ACS ENVIRONMENTAL Au
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Maleic Acid-Butanol Pretreatment to Enhance Cellulose Accessibility for Enzymatic Hydrolysis and Ethanol Production from Oil Palm Empty Fruit Bunch

Annaëlle Postiaux,* Filemon Jalu Nusantara Putra, Prihardi Kahar, Aurore Richel, and Chiaki Ogino

Cite This: ACS Environ. Au 2025, 5, 76–85
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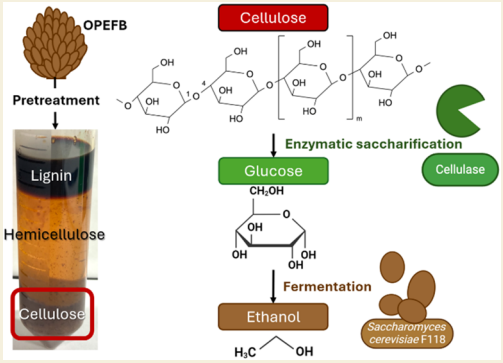
ABSTRACT: Pretreatment of lignocellulosic biomass is crucial yet challenging for sustainable energy production. This study focuses on enhancing enzymatic accessibility of cellulose in oil palm empty fruit bunches by optimizing pretreatment parameters to improve glucose and ethanol yields while reducing fermentation inhibitors. It evaluates the impact of maleic acid concentrations on biorefinery processes. High maleic acid concentrations (>25% w/w) may allow reuse and offer benefits over lower concentrations, such as enhanced delignification and increased sugar yield under milder conditions. Biomass undergoes pretreatment, enzymatic saccharification, and fermentation using *Saccharomyces cerevisiae* F118. Pretreatment with 75% maleic acid (w/w) for 60 min at 180 °C effectively removes lignin and hemicellulose, increasing cellulose accessibility but results in 74.8% crystallinity, hindering saccharification. A 50% maleic acid pretreatment yielded higher glucose (77.1%). Optimal ethanol production is achieved with 1% maleic acid pretreatment. However, the ethanol yield is negatively impacted by residual maleic acid on the solid matrix.



KEYWORDS: *oil palm empty fruit bunch, biorefinery, organosolv pretreatment, maleic acid butanol, cellulose accessibility*

1. INTRODUCTION

While the rapid population growth presents numerous challenges, including managing space, utilizing natural resources, and ensuring food production, research across diverse fields aims to discover sustainable solutions for both environmental preservation and human health.[1−4] This includes addressing our reliance on fossil fuels. This research aligns closely with the Sustainable Development Goals (SDGs) outlined by the United Nations, mainly focusing on Goals 9, 12, and 13.[5] Goal 9 is promoted by optimizing industrial waste treatment processes, encouraging sustainable industrialization, and increasing renewable energy production, such as bioethanol derived from lignocellulosic biomass. Goal 12 emphasizes responsible consumption by converting organic waste into resources and minimizing environmental impact. Finally, Goal 13 aims to combat climate change by expanding renewable energy production and implementing effective waste management strategies to reduce greenhouse gas emissions and decrease dependence on fossil fuels.

Modeled after petroleum refineries, biorefineries convert sustainable biomass into various products, including energy, chemicals, and fuels.[6] Bioethanol, derived from sources like sugar cane, corn, and wheat,[7] is proposed as a viable transportation biofuel due to its compatibility with gasoline and superior characteristics such as a higher vaporization heat temperature, and a superior octane number.[8,9] However, its production competes with food and feed resources, potentially leading to price hikes or shortages.[10,11] To mitigate these issues, utilizing crop waste such as palm oil industry residues is herein suggested.[10,12] Oil palm empty fruit bunches (OPEFB) are proposed as a cost-effective and readily available biomass source with high cellulose content[13] averaging at 43%.[13,14] Lignocellulosic biomass, the primary material in biorefineries, offers advantages like carbon capture and high cellulose content for fermentable sugars.[15−17] The production process involves pretreatment, hydrolysis, fermentation, and ethanol recovery.[18] Despite its promise, the recalcitrant nature of lignocellulosic biomass poses a significant challenge to efficient conversion into bioethanol.[19,20] Efficient pretreatment and hydrolysis methods are therefore needed to break the β-1,4 glycosidic bonds enclosing cellulose.[21] Various pretreatment techniques, including physical, chemical, physio-chemical, and biological processes, aim to break down lignocellulosic structures and remove lignin and hemicellulose from

Received: June 27, 2024
Revised: October 4, 2024
Accepted: October 4, 2024
Published: November 21, 2024

lignocellulosic biomass.[22] Pretreatment aims to enhance the enzyme's accessibility to biomass and increase the yield of fermentable sugars[22] by depolymerizing lignin, increasing porosity, and improving cellulose digestibility[23] while minimizing inhibitor production.[21−23] However, pretreatment is considered one of the most costly steps in the conversion process, potentially accounting for up to 30% of the total cost of lignocellulose-to-ethanol conversion.[24] The main inhibitors released during the pretreatment stage include 5-hydroxymethylfurfural (5-HMF), 2-furaldehyde (furfural), and a few organic acids, e.g., acetic acid, formic acid, and levulinic acid.[25] Acid and organosolv pretreatments are highlighted for their effectiveness and potential to minimize inhibitor production.[26,27] Organosolv pretreatments are promising technology that selectively isolates the various biomolecules constituting lignocellulosic biomass into three distinct and nonmiscible phases: a solid phase rich in cellulose, an aqueous phase containing hydrolyzed hemicellulose, and a hydrophobic phase containing lignin.[27] This two-phase system consists of a dilute acid and an organic solvent, mostly alcohol such as methanol, ethanol, propanol, ethylene glycol, or acetone.[28] Butanol and maleic acid are the two solvents used in this pretreatment by considerations of low cost, minimal health risks, and the efficient treatment of cellulose for the subsequent saccharification stage.[29] According to the Pfizer solvent selection guide, butanol stands out as a preferred organic solvent in the context of green chemistry.[30,31] While the basic technology for the efficient use of maleic acid has not been fully developed, choosing maleic acid for the acidic component of this pretreatment is justified for several reasons: (1) it appears not to strongly encourage the generation of inhibitors such as furfural or 5-HMF,[14] (2) after pretreatment it can be recovered and reused for further treatment (3) being an organic acid, it can be assimilated by yeast as a carbon source, (4) it is less corrosive than acids typically used in pretreatment such as sulfuric acid,[32] (5) it has a better selectivity in the hemicellulose hydrolysis,[13] and (6) it can achieve the same efficiency as pretreatment with dilute sulfuric acid.[13] Previous studies have demonstrated that pretreatment using maleic acid, a dicarboxylic acid, produces a higher yield of glucose and xylose with minimal production of sugar degradation products such as furfural and 5-HMF. To note, Risanto et al. achieved a sugar conversion yield of 89.8% by pretreating OPEFB with 1% (w/w) maleic acid for 45 min at 200 °C.[13] The principle of this pretreatment is the cleavage of internal lignin and hemicellulose bonds via weak acid hydrolysis and organosolv solubilization. Thanks to this strategy, the sugars are physically separated from the lignin and inhibitors potentially produced during pretreatment.[26] As a result, organic compounds inhibiting fermentation will be partially solubilized in the butanol phase, and their concentration will decrease in the aqueous phase.[27] Furthermore, this separation, based on solubilization, does not require high energy consumption compared to single-phase systems.[33]

Enzymatic saccharification is a crucial step in converting biomass into fermentable sugars using enzymes like cellulases and xylanases.[34] Enzymatic hydrolysis of lignocellulosic biomass is a heterogeneous biochemical reaction governed by three critical processes. First, intimate contact between reactants is reflected in the cellulose accessibility and cellulase binding to cellulose. Then reactant reactivity is reflected in cellulose crystallinity and cellulase activity, and reaction conditions, such as substrate loading, reaction time, pH, agitation, and temperature.[12,35] To improve cellulose accessibility to cellulase (CAC), a pretreatment must be applied to deconstruct the cell wall of the lignocellulosic biomass, opening up the substrate structure and making it porous.[35] The commercial enzyme preparation of Cellic CTec 2 contains cellulase, β-glucosidase, and hemicellulase, breaking down cellulose into fermentable sugars.[35] Cellulase specifically catalyzes and hydrolyzes β-1,4 glycosidic bonds in cellulose.[36] Yeast fermentation, mainly using *Saccharomyces cerevisiae*, is pivotal in converting sugars into ethanol.[37] Notably, its use in bioethanol production is accentuated by its high ethanol tolerance compared with other ethanol-producing microorganisms,[38] coupled with its generally regarded as safe (GRAS) status.[39] Moreover, yeasts exhibit optimal performance while assimilating cost-effective substrates.[40] Flocculent yeast strains like *S. cerevisiae* F118 exhibit resistance to fermentation inhibitors, enabling ethanol production in challenging conditions.[25,41]

While biorefinery and bioethanol production offer promising avenues for sustainable energy generation, overcoming challenges such as biomass recalcitrance and inhibitor presence remains paramount for their widespread adoption and economic viability. Continued research and innovation in biorefinery technologies are essential to realizing the full potential of renewable energy sources and transitioning toward a more sustainable energy future. To make the conversion of lignocellulosic biomass into bioethanol economically viable and competitive with that of fossil fuels, research efforts must be optimized. This study explores biomass fractionation techniques, particularly utilizing a mixed organosolv system comprising maleic acid and butanol. The effects of varying concentrations of maleic acid in organosolv pretreatments on different conversion stages, aiming to improve cellulose accessibility and enhance glucose production during enzymatic hydrolysis and ethanol conversion in fermentation, are analyzed. Assessments include pretreatment efficiency, cellulose recovery, inhibitor distribution, glucose yield, and ethanol yield with structural analysis. This approach aims to engineer a sustainable and economically feasible bioethanol production process. Exploring varying concentrations of maleic acid in this study, particularly above >25% w/w, presents a novel contribution, as current literature predominantly focuses on lower concentrations (e.g., 1% w/w). The use of higher concentrations in organosolv pretreatment could not only enhance delignification and hemicellulose removal but also improve cellulose accessibility and sugar yields, resulting in faster pretreatment times. Furthermore, the potential reuse of maleic acid in subsequent pretreatments and other valuable applications is another significant advantage over the traditional methods. These innovations have not yet been extensively studied and represent an important step toward making the process more efficient. Finally, this promising approach could help address several key challenges, such reducing process costs, enabling all stages to be performed in a single reactor, and developing a versatile pretreatment method applicable to various types of lignocellulosic biomass for bioethanol production.[42]

2. MATERIALS AND METHODS

2.1. Biomass, Strain, and Reagents

The Oil Palm Empty Fruit Bunches (OPEFB) samples were obtained from plantation oil palms in Sukabumi, West Java, Indonesia. OPEFB

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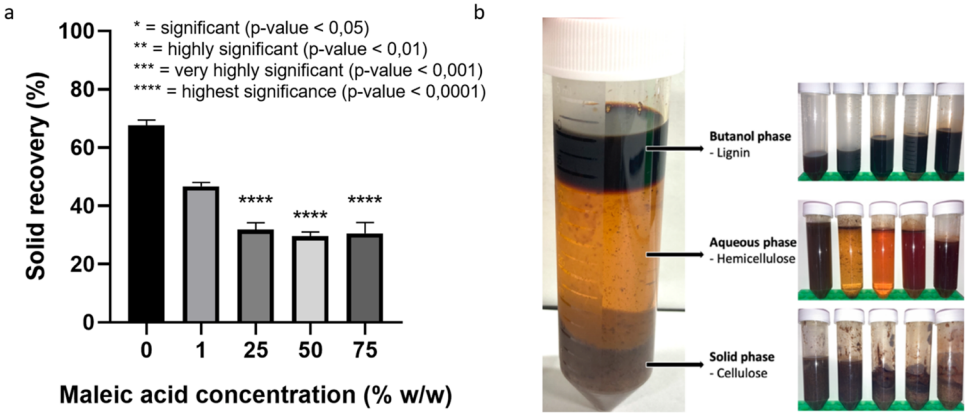

Figure 1. (a) Comparison of solid recovery after pretreatment (%) between the different maleic acid concentrations used during pretreatment. (b) Separation occurred after pretreatment of the OPEFB using different maleic acid concentrations (from the left to the right, 0, 1, 25, 50, and 75% w/w).

contains 34−44% of cellulose and around 25% of hemicellulose.[13] It contains 17.35% lignin, composed of syringyl and guaiacyl subunits, which are the lignin components present in this raw material.[43] The raw OPEFB was crushed into smaller fragments (0.7 mm mesh diameter). Afterward, the sample was dried inside a beaker covered with aluminum foil at 80 °C for 24 h. Then, the mixture was cooled for 30−40 min in a vacuum desiccator and stored in a sealed bag at room temperature. The yeast employed in this study was *S. cerevisiae* F118 obtained from Kobe University, Japan. The F118 was maintained in a 30% (w/w) glycerol stock at −80 °C. Resurrected yeast was conducted by inoculating it onto Yeast Peptone Dextrose (YPD) agar medium (10 g L^{-1} yeast extract, 20 g L^{-1} bacto peptone, 20 g L^{-1} glucose, and 15 g L^{-1} agar) at 30 °C for 24 h. Commercial reagents, including *n*-butanol, maleic acid, ethanol, glucose, xylose, glycerol, acetic acid, furfural, and 5-HMF, were purchased from Nacalai Tesque (Kyoto, Japan).

2.2. Pretreatment

Maleic acid solutions at 1, 25, 50, and 75% (w/w) were prepared using solid maleic acid dissolved in deionized water. A laboratory-scale thermostirrer, KPI THERMO-MIGHTY STIRRER model HHE-19G-U 100 mL was used for the pretreatment of the biomass. For all experiments, 20:60 mL of *n*-butanol/maleic acid solutions at concentrations of 1, 25, 50, and 75% (w/w) (according to the experiment) were added to the Teflon vessel containing 6 g of the OPEFB sample. A magnet was also added, and the mixture was stirred prior to pretreatment, 200 μL was taken for further analysis. A control test, where maleic acid was replaced with water, was carried out to confirm the influence of maleic acid on the process, hereinafter referred to as 0% maleic acid samples. The samples were pretreated at 180 °C for 60 min.

The fractions obtained contained a solid phase (the wet pulp) and a two-phase system of nonmiscible liquids. The solid phase and the two nonmiscible liquid phases, namely the butanol phase and the maleic acid phase, were separated using prior centrifugation for 5 min at 3500 rpm (KABOTA 6200). The maleic acid phase was then filtered with a vacuum pump and a Satorius Sartolab BT 150 Filter System (0.22 μm PES 150 mL). 200 μL were sampled for further analysis. The butanol phase was discarded, and the maleic acid phase was stored at 4 °C. The solid fraction was washed using deionized water until a neutral pH was attained. The wet pulp was left to dry under the fume hood at room temperature. Pretreatments were performed at least in triplicate.

2.3. Saccharification by Enzymatic Hydrolysis

Enzymatic hydrolysis of the pretreated biomass was performed in a citric acid buffered medium containing citric acid monohydrate, citrate dihydrate, and deionized water. The buffer was adjusted with NaOH to attain a pH of 4.8. The pretreated OPEFB samples were dried at 80 °C overnight (until the weight stabilized), and 2 g of this dried cellulosic residue was used for the enzymatic saccharification. A concentration of 100 mg mL^{-1} was reached by adding 17.2 g of citrate buffer to a Schott flask of 100 mL. Before enzymatic saccharification, the Schott bottle containing the OPEFB samples and the citrate buffer was incubated at 50 °C for 1 h by stirring at 160 rpm. After 1 h, 200 μL of enzymes (Cellulase, enzyme blend, SIGMA − Aldrich, SAE0020) were added into the Schott. The Schotts were incubated for 120 h at 50 °C by stirring at 160 rpm in an incubator BioShaker G.BR-300 TAITEC. 200 μL of samples were taken at 0, 3, 6, 9, 24, 48, 72, 96, and 120 h. Those samples were centrifuged at 14,500 rpm for 2 min to remove the residual biomass that could have been taken with and then stored in the freezer at −80 °C. The harvest of the product of the enzymatic saccharification was done after 120 h. The product was centrifuged at 3500 rpm for 5 min, the remaining solid fraction was kept and let dry under the fume hood at room temperature, and the liquid fraction was filtered using a Millex-GV 0.22 μm syringe filter and kept in the freezer at −30 °C. All of the preparation and sampling were done in sterile conditions to avoid contamination since the product of the enzymatic saccharification was further used for yeast fermentation.

2.4. Fermentation Condition

The flasks containing one single colony of *S. cerevisiae* F118 and 12 mL of the YPD medium were incubated for less than 24 h at 30 °C by stirring at 150 rpm in an incubator BioShaker G.BR-300 TAITEC. The yeasts were harvested and centrifuged at 3500 rpm for 5 min; the YPD medium was discarded, and as F118 is a flocculent, the initial weight of the yeast was measured before fermentation. Afterward, the yeasts were put into 12 mL of each product of the enzymatic saccharification and then incubated for 120 h at 150 rpm in an incubator BioShaker G.BR-300 TAITEC. The fermentation was conducted under microaerobic conditions, and 200 μL of samples were taken for further analysis. Due to the flocculent phenotype of the *S. cerevisiae* F118 strain, the growth was checked on a wet-weight cell basis at the initial and final times of the experiment.

2.5. Determination of Sugars and Other Byproducts

All experiments are conducted in triplicates. Statistical analysis was performed using GraphPad Prism software. All the formulas and methodology employed for the calculations in this work are detailed in the Supporting Information document.[13,27,44,45] Sugars and byproducts concentration was determined in the aqueous phase after pretreatment, enzymatic saccharification, and yeast fermentation. The compound identification and quantification were performed by high-performance liquid chromatography (HPLC) analysis associated with two detectors, RID and ultraviolet/visible (UV/vis) at 254 nm. A Coregel column (300 mm length and 7.8 mm inner diameter from Transgenomic) was used. The mobile phase used was H_2SO_4 at a concentration of 5 mM and at a flow rate of 0.6 mL min^{-1}. The temperature of the oven was 80 °C. The separation time was 40 min.

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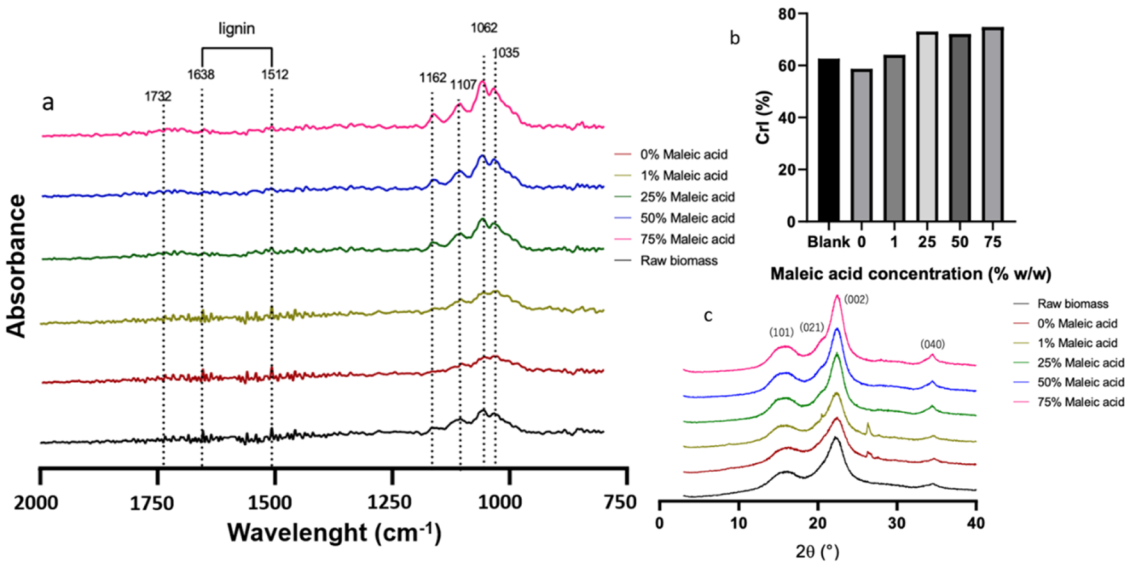

Figure 2. Effect of maleic acid concentration (0, 1, 25, 50, 75% w/w) on OPEFB pretreatment. (a) Chemical changes in OPEFB solids determined by FTIR of wavelength ranged from 750 to 2000 (cm^{-1}), the spectra are analyzed for characteristic absorption bands corresponding to cellulose, hemicellulose, and lignin, assignments are detailed in Table S1 of the Supporting Information.[46] (b, c) XRD analysis of pretreated biomass, the levels of the crystalline index (CrI) are compared between the different maleic acid concentrations applied during pretreatment.

Prior to analysis, the samples were diluted 10 times, and 200 μL was put in filtration vials (Whatman Mini-UniPrep Syringeless Filters). The data acquisition was performed using Labsolutions from Shimadzu software.

2.6. FT-IR Analysis

Solid samples collected after pretreatment and enzymatic saccharification were dried, ground, and kept dry in a desiccator until Fourier transform infrared (FT-IR) and X-ray diffraction (XRD) analysis. FT-IR spectra were recorded on a Bruker α with attenuated total reflectance (ATR) attachment and reported in wavenumbers (cm^{-1}). The prominent bands in the FT-IR spectra are assigned based on refs 40,46.

2.7. X-ray Diffraction (XRD) Analysis

X-ray diffraction (XRD) analysis was performed on raw biomass and solid residue after pretreatment and enzymatic saccharification. The method described by Segal et al. allows the calculation of crystallinity index (CrI)[47] which expresses the relative degree of crystallinity. The calculations and methods are located in the Figure S1.[48]

3. RESULTS AND DISCUSSION

3.1. Effect of Maleic Acid Concentrations on Cellulose after Pretreatment

After pretreatment, a decrease in biomass dry mass is observed, which correlates with the increasing severity of pretreatment, i.e., higher maleic acid concentration. This reduction is likely due to more effective lignin removal into the butanol phase and greater hemicellulose extraction into the aqueous phase. Figure 1a shows that solid recovery decreases with a higher pretreatment severity, indicating that increased maleic acid concentrations significantly improve delignification and hemicellulose removal.

Given that OPEFB is a lignocellulosic biomass containing between 34 and 44% cellulose,[49] the solid remaining after pretreatment with a concentration of maleic acid of 75% would therefore be composed almost entirely of cellulose. A study by Santos et al. found that pretreatment of elephant grass with 5% w/w sulfuric acid resulted in a solid recovery of 59.9% while increasing the concentration to 20% w/w reduced the solid recovery to 54.3% (121 °C and 30 min).[50] Elephant grass samples contain between 30−37% cellulose.[50] Compared with these results, the pretreatment conditions used here appear to be more effective.

After pretreatment, an aqueous maleic acid phase is obtained, containing hemicellulose and its degradation products. As the maleic acid concentration increases, the color of the aqueous phase darkens and the quantity decreases, as shown in Figure 1b. The control using water is not colored, assuming that the separation of the three parts of the lignocellulosic biomass is not achieved. This color change could be attributed to a higher concentration of the product compound. HPLC analysis of the composition of this phase will reveal which compound is more extracted or produced when the maleic acid concentration increases. On the other hand, the decrease in quantity is probably due to the extent of cross-reactions among maleic acid, butanol, and lignin, with some of the reaction products trapped in the butanol phase.

Analysis of Figure 2a reveals key findings: (1) increased maleic acid concentration leads to the disappearance of the 1732 cm^{-1} band, indicating the removal of lignin from the cellulose structure; (2) The decline in band positions between 1638 and 1512 cm^{-1} suggests effective lignin removal with rising maleic acid concentration; (3) Enhanced intensity of bands from 1162 to 1135 cm^{-1} reflects an increase in cellulose on pretreated biomass surfaces as maleic acid concentration rises, implying heightened cellulose accessibility.

The crystallinity index serves to gauge the relative crystalline content within cellulose, reflecting changes in the biomass structure postpretreatment. Increasing maleic acid concentration correlates with a rise in the crystalline index, shown in Figure 2b, suggesting potential surface removal of lignin under milder conditions and internal lignin under harsher conditions. Compared to the literature crystallinity index standard for the cellulose of 81.0%,[41] the highest crystallinity index of 74.8% is achieved with 75% maleic acid (w/w) pretreatment at 180 °C for 60 min, indicating effective cellulose isolation and presumed lignin removal. However, this assumption warrants

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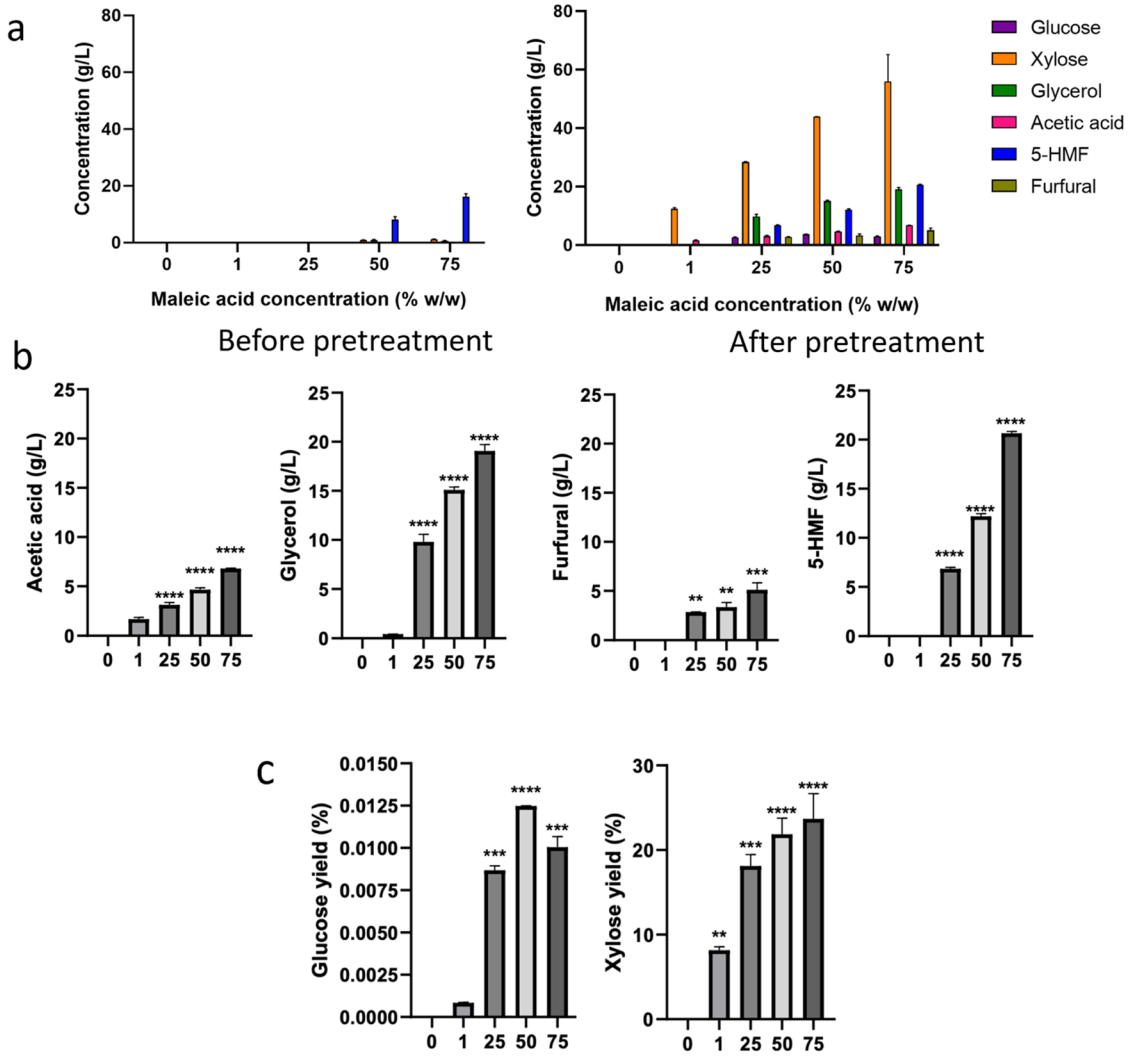

Figure 3. Sugar and byproducts formation after pretreatment. (a) Composition of the aqueous phase before and after pretreatment. (b) Detailed byproducts production after pretreatment for each maleic acid concentration applied for the pretreatment. (c) Comparison of the glucose and xylose yield after pretreatment of the OPEFB for each maleic acid concentration applied (0, 1, 25, 50, 75%).

caution, considering cellulose's two-phase structure of crystalline and amorphous regions. Cellulose crystallinity affects enzymatic saccharification with the amorphous region being more digestible. While severe pretreatment may slow reaction kinetics due to increased crystallinity, other factors like surface area and particle size also influence digestibility. Nonetheless, pretreatment with 75% maleic acid (w/w) at 180 °C for 60 min remains a viable method for enhancing glucose production, highlighting cellulose availability as the primary determinant of the hydrolysis rate.

The diffractograms of pretreated OPEFBs exhibit characteristics of semicrystalline samples. The peak (002), representing the crystalline part of cellulose, intensifies with increased maleic acid concentrations. Additionally, peaks (101) and (021) become more discernible. These peaks are obscured in raw biomass due to interferences from compounds like lignin. However, as the maleic acid concentration increases, lignin removal is significant. Consequently, the (002) peak becomes finer due to cellulose microfibril agglomeration during heat pretreatment.

Ultimately, the biphasic organosolv pretreatment appears to have successfully separated the biomass into three distinct components. This initial analysis suggests that cellulose is effectively freed from lignin and hemicellulose without sustaining damage. Additionally, this pretreatment method helps to minimize energy costs for separating these components.

3.2. Sugar Release and Byproduct Formation

As shown in Figure 3a the xylose production in the aqueous phase increases when maleic acid concentrations used during pretreatment rises. Increasing the maleic acid concentration during pretreatment enhances hemicellulose hydrolysis, releasing it into the aqueous phase. As a result, higher xylose yields postpretreatment are achieved with increased maleic acid concentration, indicating improved hemicellulose degradation. The substantial release of xylose, reaching a concentration of 55.9 g L^{-1}, into the aqueous phase, suggests successful removal of hemicellulose from cellulose, facilitated by the increased maleic acid concentration applied during pretreatment. These results seem consistent with hemicellulose being sensitive to severe pretreatment conditions due to its amorphous nature.[22]

Overdegradation of hemicellulose produces inhibitors[51] like acetic acid, furfural, and 5-HMF as shown in Figure 3b. These

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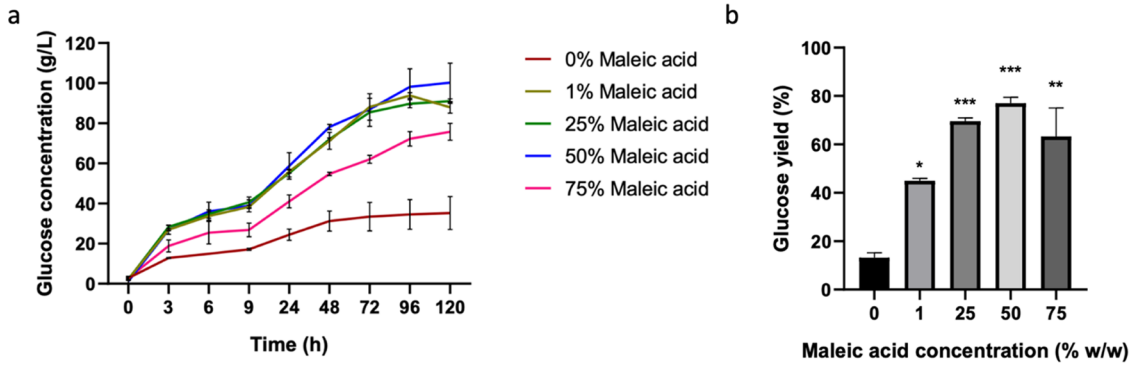

Figure 4. (a) Glucose production during enzymatic saccharification of 2 g of pretreated biomass using different maleic acid concentrations. (b) Glucose yield after 120 h of enzymatic saccharification of pretreated biomass.

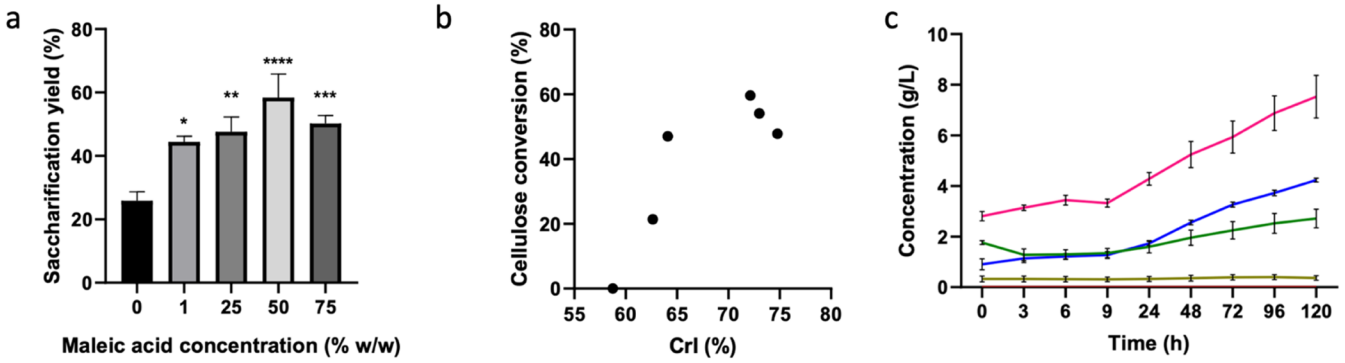

Figure 5. (a) Saccharification percentage of cellulosic residue after enzymatic saccharification. The comparison is made for each maleic acid concentration used for the pretreatment of the biomass. (b) Correlation between crystallinity index (CrI) and cellulose conversion. (c) Maleic acid is released during enzymatic saccharification.

inhibitors are found in the aqueous phase, indicating advanced hemicellulose breakdown and separation, while cellulose remains free from them. Increased maleic acid concentrations lead to increased levels of furfural and 5-HMF, further hindering subsequent biorefinery steps. However, 5-HMF can serve as a platform molecule for high-value chemical production. Despite potential challenges in enzymatic saccharification and fermentation, enriching the aqueous phase with inhibitors poses no significant issue, as these steps mainly involve the solid phase.

The glucose yield after pretreatment shown in Figure 3c indicates that the cellulose fibers remained intact and that there has been no loss of monomers in the aqueous phase. When high concentrations of maleic acid are applied during pretreatment, some glucose is recovered in the aqueous phase after pretreatment, suggesting that the amorphous part of the cellulose has been degraded into its sugars. Even so, this conversion remains negligible, less than 0.015%, allowing for the stipulation that the cellulose remained intact after pretreatment even when using high concentrations of maleic acid.

After pretreatment using severe conditions, cellulose appears to be effectively separated from lignin and hemicellulose. In addition, an aqueous phase rich in compounds of interest, such as xylose and 5-HMF, is obtained.

3.3. Enzymatic Hydrolysis of the Cellulose

This study employs the solid fraction obtained after pretreatment for enzymatic saccharification. Figure 4a shows the evolution of glucose production during enzymatic saccharification of pretreated OPEFB. Glucose production increases throughout the process, with concentration variations depending on the pretreated biomass. One obvious observation is that the use of maleic acid, at any concentration, increases glucose production; therefore, an acid is at first sight necessary to separate cellulose from the rest of the lignocellulosic biomass. The glucose yield is significantly higher for biomass pretreated with maleic acid at a concentration of 50% (w/w), as shown in Figure 4b, with a yield of 77.1%. It suggests that some of the cellulose was hydrolyzed during pretreatment at high concentrations, creating a loss in glucose yield. This hypothesis cannot be based on glucose production after pretreatment, which is very low, but on 5-HMF production. Indeed, under overly severe pretreatment conditions, glucose would be directly degraded to 5-HMF. Another hypothesis is based on the increasing crystallinity of the pretreated biomass. FT-IR and XRD results confirm that pretreatment with increasing maleic acid concentrations will make cellulose more crystalline. This increased crystallinity will slow enzymatic breakdown and lead to lower glucose yields.

The saccharification yield indicates the amount of solid hydrolyzed during the enzymatic saccharification stage. Pretreated biomass with 50% maleic acid (w/w) is the most hydrolyzed by enzymes and, therefore, the most transformed into sugars, as shown in Figure 5a. This result diverges slightly from previous interpretations. Indeed, pretreatment results indicated that biomass pretreated with 75% maleic acid (w/w) provided more accessible cellulose. A first hypothesis would be that cellulose, although more accessible, is far too crystalline to be attacked by enzymes. A longer saccharification time could

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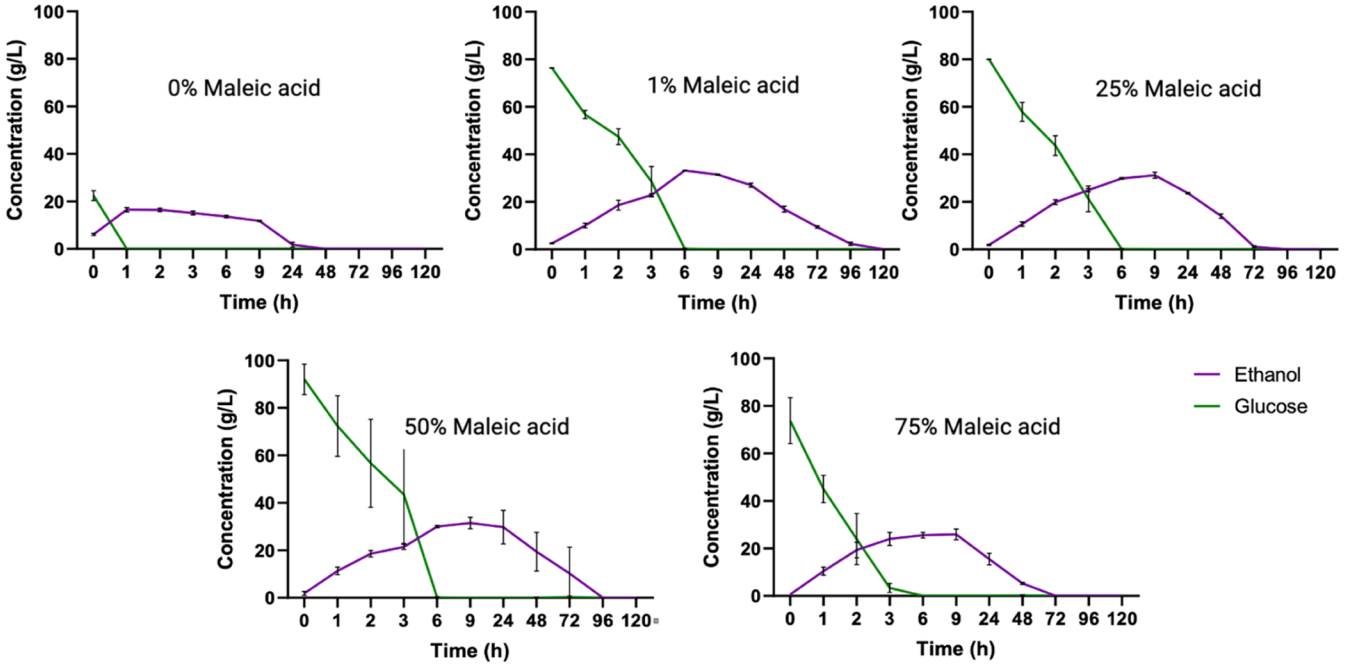

Figure 6. Ethanol production and glucose consumption during yeast fermentation for each hydrolysate obtained after enzymatic saccharification of the pretreated biomass with different concentrations of maleic acid.

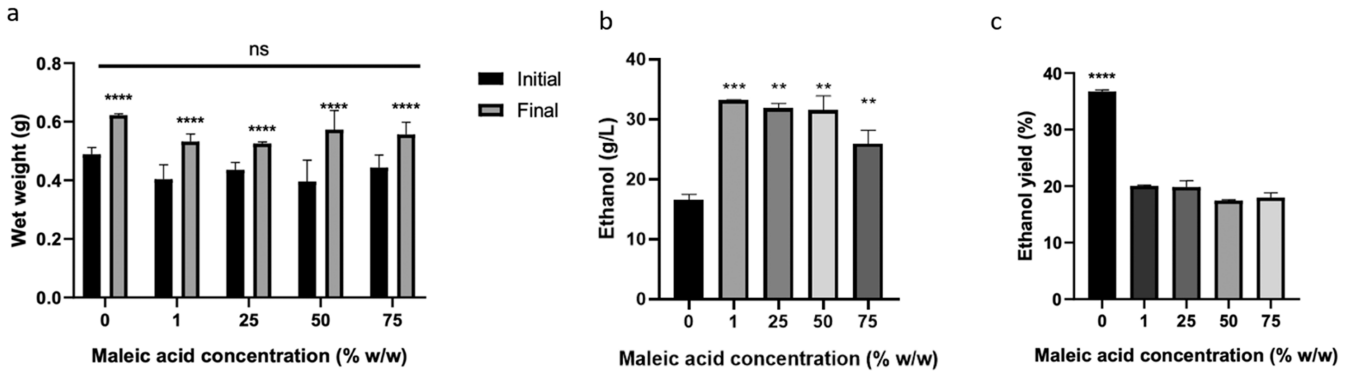

Figure 7. (a) Yeast growth represented by the initial and the final wet weight before and after fermentation, respectively. (b) Maximum ethanol production during yeast fermentation for each hydrolysate obtained after enzymatic saccharification of the pretreated biomass with different concentrations of maleic acid. (c) Ethanol yield: the comparison is done between the different concentrations used in pretreatment.

solve this problem but would increase energy costs. Enzymes can therefore handle the increasing crystallinity of cellulose up to an apparent crystallinity of 72.1%, as shown in Figure 5b. In the literature, Cui et al. found that the cellulose conversion ratio varies between 43, and 99% after 72 h of reaction.[44] It has already been reported that there is a negative correlation between cellulose conversion ratio and crystallinity.[44] In the conducted experiment, lignocellulosic biomass, rather than pure cellulose, was utilized. Since the crystallinity of the biomass is mainly due to cellulose, an increase in crystallinity is due to the removal of lignin and hemicellulose, resulting in enhanced cellulose accessibility. As the cellulose approaches near purity, it undergoes increased crystallization during pretreatment, consequently decreasing its conversion rate. When biomass is pretreated with 75% maleic acid (w/w), cellulose is more accessible, and maleic acid reacts with it, entering the biomass. A wash was performed to neutralize the solid fraction, preventing the inhibition of subsequent steps. However, the maleic acid trapped in the cellulose could not be washed out. During enzymatic saccharification, the enzymes degraded cellulose, releasing maleic acid into the medium. Figure 5c shows the evolution of maleic acid in the medium during the enzymatic saccharification. When maleic acid at a concentration of 75% (w/w) is used, more maleic acid is released, reaching a concentration of 7.5 g L^{-1}.

Figure S2 details the FT-IR spectra and diffractograms explained in this section. The FT-IR analysis of the solid fraction postenzymatic saccharification reveals key insights: (1) β-glycosidic bonds and residual glucose, consistent with cellulase activity, are observed in the spectrum (peak at 900 cm^{-1});[52] (2) After enzymatic saccharification and 75% maleic acid (w/w) pretreatment, peaks are less distinct but more intense, particularly the C = O vibration of cellulose, indicating significant cellulose detection (peaks ranging from 1162 to 1035 cm^{-1}); (3) The peak at 900 cm^{-1} suggests cellulose breakdown into glucose, potentially leading to higher glucose yield with prolonged saccharification. In contrast, biomass pretreated with 1% maleic acid (w/w) shows less distinct and

https://doi.org/10.1021/acsenvironau.4c00045
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intense peaks, possibly due to extensive cellulose conversion or limited accessibility, supported by lower glucose yield compared with 50% maleic acid concentration (w/w).

X-ray diffractograms show that OPEFB solid samples after enzymatic saccharification are typical of crystalline samples.[53] Peaks representing cellulose (101), (021), and (002) have decreased in intensity postsaccharification.

The study by Lakshmipriya et al. identified the apparent peak at 26.6° (−161) as belonging to the crystalline structure of maleic acid[54] adsorbed onto the cellulose structure. The peak (044) at 27.84° also seems part of the maleic acid XRD pattern.[54] Although this peak appears to be present in OPEFB pretreated without maleic acid and therefore with water, generally an isolated and intense peak attest to the crystallinity of a chemical compound,[53] and maleic acid is capable of forming a crystal after evaporation.[54]

When biomass is pretreated with high concentrations of maleic acid, it adsorbs to the cellulose surface and crystallizes after drying. This peak is not visible on the diffractogram of the biomass after pretreatment, as the maleic acid would have entered the solid matrix and been released only after the decomposition of the cellulose into glucose and, therefore, after enzymatic hydrolysis.

3.4. Fermentation of Hydrolysis Product by *S. cerevisiae* F118

Figure 6 shows the evolution between glucose consumption and ethanol production for each hydrolysate obtained after enzymatic saccharification of each biomass pretreated with different maleic acid concentrations. The higher the initial glucose concentration, the longer it takes yeast to assimilate it. When all the glucose is consumed, a maximum of ethanol production is observed before the yeasts switch regimes and consume the product.

As shown in Figure 7a, yeast growth did not differ between the hydrolysates obtained after enzymatic saccharification of different biomasses pretreated with varying concentrations of maleic acid. In addition, the wetted yeast weight at the end of fermentation is significantly different and greater than the initial wetted yeast weight, demonstrating yeast growth during fermentation. These preliminary results suggest that severe pretreatment conditions have no downstream influence on yeast growth, and, therefore, perhaps no negative impact on fermentation.

Figure 7b shows the maximum ethanol production. In this case, it was the biomass pretreated with 1% maleic acid that produced the highest ethanol production. However, the initial sugar content was higher for the hydrolysate obtained from biomass pretreated with 50% maleic acid. A first hypothesis is that this high maleic acid concentration still affects the environment and, therefore, the yeast metabolism by creating stress, for example, and the glucose consumed is used for other metabolic pathways.

The calculation of ethanol yield is based on the yeast's ability to transform the initial sugar into ethanol and, therefore, to see whether a high concentration of maleic acid impacts ethanol yield. Although ethanol production seems higher for pretreatments using maleic acid, Figure 7c shows that ethanol yield is not as expected and is higher for the control using water instead of maleic acid. If the process yield is less than 100%, the sugar is not transformed into ethanol but is used for other metabolic activities due to potential stress or to produce other products. In this case, no other products are made, so the first hypothesis seems to be correct.

4. CONCLUSIONS

First, analysis of the aqueous phase composition demonstrated that maleic acid played an essential role in the delignification of lignocellulosic biomass. No hemicellulose or lignin degradation products were found in the aqueous phase when water was used instead of maleic acid for pretreatment. Pretreatment with 75% maleic acid produced a more affluent aqueous phase with xylose (55.9 g/L) and 5-HMF (20.7 g/L), beneficial for other applications while maintaining low cellulose degradation (3 g/L of glucose). Structural analysis revealed that 75% maleic acid pretreatment resulted in more accessible and crystalline cellulose, with a crystallinity index of 74.8%, indicating improved lignin and hemicellulose removal. However, 50% maleic acid pretreatment achieved higher cellulose conversion (59.6%) and glucose yield (77.1%) after enzymatic saccharification compared to those of the other maleic acid pretreatments. Increased maleic acid concentration boosted glucose yields, until cellulose crystallinity hindered conversion. Despite lower glucose yields, 1% maleic acid pretreatment yielded the highest ethanol concentration (33.2 g/L). Fermentation inhibition was observed with higher maleic acid concentrations, as evidenced by lower ethanol yields. High maleic acid concentrations efficiently degraded hemicellulose into xylose. However, the crystallinity of cellulose postpretreatment impeded glucose conversion. The study highlights the complexity of achieving uniform bioethanol conversion and emphasizes the importance of the acid concentration during organosolv pretreatment. While high maleic acid concentrations yield high xylose and glucose yields, improved purification processes are required. Rigorous cellulose washing is essential when high acid concentrations are used, necessitating further research to optimize maleic acid removal without cellulose loss.

In essence, the study underscores the efficacy of biphasic organosolv pretreatment using maleic acid and butanol in separating biomass constituents, with implications for cellulose accessibility and downstream biorefinery processes. Given the promise of these solvents and the pretreatment system, which align with the principles of green chemistry, further research may be warranted to develop a uniform and consistent process applicable to all exploitable lignocellulosic biomasses. Factors such as cellulose crystallinity, inhibitor byproduct generation, and maleic acid release require meticulous scrutiny for process refinement. As a concentration of 50% (w/w) maleic acid proves effective in enhancing glucose production, the potential for circular recycling of maleic acid for other valuable applications highlights a promising avenue for sustainable biorefinery processes. Further investigation into the feasibility and benefits of reusing maleic acid in a circular economy framework could yield valuable insights for enhancing both economic and environmental sustainability in biomass conversion technologies. This approach not only maximizes resource efficiency but also aligns with the principles of green chemistry by minimizing waste and optimizing resource utilization throughout the biomass valorization chain.

https://doi.org/10.1021/acsenvironau.4c00045
ACS Environ. Au 2025, 5, 76−85

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsenvironau.4c00045.

Equations used in research; assignment table for the FTIR analysis; method for calculating the crystallinity index; and FTIR spectra and XRD spectra after enzymatic saccharification (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Annaëlle Postiaux − *Laboratory of Biomass and Green Technologies, University of Liège, B-5030 Gembloux, Belgium;* orcid.org/0009-0008-4274-1671; Phone: +32.81.622.633; Email: annaelle.postiaux@uliege.be

Authors

Filemon Jalu Nusantara Putra − *Department of Chemical Science and Engineering, Graduate School of Engineering, Kobe University, Kobe 657-8501, Japan*

Prihardi Kahar − *Department of Chemical Science and Engineering, Graduate School of Engineering, Kobe University, Kobe 657-8501, Japan*

Aurore Richel − *Laboratory of Biomass and Green Technologies, University of Liège, B-5030 Gembloux, Belgium;* orcid.org/0000-0002-4352-6542

Chiaki Ogino − *Department of Chemical Science and Engineering, Graduate School of Engineering, Kobe University, Kobe 657-8501, Japan;* orcid.org/0000-0002-8906-7724

Complete contact information is available at: https://pubs.acs.org/10.1021/acsenvironau.4c00045

Author Contributions

A.P.: conceptualization, methodology, data curation, formal analysis, validation, and writing-original draft; F.J.N.P.: conceptualization, supervision, and writing-review; P.K.: conceptualization and supervision; and O.C. and A.R.: resources and supervision.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors would like to express their gratitude to the University of Kobe for providing laboratory facilities and technical support during the experiments conducted for this study.

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