and P8 was loaded, respectively. The hydrolysis experiment without any OA addition was set as the control. The total working volume of 10 mL was supplemented by a 20 mM HAc-NaAc (pH 5.0) buffer. After being sealed with taps, all flasks were kept at 50 °C and 150 rpm for 72 h. Each sample was

evaluated in triplicate, and the results were expressed as an average value. All the samples were taken at 24 h, 48 h, and 72 h, and were used for determining the sugar contents via HPLC.

2.3.3. Synergistic Effect of Different Compound Additives

Based on the OA screening results, the compound additives of 0.1% (*v*/*v*) C1, C4, C5, I5, or C10 mixed with 1% (*v*/*v*) SLs were used in the Avicel hydrolysis process. The mixing formula of each compound additive is shown in Table 1. Additionally, the enzymatic hydrolysis was conducted under the same conditions as described in Section 2.3.2.

Table 1. The mixing formulas of compound additives.

Mixture	T1	T2	T3	T4	T5
Additive 1 (0.1% *v*/*v*)	C1	C4	C5	I5	C10
Additive 2 (1% *v*/*v*)	SL	SL	SL	SL	SL

2.3.4. Enzymatic Hydrolysis Effect of Poplar with Different Compound Additives

Acid-alkali-treated poplar, in an amount of 0.1 g, was placed into a 25 mL Erlenmeyer flask supplemented with the 10 FPU/g LLC02 commercial cellulase substrate and 40 m/g W_{63} crude enzyme protein substrate that is rich in BGL and XYL. The pretreated poplar enzymatic hydrolysis process with different compound additives is described in Table 1. The total enzymatic working volume was 5 mL and was supplemented with a 20 mM HAc-NaAc (pH: 5.0) buffer. Additionally, the enzymatic hydrolysis was conducted under the same conditions as described in Section 2.3.2.

2.3.5. Activity and Stability Effect of LLC02 and W_{63} with or without the Addition of Compound Additives

LLC02 and W_{63} were diluted to appropriate concentrations to evaluate cellulase and hemicellulase activities. The filter paper activity (FPA) was investigated by using the modified method of Ghose [30]. CMCase activity was determined with 1% (*w*/*v*) carboxymethylcellulose (CMC) incubated at a pH of 5.0 and at 50 °C for 1 h [31]. β-glucosidase activity (BGL) was measured using a 5 mM 4-nitrophenyl-β-D-glucopyranoside (*p*NPG) as the model substrate [20]. Xylanase activity was estimated with 1% (*w*/*v*) xylan incubated at a pH of 5.0 and at 50 °C for 0.5 h [32]. Meanwhile, different combinations of additives, as shown in Table 1, were added to all of the above enzyme activity assay groups. The groups of LLC02 and W_{63} without the addition of additives were set as controls. The detailed information on the analysis of the FPA, CMC, BGL, and XYL activities is described in our previous study [20].

The stability of the LLC02 enzyme and W_{63} crude enzyme in the presence of different combinations of additives is shown in Table 1. It was measured by incubating a certain dilution of LLC02 or W_{63} crude enzyme solution at 50 °C and 150 rpm for 24 h in 5 mL of a 20 mM HAc-NaAc buffer (pH 5.0). Subsequently, the incubated samples of LLC02 or W_{63} mixture with different compound additives were used to analyze for the changing of enzyme activities assay.

The relative decrease/increase in activity and stability was standardized with the experiment without additives.

2.4. Analysis Methods

The reducing sugars were analyzed at 50 °C via a high-performance liquid chromatograph (HPLC; Waters 2698, Milford, MA, USA) equipped with a differential refractive index detector (Waters 2410, Milford, MA, USA) and a sugar column (SH1011, Shodex, Tokyo, Japan). The 5 mM H_2SO_4 mobile phase was set at a flow rate of 0.5 mL/min [33].

2.5. Calculation

The effects of different additives on Avicel hydrolysis was evaluated by using Equation (1):

$$\text{Relative enzymatic conversion rate\%} = \frac{\text{Glucose produced in the sample (g)}}{\text{Glucose produced in the control (g)}} \times 100\% \quad (1)$$

where the sample is the enzymatic hydrolysate with the addition of additives and the control is the one without the addition of additives.

The enzymatic hydrolysis efficiency of Avicel and poplar was calculated via Equation (2):

$$\text{Cellulose conversion rate\%} = \frac{\text{Gluose produced in enzymatic hydrolysate (g)}}{\text{Glucan amount in substrate (g)} \div 0.9} \times 100\% \quad (2)$$

where 0.9 is the dehydration coefficient of the synthesis of glucose to glucan.

The effects of different compound additives on the enzyme activity and stability of the LLC02 and W_{63} crude enzyme were calculated with Equation (3):

$$\text{Relative decreasing/increasing enzymatic conversion rate\%} = \left(\frac{\text{Glucose produced in the sample (g)}}{\text{Glucose produced in the control (g)}} \times 100\%\right) - 100\% \quad (3)$$

3. Results and Discussion

3.1. Screening of Different Straight-Chain OAs for Enhancement of Enzymatic Hydrolysis

The long-chain fatty alcohols (LFAs) of n-octanol (C8), n-decanol (C10), n-dodecanol (C12), n-hexadecanol (C16), and n-octadecanol (C18) have been well studied as nonionic surfactants for improving the enzymatic hydrolysis of lignin-cellulose films [23,25]. Low-carbon alcohols, including C1–C5, C8, and C10, were selected in this study to investigate their impact on the enzymatic hydrolysis of crystalline cellulose (Table 2). The enzymatic hydrolysis of Avicel was improved by OAs at a low concentration of 0.1% (*v*/*v*) for 48 h, but this improvement decreased after 72 h. The increase in the enzymatic hydrolysis of Avicel by C1–C5, C8, and C10 was 10.62%, 4.14%, 2.42%, 11.85%, 8.83%, 9.38%, and 12.04%, respectively. This enhancement was related to the structural change in the crystal cellulose due to the interaction between the enzyme and OA during the enzymatic hydrolysis, which might lead to the exposure of a more accessible surface area [34]. The reason for the decrease in the relative cellulose conversion rate at 72 h could be ascribed to the fact that the active side of the OA no longer responds to the collapsed crystal structure of Avicel after 48 h.

Table 2. Effects of different OAs on enzymatic hydrolysis of Avicel.

Organic Alcohol	Carbon Chain Length	Relative Conversion Rate [a]					
		0.1% (*v*/*v*) Addition			1% (*v*/*v*) Addition		
		24 h	48 h	72 h	24 h	48 h	72 h
Methanol	C1	96.87	110.62	100.17	99.34	97.93	93.62
Ethanol	C2	102.98	104.14	99.69	91.42	97.92	80.04
Glycerol	C3	102.25	102.42	98.34	96.93	95.57	95.21
n-Butanol	C4	106.27	111.85	100.62	95.13	98.45	85.95
n-Pentanol	C5	97.58	108.83	92.86	82.82	91.51	78.38
n-Octanol	C8	100.89	109.38	91.33	99.42	109.20	98.18
n-Decanol	C10	107.94	112.04	105.86	105.41	127.96	97.54

[a] Relative conversion rate was standardized by using the experiment without additives.

The relative conversion rates of Avicel at 48 h with 1% (*v*/*v*) C8 and C10 were 109.20 and 127.96%, which were 9.2 and 27.96% higher than the control groups. The promoting effect of C8 and C10 on the enzymatic hydrolysis process may be due to the positive reaction of octanol and decanol with Avicel [22], whereas the relative cellulose conversion rates of Avicel with 1% (*v*/*v*) C1–C5 supplements were inhibited. The negative effect

could be explained by the hydrophobic interactions of C1–C5 with the crystalline cellulose substrate [25].

3.2. Effect of Different OA Isomers on Enzymatic Hydrolysis of Crystalline Cellulose

The impact of molecular configuration on enzymatic hydrolysis was investigated with the organic alcohol isomers by comparing n-pentanol (C5)/isoamyl alcohol (I5) and n-octanol (C8)/phenyl ethanol (P8). The results are shown in Figure 1.

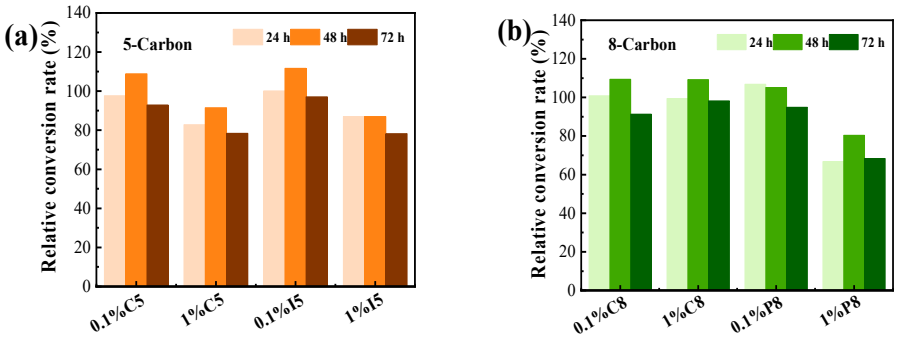

Figure 1. Effects of organic alcohol isomers on enzymatic hydrolysis of Avicel (**a**) isomers of 5-carbon; (**b**) isomers of 8-carbon. Organic alcohol isomers were supplemented at 0.1% (*v*/*v*) or 1% (*v*/*v*). Relative conversion rate was standardized by the experiment without additives. (C5—n-pentanol; I5—isoamyl alcohol; C8—n-octanol; P8—phenyl ethanol).

When C5 and I5 were supplemented at a low concentration of 0.1% (*v*/*v*), they had a similar influencing behavior on the enzymatic hydrolysis of Avicel. The results show that both C5 and I5 had a positive effect on enzymatic hydrolysis at 48 h, but a negative effect at 72 h. The highest relative conversion rate of C5 and I5 was increased by 8.83% and 11.65% at 48 h, respectively. However, it was found that both C5 and I5 impeded the enzymatic hydrolysis of Avicel at a high concentration (1% *v*/*v*) (Figure 1a). Based on the above results, C5 and I5, with the same carbon atoms, showed a similar impact on the enzymatic hydrolysis of cellulose. This indicates that a hydroxyl connected with a straight chain or branched chain does not affect the digestibility of cellulose.

On the other side, the highest relative conversion rate of Avicel with the addition of 0.1% (*v*/*v*) C8 was increased by 9.38% at 48 h, whereas that with the addition of 0.1% (*v*/*v*) P8 was increased by 6.81% at 24 h (Figure 1b). The enzymatic hydrolysis efficiency of Avicel was enhanced by 1% (*v*/*v*) C8, but was strongly inhibited by 1% (*v*/*v*) phenyl ethanol at 48 h. A similar result was found in a previous study that found that 8-carbon straight-chain alcohols were conducive to increasing the enzymatic hydrolysis, but 8-carbon isomeric alcohols impeded enzymatic hydrolysis because of the stronger steric hindrance effect [25]. Above all, the OAs of C1, C4, C5, I5, C8, and C10 at a low concentration (0.1% *v*/*v*) were suitable to be used to enhance the enzymatic hydrolysis of cellulose.

3.3. Associations between Additives in the Enzymatic Hydrolysis of Crystalline Cellulose

The addition of an additive mixture might be a feasible strategy for improving the enzymatic hydrolysis process due to its possible synergistic effect. Bio-additives of SL, a kind of surfactant, possess a hydrophilic sophorose group and hydrophobic saturated or unsaturated ω-(or ω-1) hydroxy long-chain fatty acids [3]. The synergistic effect of OAs and SL on the enzymatic hydrolysis of cellulose was investigated.

The W_{63} crude enzyme was supplemented with the 40 mg/g substrate, and the effects of all compound additives are illustrated in Figure 2. Compared to the control (without additives) and the supplementation of SLs individually, the enzymatic conversion rate of Avicel based on the association with the compound additives of T1, T2, T3, T4, and T5 at 72 h was increased by 6.7%, 4.7%, 5.2%, 5.6%, and 8.0%, respectively. The promotion effects of the compound additives of OA and SL might be due to the hydrophobicity that was

formed by the combination of different OAs and SL. Furthermore, the cellulose conversion rate at 72 h for the OA isomers was not different between T3 and T4. Here, the enzymatic hydrolysis results are close to those from the addition of the OA isomers of C5 and I5 solely. Consequently, the enzymatic conversion rate of Avicel in the presence of compound additives followed the order of T5 > T1 > T4 > T3 > T2 > SL > control. The positive effect of this association was probably due to the synergistic enhancement of the OAs and SL in the release of the unproductive binding of cellulase and hemicellulase, especially BGL and XYL [35], while also protecting the stability of the enzymes and the reduction in the protein aggregation and deactivation [36].

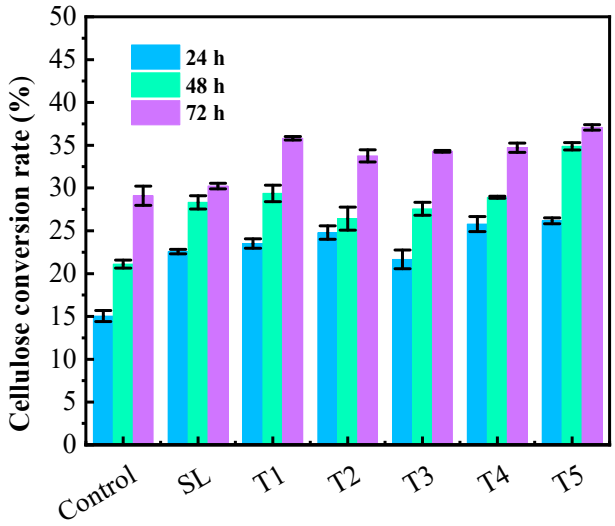

Figure 2. Effects of different compound additives on enzymatic hydrolysis of Avicel. SL—sophorolipid; T1—methanol + sophorolipid (C1 + SL); T2—n-butanol + sophorolipid (C4 + SL); T3—n-pentanol + sophorolipid (C5 + SL); T4—isoamyl alcohol + sophorolipid (I5 + SL); T5—n-decanol + sophorolipid (C10 + SL). Experaments were determined in use with 10% (*w*/*v*) Avicel as substrate with cellulase of 10 FPU/g substrate and W_{63} crude enzyme protein of 40 mg/g substrate hydrolysis for 72 h.

3.4. Synergistic Enhancement of Poplar Enzymatic Hydrolysis by Compound Additives

The outstanding performances of different OAs and SL were investigated on the pretreated poplar. The results show that all the compound additives of T1, T2, T3, T4, and T5 could dramatically increase the cellulose conversion rate, compared to the control without additives or with only the addition of SL (Figure 3).

When the dosage of the W_{63} crude enzyme protein was loaded with a 40 mg/g substrate, the mixture addition of T1, T2, T3, T4, and T5 reached the highest cellulose conversion rates of 80.4%, 77.5%, 77.1%, 69.5%, and 85%, respectively. The dramatic enhancement of the enzymatic conversion rate of pretreated poplar with the addition of different compound additives was much higher than the enhancement of the crystallinity cellulose of Avicel. The difference was because the hydrolysis concentration of pretreated poplar was 1% (*w*/*v*), whereas the hydrolysis concentration of Avicel was 10% (*w*/*v*). The promotion effects were comparable to those obtained in the enzymatic hydrolysis conversion rate when using a biosurfactant alone. This result implies that the mixture of these compound additives could enhance the enzymatic hydrolysis rate more effectively than a single additive. This could be attributed to the synergetic effect of 0.1% (*v*/*v*) OA and 1% (*v*/*v*) SL stopping the nonproductive adsorption of the enzyme in the substrate and protecting the deactivation of the enzyme proteins [5]. It was found that, with combinations of different kinds of OAs and SLs, the hydrolysis conversion rate was not only based on the OA of their carbon chain length, but was also affected by the changing of the poplar active site after the substrate pretreatment. The cellulose conversion rate of pretreated poplar in the presence of compound additives followed the order of T5 > T1 > T2 > T3 > T4 > SL. Additionally, a significant difference was observed in the OA isomers that had the cellulose

conversion rate between T3 and T4. It is obvious that the OA straight chain was superior to the side chain for the enzymatic conversion of lignocellulose.

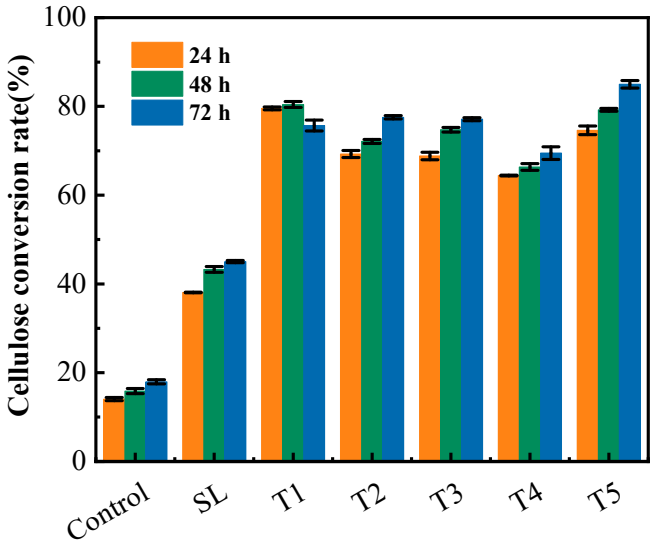

Figure 3. Effects of different compound additives on enzymatic hydrolysis of poplar. SL—sophorolipid, T1—methanol + sophorolipid (C1 + SL); T2—n-butanol + sophorolipid (C4 + SL); T3—n-pentanol + sophorolipid (C5 + SL); T4—isoamyl alcohol + sophorolipid (I5 + SL); T5—n-decanol + sophorolipid (C10 + SL). Experaments were determined in use with 2% (*w*/*v*) pretreated poplar as substrate with cellulase of 10 FPU/g substrate and W_{63} crude enzyme protein of 40 mg/g substrate hydrolysis for 72 h.

3.5. Effects of Different Compound Additives on Activity and Stability of LLC02 and W_{63}

At present, most studies take the purified enzyme as the object and do not consider the comprehensive effects on the cellulase complex [4,22,37,38]. In this study, the effects of different compound additives on the filter paper enzyme activity (FPA), endoglucanase activity (CMC), β-glucosidase activity (BGL), xylanase activity (XYL) and stability of the LLC02 and W_{63} crude enzyme were investigated to better understand the mechanism of the compound additives that affects enzymatic hydrolysis. All the results of relative decreasing/increasing activity are shown in Figure 4.

When compared with the LLC02, two compound additives of T1 and T4 (Figure 4a) had the most obvious promotion effect on the FPA, and the relative enzyme activity was increased by 62.7% and 46.5%, respectively. However, the compound additives of T2 and T5 had negative effects, which decreased the FPA by 26.8% and 33.4%, respectively. For the enzyme stability, the FPA of LLC02 was decreased by 43.6% without the supplementation of compound additives. On the contrary, the stability of the LLC02 FPA in the presence of the compound additives of T1, T2, T3, T4, and T5 was increased by 100%, 76.3%, 75.2%, 78.3%, and 93.9%, respectively. The increase in FPA stability was due to the presence of different compound additives. It can be concluded that all of these compound additives could improve the FPA, enzyme stability, and longevity, facilitating the recovery of cellulase. As shown in Figure 4b, compared to the W_{63} crude enzyme without additives, FPA activity was highly improved in the presence of the compound additives of T1, T2, T3, T4, and T5, which increased by 88.7%, 87%, 127.4%, 266.4%, and 102.1%, respectively. For FPA stability, compared to the control of W_{63}, all the samples with a supplement of different compound additives were slightly inhibited. The decreasing stability of W_{63} might due to it being a fermented crude enzyme solution that, without a stabilizer, is different from the commercial cellulase. Consequently, there are dramatic increases in the enzymatic hydrolysis for all of the compound additives compared to the control of LLC02 and W_{63} without any additives.

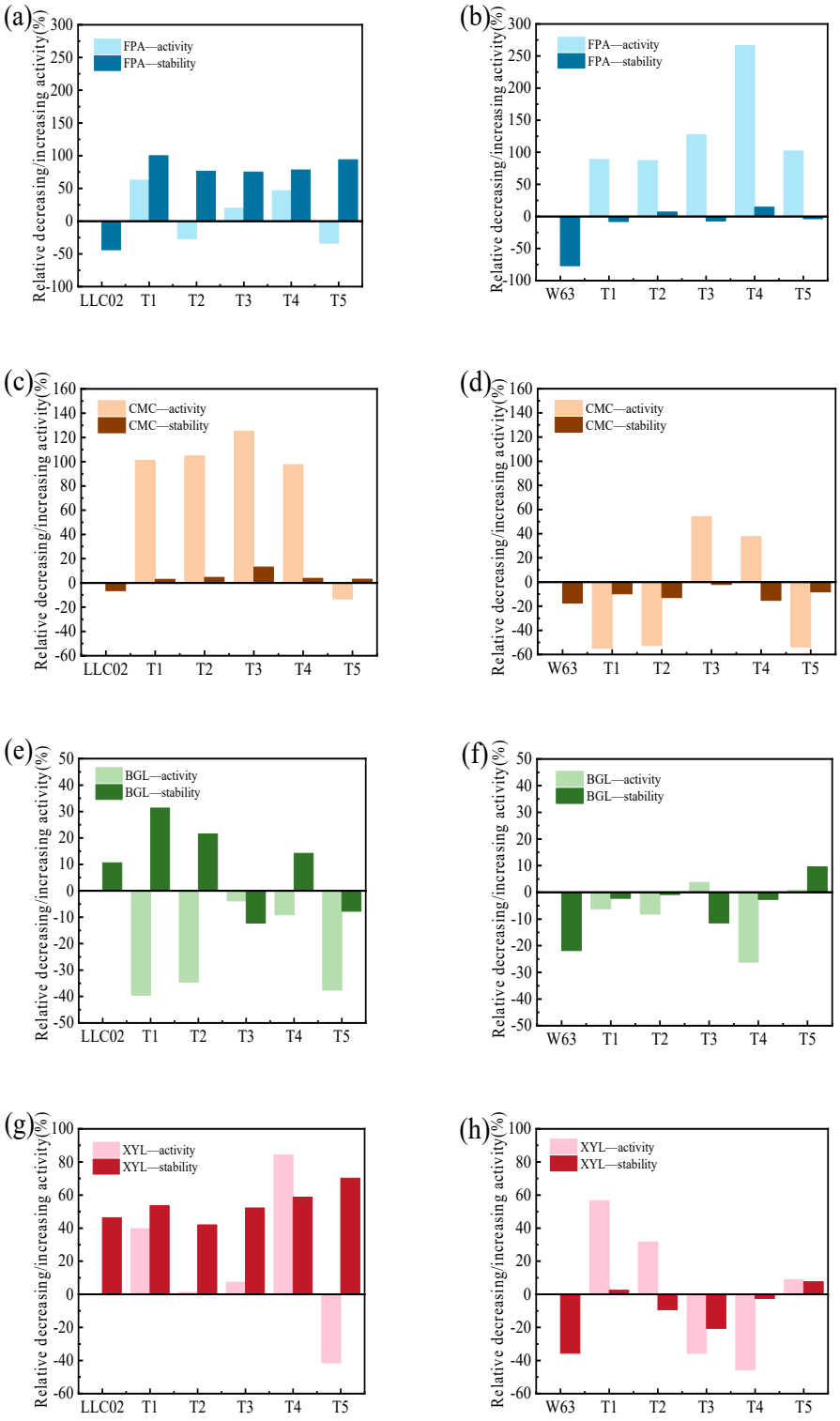

Figure 4. Effects of different compound additives on relative decreasing/increasing enzyme activities and stability of LLC02 and W_{63}. T1—methanol + sophorolipid (C1 + SL); T2—n-butanol + sophorolipid (C4 + SL); T3—n-pentanol + sophorolipid (C5 + SL); T4—isoamyl alcohol + sophorolipid (I5 + SL); T5—n-decanol + sophorolipid (C10 + SL). (**a**) Fpase activity and stability of LLC02; (**b**) Fpase activity and stability of W_{63}; (**c**) CMCase activity and stability of LLC02; (**d**) CMCase activity and stability of W_{63}; (**e**) *p*NPGase activity and stability of LLC02; (**f**) *p*NPGase activity and stability of W_{63}; (**g**) Xylanase activity and stability of LLC02; (**h**) Xylanase activity and stability of W_{63}. Relative decreasing/increasing enzyme activities was standardized by the experiment without additives.

As for the CMCase activity of LLC02 (Figure 4c), the T1, T2, T3, and T4 compound additives had greater promoting effects compared to the control, with T5 as the exception. CMCase activity from T1 to T4 sharply increased by 101.2%, 104.8%, 125.2%, and 97.6%, respectively. It can be assumed that the compound additive makes it more easy to disperse EG into the CMC substrate. However, for the CMCase stability of LLC02, all the samples merely have a slight increase when compared to the control. As shown in Figure 4d, the combination of T3 and T4 had a great promoting effect of 54.2% and 37.6% on the CMCase activity of the W_{63} crude enzyme, whereas T1, T2, and T5 significantly inhibited CMCase activity, which decreased by more than 50%. For W_{63} CMCase stability, the enzyme stability was slightly inhibited by all combinations (T1–T5).

In a previous study, it was observed that the surfactants of Tween 20, Triton X-100, and PEG4000 had a positive efficiency with regard to BGL activity [4,39,40]. This study revealed that the influence of different compound additives on LLC02 and W_{63} BGL activity and stability was different. As shown in Figure 4e,f, the results show that all of the compound additives were significantly inactivated for the BGL activity of LLC02. For the BGL stability of LLC02, the compound additives of T1, T2, and T4 could enhance the BGL stability, whereas T3 and T5's BGL stability was inhibited. For the BGL activity of W_{63}, the combination of T3 and T5 had a slight increase, whereas T1 and T2 were slightly inhibited in BGL activity. T4 had the greatest inhibition effect on W_{63} BGL activity, with the relative activity decreasing by 26.2%. The BGL stability of W_{63} slightly inhibited the relative enzyme activity with the addition of T1, T2, T3, and T4 compound additives. Meanwhile, the W_{63} BGL relative stability decreased the most, with a decrease of 21.8%, without the addition of compound additives. Surprisingly, T5 had the best stability of BGL, where the relative activity increased by 9.6%. This result indicates that the insufficiency and instability of BGL in LLC02 was replenished by the W_{63} BGL of T5, which may be the key factor that makes T5 become one of the most excellent compound additives for the promotion of enzymatic hydrolysis in lignocellulose.

It has been proved that the promotion effect of additives is due to the removal of xylan from the lignocellulose, which changes the physical structure of the substrate [41]. However, the influence of the compound additives on hemicellulase activity and stability were rarely reported. The results of XYL activity and stability are shown in Figure 4g,h. T1 and T4 show great improvement with regard to the XYL activity of LLC02, increasing by 39.7% and 84.2%, respectively, whereas T5 has the most inhibitory effect on XYL activity, which decreased by 41.4%. Surprisingly, the XYL stability of LLC02 was sharply increased by all of the compound additives. Among them, the XYL stability of T5 was dramatically increased by 70.2%. The contrast between the efficiency of XYL activity and stability for T5 might be due to the mixing of this kind of organic chemical group hindering the XYL activity at the beginning. After that, T5 developed hydrophobicity in the liquid phrase. affecting the promotion of the XYL stability. As seen with W_{63}, the compound additives of T1, T2, and T5 activated the XYL activity by 56.5%, 31.6%, and 8.9%, respectively. Moreover, the XYL stability of W_{63} with T1 and T5 was slightly increased by 2.4% and 7.6%. Based on the great promotion effect of T5 in the enzymatic hydrolysis of pretreated poplar, it can be suggested that the boosting effect of the additives during the hydrolysis of lignocellulose synergistically increased the activities of cellulases and xylanase, and promoted the interaction between the cellulases and xylanase that caused lignocellulose accessibility [42].

It has been reported that many additives could improve the digestibility of biomass significantly based on substrate properties [4]. Cellulose enzymatic hydrolysis is a complicated process, and thus, the determination of FPA, CMC, BGL, and XYL cannot represent the entirety of the enzymatic hydrolysis effect on lignocellulosic biomass. However, we can conclude that the relative decreasing/increasing activity and stability results of LLC02 and W_{63} are correlated with the enzymatic hydrolysis effects of Avicel and pretreated poplar. The results show that the association of T5 was the most efficient in the degradation of Avicel and pretreated poplar. We hypothesize that the excellent improvement in the hydrolysis of lignocellulose might be caused by the synergistic effects of 0.1% (v/v) C10 and 1%

(*v*/*v*) SL, which formed a hydrophobic environment that activated BGL and XYL activity, stabilized these two kinds of enzymes in the enzymatic hydrolysis process, and finally, played a promoting role in lignocellulosic digestion. However, further studies are needed to clarify the mechanisms and the economic analysis of the lignocellulose hydrolysis process.

4. Conclusions

The addition of OAs at a low concentration of 0.1% (*v*/*v*) could increase the enzymatic hydrolysis rate of Avicel. Among them, C1, C4, C5, I5, and C10 have higher efficiency with regard to the promotion of enzymatic hydrolysis. Subsequently, 0.1% (*v*/*v*) C1, C4, C5, I5, and C10 mixed with 1% (*v*/*v*) SL could further improve the enzymatic saccharification of Avicel and pretreated poplar. It can be concluded that all of these compound additives could enhance the FPA, enzyme stability, and longevity for LLC02 and W_{63}, helping with the recovery of the cellulolytic enzyme cocktails. The most effective compound additive of T5 containing 0.1% (*v*/*v*) C10 and 1% (*v*/*v*) SL dramatically increased the enzymatic hydrolysis of pretreated poplar from 17.9 to 85%, which was an improvement of 67.1%. The excellent improvement in the hydrolysis of lignocellulose might be caused by the synergism of T5, as it formed a hydrophobic environment that activated BGL and XYL activity and stabilized these two kinds of enzymes in the enzymatic hydrolysis process. Finally, the development of compound additives could effectively improve the hydrolysis rate of lignocellulose.

Author Contributions: C.L.: Conceptualization, Methodology, Formal analysis, Writing—original draft. Q.F.: Part experiment. S.L.: Part experiment. Q.W.: Data curation, Guidance on lignocellulosic biomass pretreatment. Y.H.: Writing—review and editing. Z.W.: Supervision. W.W.: Data curation, Methodology, Writing—review and editing. W.Q.: Project administration, Funding acquisition, Supervision. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the National Key R&D Program of China (2019YFB1503802); the National Natural Science Foundation of China (52006227 and 51976224); Guangdong Province Key Field R&D Program (2019B110209003); the Key Fundamental Research Project of Guangdong-Guangxi Joint Funding (2020B1515420005); Guangdong Basic and Applied Basic Research Foundation (2020A1515011012); Youth Innovation Promotion Association, CAS (2021351).

Data Availability Statement: The figures and tables data can be downloaded from Science Data Bank at: www.sciencedb.cn/ (accessed on 17 October 2022).

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Zhao, X.X.; Meng, X.Z.; Ragauskas, A.J.; Lai, C.H.; Ling, Z.; Huang, C.X.; Yong, Q. Unlocking the secret of lignin-enzyme interactions: Recent advances in developing state-of-the-art analytical techniques. *Biotechnol. Adv.* **2022**, *54*, 107830. [CrossRef] [PubMed]
2. Brondi, M.G.; Pinto, A.S.; Farinas, C.S. Combining additives improves sugars release from hydrothermally pretreated sugarcane bagasse in integrated 1G-2G biorefineries. *Bioresour. Technol. Rep.* **2021**, *15*, 100819. [CrossRef]
3. Xu, C.; Alam, M.A.; Wang, Z.M.; Chen, H.G.; Zhang, J.; Huang, S.S.; Zhuang, W.; Xu, J.L. Mechanisms of bio-additives on boosting enzymatic hydrolysis of lignocellulosic biomass. *Bioresour. Technol.* **2021**, *337*, 125341. [CrossRef] [PubMed]
4. Rocha-Martín, J.; Martinez-Bernal, C.; Pérez-Cobas, Y.; Reyes-Sosa, F.M.; García, B.D. Additives enhancing enzymatic hydrolysis of lignocellulosic biomass. *Bioresour. Technol.* **2017**, *244*, 48–56. [CrossRef]
5. Zhang, J.; Li, K.T.; Liu, S.C.; Huang, S.S.; Xu, C. Alkaline hydrogen peroxide pretreatment combined with bio-additives to boost high-solids enzymatic hydrolysis of sugarcane bagasse for succinic acid processing. *Bioresour. Technol.* **2022**, *345*, 126550. [CrossRef] [PubMed]
6. Li, Y.; Sun, Z.; Ge, X.; Zhang, J. Effects of lignin and surfactant on adsorption and hydrolysis of cellulases on cellulose. *Biotechnol. Biofuels.* **2016**, *9*, 20. [CrossRef] [PubMed]
7. Bhagia, S.; Kumar, R.; Wyman, C.E. Effects of dilute acid and flow through pretreatments and BSA supplementation on enzymatic deconstruction of poplar by cellulase and xylanase. *Carbohydr. Polym.* **2017**, *157*, 1940–1948. [CrossRef]
8. Tan, L.; Liu, Z.; Zhang, T.; Wang, Z.; Liu, T. Enhanced enzymatic digestibility of poplar wood by quick hydrothermal treatment. *Bioresour. Technol.* **2020**, *302*, 122795. [CrossRef]

9. Mcintosh, S.; Zhang, Z.; Palmer, J.; Wong, H.H.; Doherty, W.O.S.; Vancov, T. Pilot-scale cellulosic ethanol production using eucalyptus biomass pre-treated by dilute acid and steam explosion. *Biofuel. Bioprod. Biorefin.* **2016**, *10*, 346–358. [CrossRef]
10. Su, Y.; Huang, C.X.; Lai, C.H.; Yong, Q. Green solvent pretreatment for enhanced production of sugars and antioxidative lignin from poplar. *Bioresour. Technol.* **2021**, *321*, 124471. [CrossRef]
11. Ying, W.J.; Xu, Y.; Zhang, J.H. Effect of sulfuric acid on production of xylooligosaccharides and monosaccharides from hydrogen peroxide-acetic acid-pretreated poplar. *Bioresour. Technol.* **2021**, *321*, 124472. [CrossRef] [PubMed]
12. Wang, Q.; Liu, Y.Y.; Zhang, Y.; Chen, Y.; Feng, Q.F.; Wang, W.; Liang, C.Y.; Hu, Y.Z.; Qi, W. Enhanced enzymatic hydrolysis of poplar cellulosic residue fractionated by a magnetic carbon-based solid-acid catalyst in the γ-valerolactone–water system. *Ind. Crops Prod.* **2022**, *176*, 114397. [CrossRef]
13. Abdella, A.; Mazeed, T.E.-S.; El-Baz, A.F.; Yang, S.T. Production of β-glucosidase from wheat bran and glycerol by *Aspergillus niger* in stirred tank and rotating fibrous bed bioreactors. *Process Biochem.* **2016**, *51*, 1331–1337. [CrossRef]
14. Bai, A.; Zhao, X.; Jin, Y.; Yang, G.; Feng, Y. A novel thermophilic β-glucosidase from *Caldicellulosiruptor bescii*: Characterization and its synergistic catalysis with other cellulases. *J. Mol. Catal B Enzym.* **2013**, *85*, 248–256. [CrossRef]
15. Kalim, B.; Böhringer, N.; Ali, N.; Schäberle, T.F. Xylanases from microbial origin to industrial application. *Br. Biotechnol. J.* **2015**, *7*, 1–20. [CrossRef]
16. Olsen, S.N.; Bohlin, C.; Murphya, L.; Borch, K.; McFarland, K.C.; Sweeny, M.D.; Westh, P. Effects of non-ionic surfactants on the interactions between cellulases and tannic acid: A model system for cellulase–poly-phenol interactions. *Enzyme. Microb. Technol.* **2011**, *49*, 353–359. [CrossRef]
17. Wang, S.W.; Liu, G.; Wang, J.; Yu, J.T.; Huang, B.Q.; Xing, M. Enhancing cellulase production in *Trichoder mareesei* RUT C30 through combining manipulation of activating and repressing genes. *J. Ind. Microbiol. Biotechnol.* **2013**, *40*, 633–641. [CrossRef]
18. Park, S.; Kazlauskas, R.J. Biocatalysis in ionic liquids-advantages beyond green technology. *Curr. Opin. Biotech.* **2003**, *14*, 432–437. [CrossRef]
19. Xu, W.J.; Huang, Y.K.; Li, F.; Wang, D.D.; Yin, M.N.; Wang, M.; Xia, Z.N. Improving β-glucosidase biocatalysis with deep eutectic solvents based on choline chloride. *Biochem. Eng. J.* **2018**, *138*, 37–46. [CrossRef]
20. Liang, C.Y.; Xu, Z.H.; Wang, Q.; Wang, W.; Xu, H.J.; Guo, Y.; Qi, W.; Wang, Z.M. Improving β-glucosidase and xylanase production in a combination of waste substrate from domestic wastewater treatment system and agriculture residues. *Bioresour. Technol.* **2020**, *318*, 124019. [CrossRef]
21. Wen, P.Y.; Chu, J.; Zhu, J.J.; Xu, Y.; Zhang, J.H. Highly selective delignification of poplar by hydrogen peroxide-ethylacetate pretreatment at room temp. *Renew. Energ.* **2022**, *188*, 1022–1028. [CrossRef]
22. Hsieh, C.W.C.; Cannella, D.; Jørgensen, H.; Felby, C.; Thygesen, L.G. Cellobiohydrolase and endoglucanase respond differently to surfactants during the hydrolysis of cellulose. *Biotechnol. Biofuels.* **2015**, *8*, 52. [CrossRef] [PubMed]
23. Lou, H.M.; Yuan, L.; Qiu, X.Q.; Qiu, K.X.; Fu, J.G.; Pang, Y.X.; Huang, J.H. Enhancing enzymatic hydrolysis of xylan by adding sodium lignosulfonate and long-chain fatty alcohols. *Bioresour. Technol.* **2016**, *200*, 48–54. [CrossRef]
24. Wei, W.Q.; Jin, Y.C.; Wu, S.B.; Yuan, Z.Y. Improving corn stover enzymatic saccharification via ferric chloride catalyzed dimethyl sulfoxide pretreatment and various additives. *Ind. Crops Prod.* **2019**, *140*, 111663. [CrossRef]
25. Guo, Z.W.; Zhang, L.M.; Zhang, L.; Yang, G.H.; Xu, F. Enhanced enzymatic hydrolysis by adding long-chain fatty alcohols using film as a structure model. *Bioresour. Technol.* **2018**, *249*, 82–88. [CrossRef] [PubMed]
26. Mesquita, R.; Hallwass, F.; Santana, A.; Baudel, H.; Ribeiro, E. Influence of dirhamnolipids on the enzymatic hydrolysis of steam-pretreated eucalyptus wastes. *Ind. Crops Prod.* **2019**, *141*, 111835. [CrossRef]
27. Sha, R.Y.; Yu, Z.; Wang, Z.Z.; Gbor, E.M.; Jiang, L.G.; Bi, Y.X.; Fang, S.; Meng, Q.; Mao, J.W. Effects of Rhamnolipids on Enzymatic Hydrolysis on Bamboo Biomass and Mechanism. *J. Biobased. Mater. Bio.* **2020**, *14*, 453–460. [CrossRef]
28. Rose, M.M.; Hu, J.; Zhao, X.; Sun, S.F.; Pascal, K.; Ren, H.; Zhang, J.; Sun, F.F.; Pascal, K.; Ren, H.; et al. Enhanced high-solids fed-batch enzymatic hydrolysis of sugar cane bagasse with accessory enzymes and additives at low cellulase loading. *ACS Sustain. Chem. Eng.* **2018**, *6*, 12787–12796.
29. Sluiter, A.; Hanmes, B.; Ruiz, R.; Scarlata, C.; Sluiter, J.; Templeton, D.; Crocker, D. Determination of Structural Carbohydrates and Lignin in Biomass. Golden: Laboratory Analytical Procedure of National Renewable Energy Laboratory (NREL). 2012. Available online: https://www.nrel.gov/docs/gen/fy13/42618.pdf (accessed on 14 October 2022).
30. Ghose, T.K. Measurement of cellulase activities. *Pure Appl. Chem.* **1987**, *59*, 257–268. [CrossRef]
31. Sarsaiya, S.; Awasthi, S.K.; Awasthi, M.K.; Mishra, S.; Chen, J.S. The dynamic of cellulase activity of fungi inhabiting organic municipal solid waste. *Bioresour. Technol.* **2018**, *251*, 411–415. [CrossRef]
32. Sharma, B.; Agrawal, R.; Singhania, R.R.; Satlewal, A.; Mathur, A.; Tuli, D.; Adsul, M. Untreated wheat straw: Potential source for diverse cellulolytic enzyme secretion by *Penicillium janthinellum* EMS-UV-8 mutant. *Bioresour. Technol.* **2015**, *196*, 518–524. [CrossRef] [PubMed]
33. Qi, W.; Liu, G.F.; He, C.; Liu, S.N.; Lu, S.; Yue, J.; Wang, Q.; Wang, Z.M.; Yuan, Z.H.; Hu, J.H. An efficient magnetic carbon-based solid acid treatment for corncob saccharification with high selectivity for xylose and enhanced enzymatic digestibility. *Green Chem.* **2019**, *21*, 1292. [CrossRef]
34. Nakagame, S.; Chandra, R.P.; Kadla, J.F.; Saddler, J.N. Enhancing the enzymatic hydrolysis of lignocellulosic biomass by increasing the carboxylic acid content of the associated lignin. *Biotechnol. Bioeng.* **2011**, *108*, 538–548. [CrossRef] [PubMed]

35. Eriksson, T.; Börjesson, J.; Tjerneld, F. Mechanism of surfactant effect in enzymatic hydrolysis of lignocellulose. *Enzyme Microb. Technol.* **2002**, *31*, 353–364. [CrossRef]
36. Ko, J.K.; Ximenes, E.; Kim, Y.; Ladisch, M.R. Adsorption of enzyme onto lignins of liquid hot water pretreated hardwoods. *Biotechnol. Bioeng.* **2015**, *112*, 447–456. [CrossRef]
37. Okino, S.; Ikeo, M.; Ueno, Y.; Taneda, D. Effects of Tween 80 on cellulase stability under agitated conditions. *Bioresour. Technol.* **2013**, *142*, 535–539. [CrossRef]
38. Boyce, A.; Walsh, G. Characterisation of a novel thermostable endoglucanase from Alicyclobacillus vulcanalis of potential application in bioethanol production. *Appl. Microbiol. Biotechnol.* **2015**, *99*, 7515–7525. [CrossRef]
39. Liu, J.; Shi, J.; Li, J.; Yuan, X. Characterization of the interaction between surfactants and enzymes by fluorescence probe. *Enzyme Microb. Technol.* **2011**, *49*, 360–365. [CrossRef]
40. Ding, D.Y.; Li, P.Y.; Zhang, X.M.; Ramaswamy, S.; Xu, F. Synergy of hemicelluloses removal and bovine serum albumin blocking of lignin for enhanced enzymatic hydrolysis. *Bioresour. Technol.* **2019**, *273*, 231–236. [CrossRef]
41. Wang, W.; Wang, C.J.; Zahoor; Chen, X.Y.; Yu, Q.; Wang, Z.M.; Zhuang, X.S.; Yuan, Z.H. Effect of a Nonionic Surfactant on Enzymatic Hydrolysis of Lignocellulose Based on Lignocellulosic Features and Enzyme Adsorption. *ACS Omega.* **2020**, *5*, 15812–15820. [CrossRef]
42. Li, K.N.; Wang, X.; Wang, J.F.; Zhang, J.H. Benefits from additives and xylanase during enzymatic hydrolysis of bamboo shoot and mature bamboo. *Bioresour. Technol.* **2015**, *192*, 424–431. [CrossRef] [PubMed]