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Phosphoric Acid Pre-Treatment Improves Enzymatic Hydrolysis of Weedy Biomass (*Thysanolaena latifolia*) for Bioethanol Production

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Abstract: Lignocellulosic biomass has garnered attention as an abundant and sustainable alternative energy source that can facilitate reliable and environmentally friendly energy generation. Energy security improves, and environmental impacts diminish when bioethanol, a biofuel produced via lignocellulose-based processes, is utilized. The lignocellulosic biomass of *Thysanolaena latifolia* has considerable potential as a bioethanol feedstock due to its high carbohydrate content (62.4 ± 0.7%). In this study, feedstock derived from *T. latifolia* was pretreated with various concentrations of H_3PO_4 to determine the ideal conditions for enzymatic hydrolysis to convert the feedstock to fermentable sugar. The findings revealed that hydrolysis efficiency and glucose recovery yields were substantially improved compared to those of the untreated sample. Pretreated samples were enzymatically digested to produce a liquid hydrolysate. This hydrolysate was fermented without detoxification using *Saccharomyces cerevisiae* TISTR 5339 to produce ethanol. The results indicated that *T. latifolia* biomass hydrolysate is a promising long-term carbon source for ethanol production from cellulosic biomass. Furthermore, the morphological and crystallographic characteristics of the treated *T. latifolia* biomass were influenced by H_3PO_4 concentration, as indicated by the SEM images, X-ray diffractogram patterns, and crystallinity index values. Therefore, utilizing *T. latifolia* feedstock to produce bioethanol can enhance bioenergy sustainability.

Keywords: weed biomass; *Thysanolaena latifolia*; renewable resource; glucose recovery; bioethanol; phosphoric acid pretreatment

1. Introduction

"Alternative renewable energy sources such as bioethanol have been gaining popularity because of the escalating energy demands, adverse environmental effects, and energy security concerns associated with conventional energy sources" [1–4]. Bioethanol is a biofuel derived from a sustainable lignocellulosic material that possesses the capacity to substantially reduce the release of detrimental pollutants owing to its exceptional combustion efficiency [5,6]. Bioethanol production using lignocellulose-based processes can reduce environmental impacts and enhance energy security [2,7,8]. Lignocellulosic biomass has been recognized as a plentiful and sustainable alternative energy resource with the potential to supply environmentally friendly clean energy [9–11]. "Lignocellulosic materials refer to plant biomass such as agricultural waste, hardwood, softwood, non-food crop plants, and weed biomass, which are composed of cellulose (30–50%), hemicellulose (20–35%), and lignin (5–25%) [5,7]."

"Various lignocellulosic feedstocks, including rice straw, wheat straw, corn stover, sugar cane, and bamboo [4], as well as weed biomass, such as *Miscanthus × giganteus* [12], *Panicum virgatum* [13] and *Vietnamosasa pusilla*" [14], show bioethanol production potential. *Thysanolaena latifolia,* commonly referred to as tiger grass or broom grass, is indigenous to Southeast Asia and belongs to the Poaceae

family. It grows in dense clumps and has a lifecycle of up to ten years. It thrives in temperate and subtropical regions and can adapt to various environments, such as hillsides and valleys, among rocks, degraded areas, and wastelands, and is also successfully cultivated in agricultural areas [15,16]. *Thysanolaena latifolia* is used in various ways—its leaves and tips are used as animal feed, "while its bushy, fox-tail-like panicles are utilized for broom-making. However, after cutting the panicles, the stem portion is disposed of by burning, resulting in the release of significant quantities of air pollutants such as carbon monoxide (CO) and nitrogen dioxide (NO_2)" [17,18]. *Thysanolaena latifolia* is synonymous with *T. maxima* [19], which is a common weed in the Northern part of Thailand. "This weed can grow and produce a large amount of biomass even with limited nutrition and water" [20]. Initial analysis indicates that *T. latifolia's* biomass contains approximately 60% polysaccharide. The substantial polysaccharide content of this biomass suggests that it has considerable potential as a sustainable feedstock for bioethanol production. However, the complex polysaccharide structure significantly affects saccharification, hindering the conversion of biomass into biofuels. Consequently, pretreatment is indispensable for overcoming the intrinsic resistance of lignocellulose to fermentation. "This process improves the enzymatic digestion of the treated substance, contributing to the generation of monomeric sugars that can be subsequently fermented to produce bioethanol" [4,5,21].

"Different approaches, including physical, chemical, physicochemical, and biological reactions, can be utilized for the pretreatment of lignocellulosic materials" [7,9]. "Acid-based pretreatment is one of the most commonly used chemical pretreatment methods because it is effective in breaking down the structure of lignocellulosic materials and promoting cellulose conversion" [9]. "In particular, phosphoric acid (H_3PO_4) has shown great potential as a pretreatment agent because of its ability to break the linkages among lignocellulose components and disrupt the orderly hydrogen bonds in cellulose fibers under mild conditions" [7,22,23]. "Different types of biomasses, including agricultural residues [24–27], grasses [14,28,29], hardwood [30], and softwood [31], have shown a significant improvement in the yield efficiency of enzymatic hydrolysis following their treatment with H_3PO_4."

The present study aimed to examine the most effective H_3PO_4 pretreatment conditions and emphasize the potential of *T. latifolia* as an environmentally friendly material source for producing alternative bioethanol.

2. Materials and Methods

2.1. Raw Materials

A fresh sample of *T. latifolia* was collected from the upper ground in the Bo Kluea District, Nan Province, Thailand (19° 19 33.7 N, 101° 11 41.1 E) in August 2022. "The herbarium of the Biology Department of Naresuan University verified the authenticity of the specimen. This specimen was conserved, and the record 05935 was assigned as a reference for the herbarium."

"The raw biomass was initially washed with water to remove soil particles and dried in the shade for 7 days. It was cut into approximately 5 cm long segments and ground into a powdered form using a milling machine (Retsch, Haan, Germany). The samples were then passed through a 150–300 μm laboratory test sieve before being deposited in containers at 25 °C for subsequent assessment and testing" [29].

2.2. Chemical Analytical Methods

"The chemical compositions of both raw and treated *T. latifolia* feedstocks were analyzed using a standard method developed by the National Renewable Energy Laboratory to evaluate monomer sugars, acid-soluble lignin (ASL), acid-insoluble lignin (AIL), ash, and ethanol extracts" [32–34].

"The quantities of each monomer sugar and ethanol were measured using a high-performance liquid chromatography system (HPLC; Agilent 1100; Agilent Technologies, Waldbronn, Germany) with a refractive index detector (RID; G1362A; Agilent Technologies). The RID was maintained at 55 °C. An Aminex HPX-87H column (300 mm × 7.8 mm; Hercules, CA, USA) was used to separate the

ethanol and sugar. The column was operated at 60 °C, and 20 μL samples were injected with a mobile phase of 0.005 M sulfuric acid, flowing at 0.6 mL/min" [26].

2.3. Pretreatment Procedure

"The raw biomass of *T. latifolia* was pretreated according to the procedure outlined by Premjet et al." [25]. "A 300 mg dry sample was mixed with 24 mL of 70%, 75%, 80%, and 85% H_3PO_4 (v/v) in a 50 mL centrifuge tube. The mixture was stirred thoroughly, and the tubes were covered and heated to 60 °C in a water bath (Grant W28; Grant Instruments, Ltd., Sepreth, UK) for 60 min. The process was completed by adding approximately 20 mL of acetone to the treated sample in tubes and vigorously mixing it with a stirring rod. The liquid mixture was separated using a fixed-angle centrifuge (ScanSpeed 1248; Labogene ApS, DK-3450 Lynge, Denmark) at 7000× g for 10 min, and the supernatant was discarded. This process was repeated thrice. Once completed, the solid fraction was washed with deionized water until the pH reached approximately 6.5–7."

"Lignin removal (%) and recovery yield (%) were calculated using Equations (1) and (2), respectively:

$$\text{Lignin removal (\%)} = 100 - \text{lignin recovery"} \tag{1}$$

"Recovery yields of each content
$$= \frac{[\text{Recovery of each content (\%)} \times \text{Treated component of each content (\% dw)}]}{\text{Untreated component of each content (\% dw)"}} \tag{2}$$

2.4. Crystallinity and Morphology Analysis

"Analysis was performed according to the procedure suggested by Premjet et al." [25]. "Both treated and untreated *T. latifolia* biomasses were rinsed three times with acetone and dried overnight at 25 °C. The samples then underwent crushing and screening using a 150 μm sieve. An X-ray diffractometer (PANalytical X'pert Pro, PW 3040/60; Almelo, the Netherlands) was used to examine the crystallinity of the biomass. The sample was scanned between 10° and 40° at a speed of 0.02° s⁻¹/min."

"The crystallinity index (CrI) was calculated using the Segal equation [35]:

$$\text{CrI} = (I_{002} - I_{am}) / I_{002} \times 100\%" \tag{3}$$

"where I_{002} is the highest intensity of the crystallinity region at $2\theta = 22.0°$, and I_{am} corresponds to the lowest intensity of the amorphous region at $2\theta = 18.2°$."

"The raw and pretreated *T. latifolia* materials were freeze-dried and attached to stubs with a double-sided adhesive for visualization. The biomass was subsequently coated with a fine layer of gold and analyzed using field-emission scanning electron microscopy (SEM) (Apero S; Thermo Fisher Scientific, Waltham, MA, USA)" [29].

2.5. Enzymatic Hydrolysis

"Enzymatic hydrolysis was performed according to the procedure described by Premjet et al." [25]: "Both treated and untreated *T. latifolia* samples underwent enzymatic hydrolysis. The reaction was carried out in a 50 mL Erlenmeyer flask containing 0.1 g of biomass (dry weight). The reaction mixture consisted of 0.05 M sodium citrate buffer with a pH of 4.8 and 0.1 mL of a 2% (w/v) sodium azide. The total volume of the reaction mixture was 10 mL. The enzyme mixture contained 30 filter paper units of cellulase (*Trichoderma reesei* C2730; Sigma-Aldrich, St. Louis, MO, USA) and 60 units of β-glucosidase (Oriental Yeast Co., Ltd., Tokyo, Japan) per gram of dry biomass. The reaction mixture was incubated for 72 h on a rotary shaker (Innova 4340; New Brunswick Scientific Company, Edison, NJ, USA) at 50 °C and 150 rpm. The hydrolysates (20 μL) were collected at various time intervals (12, 24, 48, and 72 h) for HPLC analysis of monomer sugars."

"Hydrolysis efficiency (HE) and glucose recovery (GR) were determined using Equations (4) and (5), respectively.

$$\text{HE (\%) = [(Glucose released (g)} \times 0.9) \times \text{Glucan in the initial biomass (g)]} \times 100 \quad (4)$$

$$\text{GR (\%) = [(Solid recovery (\%)} \times \text{Glucan content (\%)} \times 1.11 \times \text{HE (\%)]} \times 100 \quad (5)$$

where 0.9 and 1.1 are the conversion factors for glucan to glucose."

2.6. Yeast Strain

"A yeast strain purchased from the Thailand Institute of Scientific and Technological Research (TISTR), *Saccharomyces cerevisiae* TISTR 5339, was used for bioethanol fermentation" [36].

2.7. Preparation of Seed Culture

"Yeast was propagated as a seed culture in a 10 mL liquid yeast malt medium. The culture was incubated at 30 °C for 18 h at 180 rpm in a rotary shaker incubator (Innova 4340; New Brunswick Scientific)" [36].

2.8. Preparation of T. latifolia Biomass Hydrolysate

"*Thysanolaena latifolia* biomass hydrolysate (TBH) was prepared according to the method described by Premjet et al. [36]. The TBH produced from enzymatic saccharification was subjected to heat treatment at 100 °C for 20 min in a water bath (Grant W28; Grant Instruments, Ltd.). Subsequently, the TBH was precipitated by centrifugation at 12,000× g for 2 h and filtered through a glass microfiber filter to remove plant matter. Then, it was concentrated to obtain approximately 20 g/L glucose, and the pH of the TBH was adjusted to 6 using 1 M NaOH. The TBH was stored at 4 °C for further investigation" [36].

2.9. Medium for Bioethanol

"Ethanol was produced using control (CT) and TBH media. The TBH medium contained glucose derived from *T. latifolia* biomass, whereas commercial glucose was used as the carbon source in the CT medium. Both media contained 20 g/L glucose, 10 g/L yeast extract, 10 g/L peptone, 2 g/L K_2HPO_4, and 2 g/L $MgSO_4$, and pH was adjusted to 6. Before use, both media were aseptically filtered using a 0.02 μm Millipore filter" [36].

2.10. Ethanol Production

"Ethanol production by *S. cerevisiae* TISTR 5339 was conducted according to the methods described by Premjet et al." [36]: "In order to produce ethanol, 50 mL of TBH and the CT medium were mixed with 2% seed culture and placed in a shaker incubator (Innova 4340; New Brunswick Scientific) at 150 rpm and 30 °C. After 3, 6, 9, 12, 15, 18, 21, and 24 h of incubation, the liquid fraction was collected to measure sugar consumption and ethanol production using HPLC."
"The ethanol yield (%) was calculated using Equation (6):

$$\text{Ethanol yield (\%) = [Ethanol released (g)]} \times 0.511 \times \text{Glucose initial (g)"} \quad (6)$$

2.11. Determination of Yeast Cell Growth

"Yeast cell growth was monitored using a UV spectrophotometer (SP-830 Plus; Metertech, Taipei, Taiwan), and the optical density was measured at 600 nm. An optical density of 1.0 (at 600 nm) corresponds to approximately 1.5×10^7 cells/mL" [37].

2.12. Statistical Analysis

"Each experiment was performed in triplicate, and the resulting data were presented in the form of the average and standard deviation (±SD). A Tukey's test at a 5% level of significance was

conducted for the comparison of treatment means and Analysis of Variance using SPSS version 26.0 (SPSS Inc., Chicago, IL, USA)" [24].

3. Results

3.1. Characterization of T. latifolia Biomass

The composition of the *T. latifolia* raw biomass employed in this investigation is listed in Table 1.

Table 1. Raw material composition of *T. latifolia* biomass.

Composition (% dw)	*T. latifolia* (%)
Glucan	32.9 ± 0.5
Xylan	23.8 ± 0.2
Arabinan	5.7 ± 0.0
AIL*	22.2 ± 0.5
ASL**	5.9 ± 0.1
Ash	9.4 ± 0.1
Extractive	4.3 ± 0.2

"AIL* = acid-insoluble lignin, ASL** = acid-soluble lignin".

3.2. Effect of H_3PO_4 Concentrations on Chemical Composition

"Table 2 shows the differences in the chemical structures of the biomass before and after pretreatment under various H_3PO_4 concentrations". This was done to determine the optimum pretreatment condition of the *T. latifolia* feedstock. When the concentration of H_3PO_4 enhanced, both the relative xylan and arabinan contents decreased substantially. The lowest yield of xylan content ($8.5 \pm 0.1\%$) was obtained at 85% H_3PO_4, whereas arabinan became undetectable. In contrast, samples treated with 70%, 75%, 80%, and 85% H_3PO_4 exhibited a progressive increase in relative glucan percentage. The maximum relative glucan content ($64.4 \pm 0.9\%$) was generated with 85% H_3PO_4 (Table 2). In addition, an increase in the H_3PO_4 concentration led to a significant ($p < 0.05$) decline in the relative amounts of AIL, ASL, and total lignin. Although the relative ASL content was reduced with 70% ($3.7 \pm 0.0\%$) and 75% ($3.6 \pm 0.1\%$) H_3PO_4, this effect was not statistically significant ($p < 0.05$). However, relative ASL decreased significantly with treatment at 80% ($3.3 \pm 0.1\%$) and 85% ($3.0 \pm 0.1\%$) H_3PO_4. Meanwhile, total lignin removal ($81.0 \pm 0.8\%$) was most effective at a concentration of 85% H_3PO_4 (Figure 1).

Table 2. Chemical composition of *T. latifolia* biomass after H_3PO_4 pretreatment.

Composition (% dw)	Raw material	H_3PO_4 concentration (% v/v)			
		70	75	80	85
Glucan	32.9 ± 0.5^e	50.4 ± 0.3^d	54.9 ± 0.2^c	59.7 ± 0.5^b	64.4 ± 0.9^a
Xylan	23.8 ± 0.2^a	12.4 ± 0.1^b	10.2 ± 0.2^c	9.0 ± 0.0^d	8.5 ± 0.1^e
Arabinan	5.7 ± 0.0^a	3.4 ± 0.0^b	3.3 ± 0.0^c	3.2 ± 0.0^d	n.d.
AIL*	22.2 ± 0.5^a	18.0 ± 0.6^b	13.6 ± 0.2^c	12.0 ± 0.6^d	9.2 ± 0.5^e
ASL**	5.9 ± 0.1^a	3.7 ± 0.0^b	3.6 ± 0.1^b	3.3 ± 0.1^c	3.0 ± 0.1^d
Total lignin	28.2 ± 0.4^a	21.7 ± 0.6^b	17.1 ± 0.1^c	15.3 ± 0.7^d	12.2 ± 0.5^e

AIL* = acid-insoluble lignin, ASL** = acid-soluble lignin.

"The superscripted characters within rows indicate the statistical significance differences ($p < 0.05$) between them, n.d. = not determined."
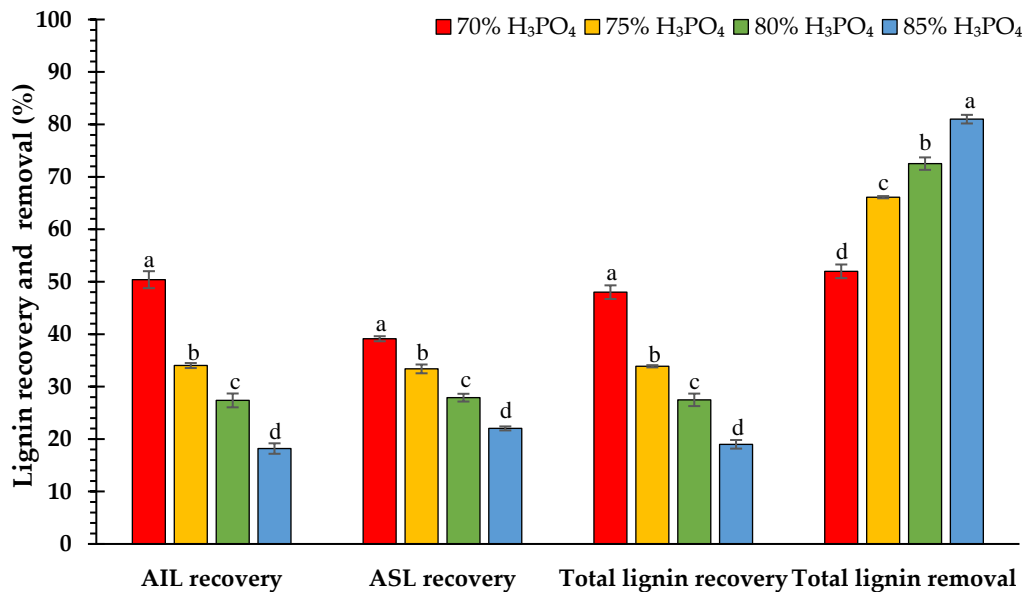
Figure 1. "Lignin recovery and removal after pretreatment at different H_3PO_4 concentrations." The superscripted characters indicate statistically significant differences in the variation of each parameter ($p < 0.05$), n.d. = not determined.

Additionally, substantial declines in the recovery yields of total lignin, glucan, xylan, arabinan, AIL, and ASL were observed as the concentration of H_3PO_4 increased (Figures 1 and 2). When the feedstock was treated in 70% H_3PO_4, the highest recovery yields were obtained for solids (62.2 ± 0.7%), glucan (95.3 ± 0.5%), xylan (32.4 ± 0.2%), arabinan (37.1 ± 0.2%), AIL (50.4 ± 1.6%), ASL (39.1 ± 0.5%), and total lignin (48.0 ± 1.3%). Nevertheless, the decline in glucan recovery yield was not significantly different ($p < 0.05$) at H_3PO_4 concentrations of 75% (93.0 ± 0.3%) and 80% (91.9 ± 0.8%). However, the sample subjected to treatment with 85% H_3PO_4 resulted in the lowest recovery yields for solids (43.8 ± 0.9%), glucan (85.8 ± 1.2%), xylan (16.2 ± 0.2%), AIL (18.2 ± 1.0%), ASL (22.0 ± 0.4%), and total lignin (19.0 ± 0.8%) whereas arabinan recovery was undetectable (Figures 1 and 2).
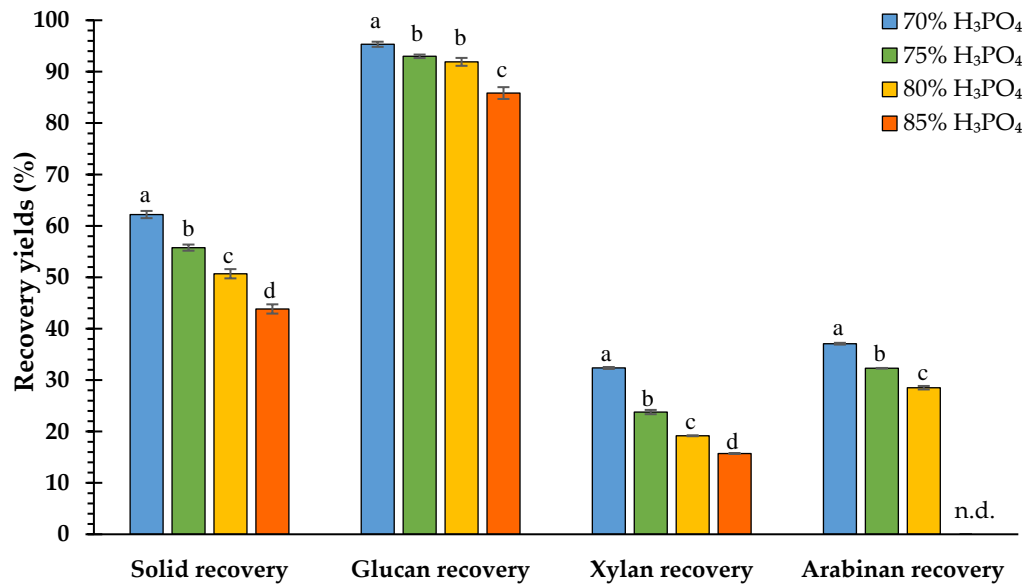

Figure 2. "Recovery yields after pretreatment at different H_3PO_4 concentrations." The superscripted characters indicate statistically significant differences in the variation of each parameter ($p < 0.05$), n.d. = not determined.

3.3. Impact of H₃PO₄ Concentration on Cellulose Crystallinity

The impact of H_3PO_4 pretreatment on cellulose crystallinity in *T. latifolia* feedstock was assessed using X-ray diffraction (XRD). The relative CrIs of both the untreated and treated feedstocks are listed in Table 3. The XRD patterns and CrI values demonstrated modifications in cellulose crystallinity when the raw material was treated with H_3PO_4 at various concentrations. The XRD pattern of the raw material was classified as cellulose I because it comprised three prominent peaks at $2\theta = 15.9°$, $22.0°$, and $34.5°$ (Figure 3), and its relative CrI value was 54.1%. The CrI increased to 57.7% and 61.1% when the sample was treated with 70% and 75% H_3PO_4, respectively. Following treatment with 80% and 85% H_3PO_4, the CrI of this material progressively declined to 59.1% and 49.1%, respectively. Moreover, the XRD patterns of the sample treated with 70%, 75%, and 80% H_3PO_4 remained identical to those of cellulose I crystallinity. After treatment of this biomass with 85% H_3PO_4, the primary peak at $2\theta = 22.0°$ transitioned to an asymmetric peak consisting of a doublet at $2\theta = 19.9°$ and $21.8°$, while the broad peak at $2\theta = 15.9°$ disappeared. Additionally, a new smaller peak emerged at about $2\theta = 12.1°$. These results indicate that cellulose I was transformed into cellulose II.

Table 3. "The crystallinity index of the untreated" and H_3PO_4-treated *T. latifolia* feedstock.

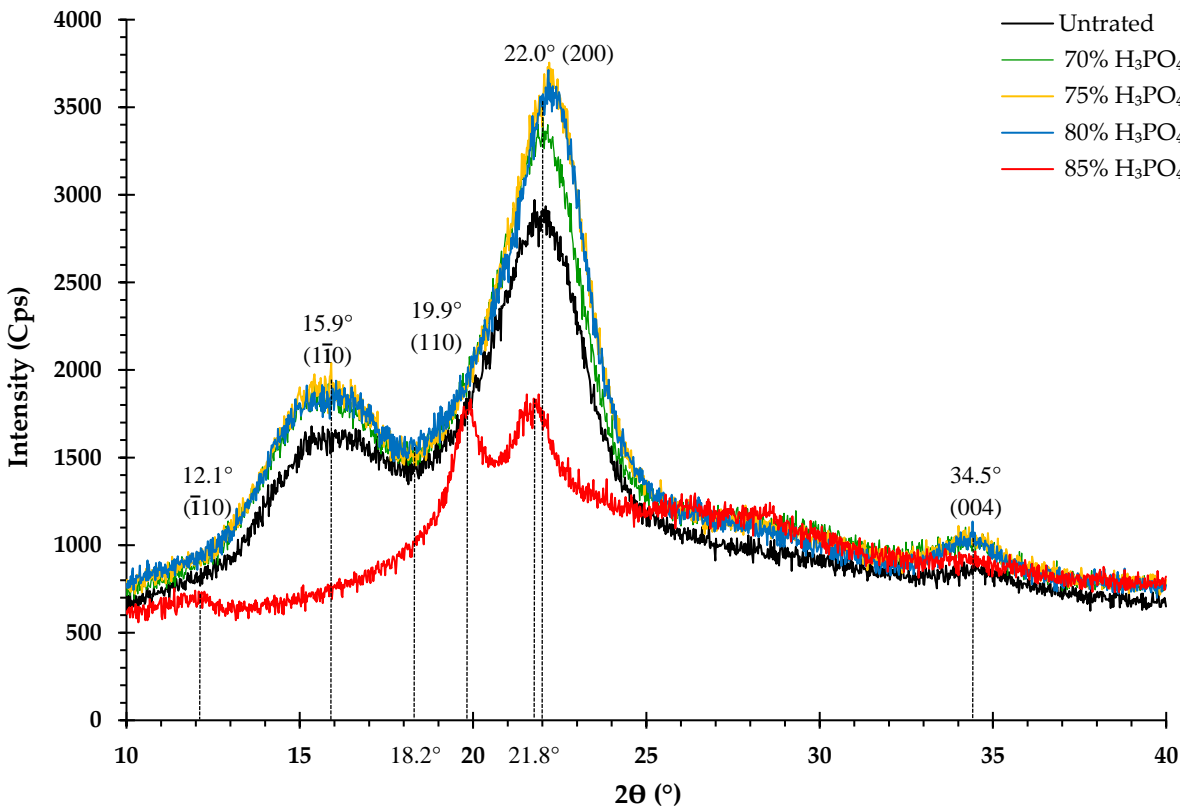| CrI (%)	Concentration of phosphoric acid				
	Untreated	**70%**	**75%**	**80%**	**85%**
	54.1	57.7	61.2	59.1	49.1

Figure 3. "X-ray diffraction pattern of untreated and pretreated *T. latifolia* biomass with different concentrations of H_3PO_4."

3.4. Impact of H₃PO₄ Concentration on Biomass Morphology

The morphology of untreated and pretreated *T. latifolia* was analyzed using SEM to evaluate the physical changes that occurred during pretreatment with various concentrations of H_3PO_4. The SEM images provided a detailed depiction of the untreated *T. latifolia* ultrastructure. The morphological

surface structure was uniform, intact, and characterized by tightly arranged and inflexible bundles (Figure 4A). The alteration in the surface structure was observed after the pretreatment of this feedstock with varying concentrations of H_3PO_4. When the sample was exposed to 70% H_3PO_4, the surface structure initially displayed roughening, splitting, expanding, and loosening (Figure 4B). Samples treated with 75% H_3PO_4 exhibited greater peeling, delamination, loosening, and disintegration on their surfaces (Figure 4C). An increase in the H_3PO_4 concentration to 80% caused the disintegration of the cellulose fibers and a more pronounced deterioration in their morphological structures (Figure 4D). The use of 85% H_3PO_4 as a feedstock treatment caused the most severe deterioration and modification of the cellulose fiber structure, resulting in a transformation of the morphological characteristics of the treated material (Figure 4E).
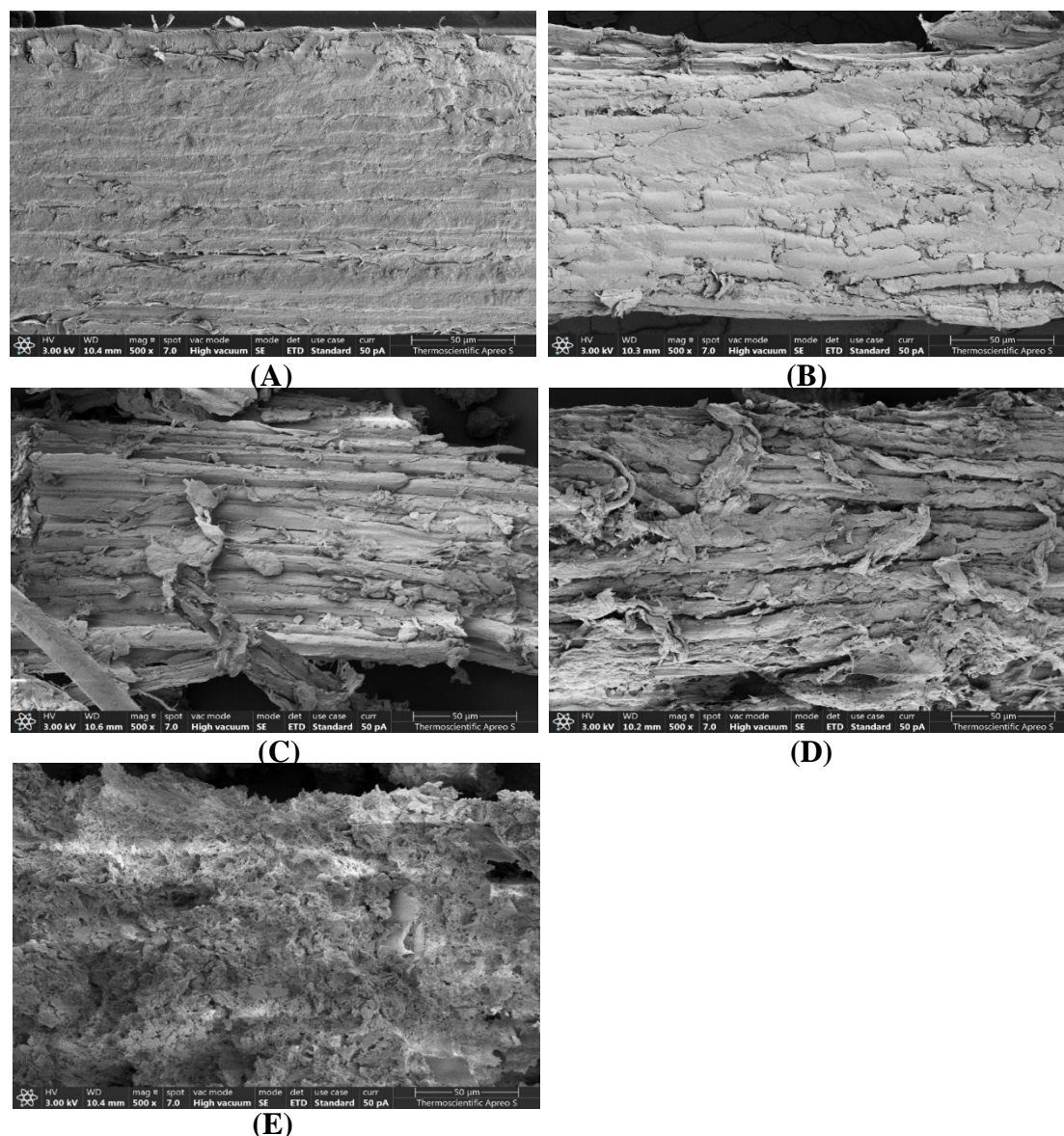

Figure 4. Surface morphological structure of *T. latifolia* samples, both (A) untreated and pretreated samples with varying concentrations of H_3PO_4," including (B) 70%, (C) 75%, (D) 80%, and (E) 85%.".

3.5. Enzymatic Saccharification Yields

To determine the optimal conditions for *T. latifolia* biomass pretreatment, both untreated and treated samples were used as substrates for enzymatic hydrolysis. After treating the feedstock with varying concentrations of H_3PO_4, the HE and GR yields of both the untreated and treated samples improved dramatically after 12 h of incubation, increasing progressively after 24, 48, and 72 h (Figure

5A). The maximum HE and GR yields of both untreated and treated samples were observed within a 72 h incubation period. However, all the treated samples generated greater amounts of HE and GR than the raw material. The untreated *T. latifolia* sample generated the least yields of HE (29.5 ± 0.7%) and GR (12.0 ± 0.3%). After treating the sample with 70% and 75% H_3PO_4, the HE yields improved substantially to 65.3 ± 0.6% and 86.9 ± 0.7%, respectively, and increased slightly to 87.2 ± 0.2% and 88.2 ± 0.2%, when treated with 80% and 85% H_3PO_4, respectively (Figure 5A). However, the HE yields among the specimens treated with 75%, 80%, and 85% H_3PO_4 did not differ significantly ($p < 0.05$). In addition, the GR yield improved dramatically to 25.3 ± 0.2%, 32.8 ± 0.3%, and 32.5 ± 0.1% after treatment with 70%, 75%, and 80% H_3PO_4, respectively. Similarly, the GR yields between the two treatment procedures using 75% and 80% H_3PO_4 did not differ significantly ($p < 0.05$). Conversely, the GR yield was reduced considerably (30.7 ± 0.1%) by treatment with 85% H_3PO_4 (Figure 5B).
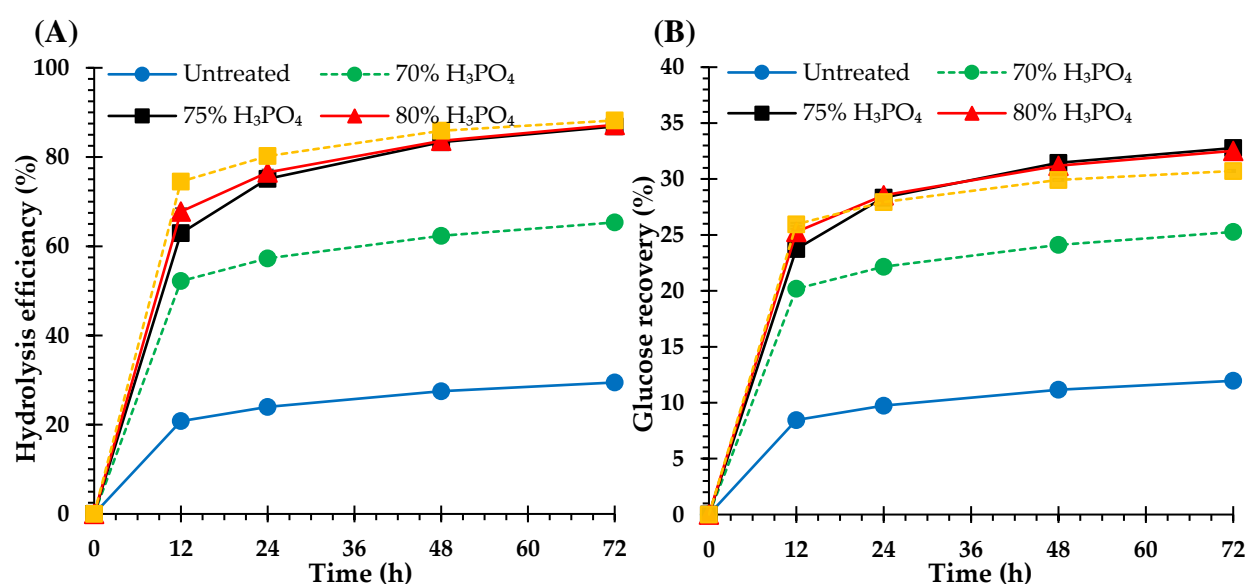

Figure 5. "(A) Hydrolysis efficiency and (B) glucose recovery of untreated" and pretreated *T. latifolia* biomass.

3.6. Bioethanol Fermentation

The TBH medium produced by enzymatic hydrolysis without a detoxification process was subjected to fermentation using *S. cerevisiae* TISTR 5339 to assess its potential for bioethanol production from lignocellulose biomass. The results of this study indicated that glucose consumption, ethanol synthesis, yeast cell proliferation, and pH levels exhibited similar trends in both the CT and TBH media, as shown in Figures 6 and 7. During fermentation, the cell growth profiles of the yeast and the pH observed in both CT and TBH media were slightly different; the CT medium demonstrated rapid growth, reaching the stationary phase at 15 h, while the growth rate in the TBH medium was slower than that in the CT medium (Figure 6). The pH of the CT and TBH media decreased from 6.0 to 5.2. In addition, the yeast strain *S. cerevisiae* TISTR 5339 rapidly utilized the glucose while concurrently producing ethanol. In both CT and TBH media, glucose was entirely consumed in 15 h. The observations of ethanol production revealed that the CT medium produced ethanol (1.0 ± 0.1 g/L or 26.7 ± 1.7% of the ethanol yield) within 6 h, whereas the TBH medium delayed ethanol production (1.2 ± 0.0 g/L or 29.0 ± 0.9% of the ethanol yield) to 9 h. Nevertheless, after 15 h of fermentation, the maximum ethanol yields were produced from both the CT (9.4 ± 0.0 g/L or 91.5 ± 0.3% of the ethanol yield) and TBH (8.9 ± 0.0 g/L or 86.6 ± 0.1% of the ethanol yield) media. Following that, ethanol yields declined steadily in both media (Figure 7).
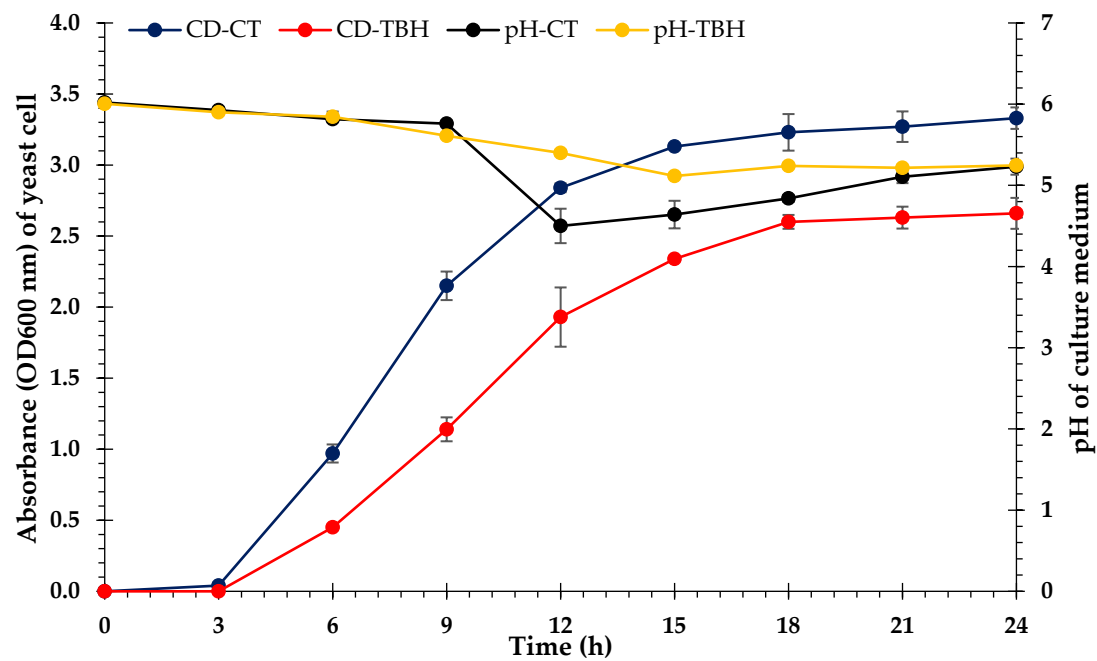
Figure 6. Cell density of *S. cerevisiae* TISTR 5339 in control (CD-CT) medium and *T. latifolia* biomass hydrolysate (CD-TBH) medium. The pH in control (pH-CT) medium and *T. latifolia* biomass hydrolysate (pH-TBH) medium.
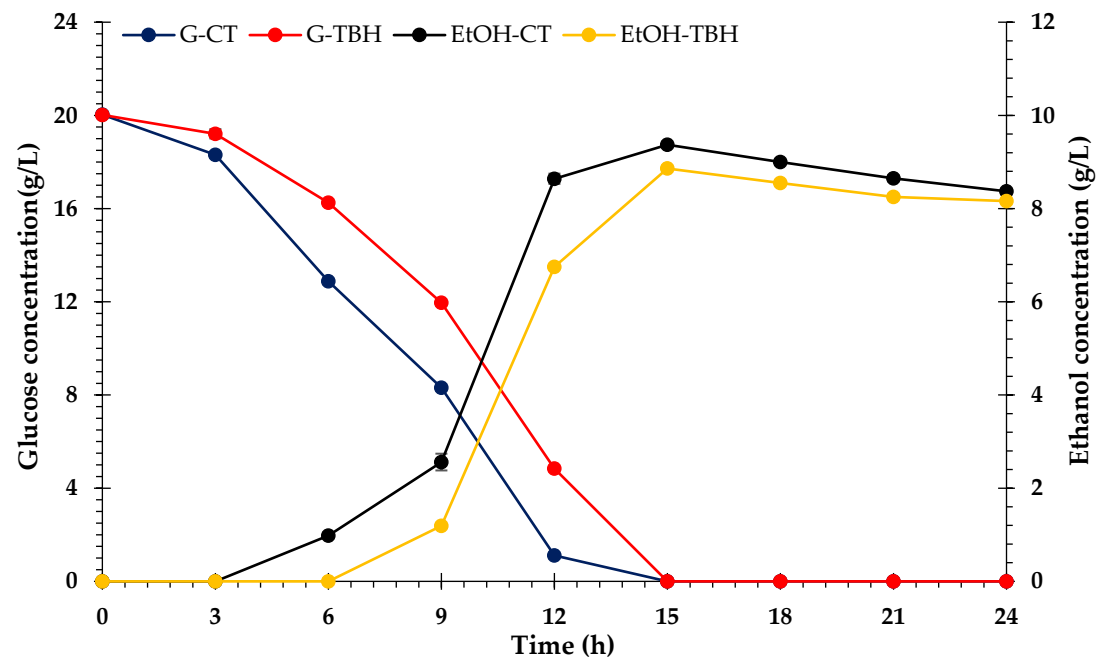

Figure 7. Profiles of glucose consumption and ethanol production of *S. cerevisiae* TISTR 5339 in control and *T. latifolia* biomass hydrolysate medium. Glucose consumption in control (G-CT) medium and *T. latifolia* biomass hydrolysate (G-TBH) medium. Ethanol in the control (EtOH-CT) medium and *T. latifolia* biomass hydrolysate (EtOH-TBH) medium.

4. Discussion

4.1. Characterization of T. latifolia Biomass

The chemical composition of biomass has a substantial effect on the efficacy of bioenergy production [38]. Consequently, determining the suitability of lignocellulose as an alternative resource necessitates an analysis of its chemical composition. According to the results, lignin, hemicellulose, and cellulose are the three primary building blocks of the *T. latifolia* biomass. The total lignin level was substantially higher than that previously reported, whereas the glucan and xylan quantities were comparable [20]. Variations in the composition of lignocellulosic biomass were attributed to factors such as growing circumstances, diverse sources, harvesting season/stage, and other associated variables [4,39,40]. Nevertheless, *T. latifolia* had a lower lignin content than both switchgrass (31.2%) and eucalyptus (29.4%) [41], and several agricultural wastes, such as sorghum straw (30.4%) [39], cotton stalk (30.0%), and walnut shell (37.5%) [1]. Additionally, the total carbohydrates (62.4 ± 0.7%) in the *T. latifolia* biomass were higher than those of lignocellulosic materials such as banana pseudo-stems (51.5%) [42], bean straw (55.0%) [43], switchgrass (56.8%) [44], olive tree (57.8%) [45], and sugar cane (60.7%) [46], which are promoted as feedstock to produce biofuels. These total carbohydrate percentages demonstrate that *T. latifolia* biomass could be utilized to generate cellulosic ethanol.

4.2. Effect of H_3PO_4 Concentrations on Chemical Composition

The utilization of lignocellulosic biomass exhibits potential as a viable non-edible resource in sugar-centric biorefineries. However, the inherent resistance of lignocellulose to enzymatic degradation is a significant challenge, necessitating a pretreatment procedure to modify the chemical and physical properties of the biomass [8,47–49]. This study demonstrated that an increase in H_3PO_4 concentration led to an increase in the relative glucan content while simultaneously causing a substantial decrease in the relative xylan, arabinan, AIL, ASL, and total lignin contents. "This can be attributed to the natural characteristics of hemicellulose and lignin; hemicellulose has a low degree of polymerization because of its amorphous nature and limited polymerization" [8,50]. Meanwhile, "lignin is an amorphous heteropolymer comprising three cinnamyl alcohol precursors (sinapyl alcohol, coniferyl alcohol, and p-coumaryl alcohol) interconnected by different types of links" [50,51]. Consequently, under mild reaction conditions, hemicellulose is more prone to dissolution and removal than lignin [8,50]. In contrast, cellulose has a robust and efficiently organized molecular structure characterized by inter- and intra-molecular hydrogen bonding, "which contribute to the formation of a highly organized crystalline region inside cellulose, rendering it more resistant to breakdown under mild reaction conditions" [1,5,23,48]. The removal of hemicellulose and lignin also increases the relative glucan content in the treated sample [48,52]. Furthermore, H_3PO_4 disrupts the glycosidic linkages within the lignin–carbohydrate complex structure of the lignocellulosic biomass during the pretreatment process, "which causes the partial solubilization of hemicellulose, lignin, and cellulose components" [4,53,54].

In addition, the recovery yield of solid, glucan, xylan, arabinan, and total lignin gradually decreased with increasing H_3PO_4 concentration. These modifications indicated that an increase in the H_3PO_4 concentration from 70% to 80% caused a gradual release of xylan, arabinan lignin, and glucan due to the breaking of glycosidic linkages within the lignin–carbohydrate complex structure of *T. latifolia* biomass. Consequently, the recovery yields of xylan, arabinan, and total lignin were all <50%. In contrast, the recovery yields of solids and glucans exceeded 50% and 90%, respectively. However, treating the feedstock with the maximum concentration (85% H_3PO_4) can not only damage the structure of the lignin–carbohydrate complex but also disrupt the organized hydrogen bond network that exists in crystalline cellulose. "Because of this structural alteration, the carbohydrate and lignin constituents dissolve more readily, leading to a substantial decrease in the recovery yields of solids, glucan, xylan, and total lignin, whereas arabinan is eliminated entirely" [23,25,53–57]. These phenomena exhibit parallels to those reported in several studies conducted on different biomass sources, including *Glycyrrhiza glabra* [57], *V. pusilla* [14], *Corchorus capsularis* [58], poplar [30], *Hibiscus cannabinus* [26], *Durio zibethinus* [24], *Hibiscus sabdariffa* [25], wheat straw [55], and *Luffa cylindrica* [59].

4.3. Impact of H₃PO₄ Concentration on Cellulose Crystallinity

Cellulose comprises both intra- and intermolecular hydrogen bonds, leading to exceptional stability in terms of its crystalline structure. This stability, however, has a substantial effect on enzymatic hydrolysis efficiency [23,60,61]. The degradation of cellulose in H_3PO_4 typically corresponds to esterification, which prevents the formation of hydrogen bonds throughout the cellulose chain [23,62]. Consequently, H_3PO_4 induces the swelling and disintegration of the crystalline region inside the cellulose structure [63]. "According to this study, the treatment of *T. latifolia* feedstock with 70%, 75%, and 80% H_3PO_4 increased the relative CrI value owing to the partial removal of amorphous fractions, such as hemicellulose and lignin, during the pretreatment process" [52,63–66]. The feedstock of *T. latifolia* exhibited a substantial reduction in relative CrI after treatment with 85% H_3PO_4 (Table 3). This was attributed to the disintegration of cellulose I crystalline sections, which caused the highly structured repeating units to become less rigid, forming cellulose II, also known as amorphous cellulose [67]. The findings of this study confirmed that the application of 85% H_3PO_4 to *T. latifolia* biomass alters the crystal structure of cellulose I to crystalline cellulose II. As several studies reported, crystalline cellulose is transformed into amorphous cellulose through pretreatment with above-critical (>80.5%) concentrated H_3PO_4 [14,67–69].

4.4. Impact of H3PO4 Concentration on Biomass Morphology

The SEM images demonstrate the recalcitrant nature of the untreated *T. latifolia* feedstock, which emerged from a complex of cellulose and hemicellulose biopolymers, together with heteropolymers of lignin [8,50,51]. After treating the sample with H_3PO_4, the images revealed that the morphological structure of *T. latifolia* feedstock gradually loosened, delaminated, and fibrillated, resulting in a highly disorganized arrangement. The severity of these modifications was dependent on the concentrations of H_3PO_4, suggesting that the alterations in the resistance structure of *T. latifolia* biomass were due to the influence of H_3PO_4, which caused the disruption of the intermolecular connections among cellulose, hemicellulose, and lignin. Consequently, hemicellulose and lignin were separated from the cellulose microfibers [23,48,54,56,62]. During the pretreatment process, cellulose swelling occurs as a consequence of the esterification reaction between the hydroxyl group of cellulose and phosphate ions derived from H_3PO_4 to form cellulose phosphate (cellulose-*O*-PO_3H_2), which extends cellulose chains, also leading to fiber structure disruption [23,62,69,70]. In addition to modifying the physical structure of the feedstock, H_3PO_4 enhances its surface area and porosity, thereby facilitating enzymatic hydrolysis [29,71–75]. Moreover, the SEM micrographs of *T. latifolia* treated with 85% H_3PO_4 revealed the most severe cellulose fiber degradation and structural alterations. This effect is attributable to the critical concentration of H_3PO_4 (80%), which destroys the ordered hydrogen bond network within crystalline cellulose, resulting in a transition from cellulose swelling to cellulose degradation [23,48,69,70].

The implications of the H_3PO_4 concentration on modifying the morphological structure and cellulose crystalline pattern in this biomass correspond to alterations in the chemical composition, as discussed in Sections 4.2. Furthermore, these effects correspond to those observed in various types of lignocellulosic biomass, including *V. pusilla* [14], *H. cannabinus* [26], *Pennisetum purpureum* [76], *D. zibethinus* [24], *Achyranthes aspera* and *Sida acuta* [29], and cotton fibers [72].

4.5. Enzymatic Saccharification Yields

The current study proved that the raw biomass of *T. latifolia* exhibited the lowest HE and GR yields, which can be attributed to the recalcitrance of lignocellulosic biomass, which hinders interactions between enzymes and plant matter [49,77]. When the feedstock was treated with 70%, 75%, 80%, and 85% H_3PO_4, the HE and GR yields substantially improved compared with those of the untreated sample. However, the level of improvement varied depending on H_3PO_4 concentration in the pretreatment process. These results suggested that the chemical linkages present in the lignin–carbohydrate complex structure were disrupted when the feedstock was treated with 70–80% H_3PO_4. "Owing to this disintegration, hemicellulose and lignin were partially eliminated during the

pretreatment process, indicating that the physical barriers (hemicellulose and lignin) surrounding the cellulose were removed" [23,48,54]. Additionally, these effects increased the pore size and cellulose accessibility surface, leading to improved HE and GR yields for each treated sample [14,23,24,62,66]. Consequently, the greatest amounts of HE and GR were achieved through treatment with 75% and 80% H_3PO_4. Pretreatment of the sample with 85% H_3PO_4 facilitated a more rapid degradation of the linkages between lignin and carbohydrates in complex structures, as well as the destruction of the crystalline framework of cellulose under harsh conditions. The cellulose therefore underwent a complete transformation into the amorphous form. This increased the dissolution of cellulose, hemicellulose, and lignin substantially [23,63,69]. The degradation of amorphous cellulose occurs faster and more easily than that of crystalline cellulose [62,78]. These modifications significantly reduce the solid and glucan recovery yields of the treated biomass, leading to a substantial decrease in the GR yield following enzymatic hydrolysis [14,23,55,57,79,80].

Although the percentage of HE yields was most likely the maximum when treated with 85% H_3PO_4, the HE yields between the specimens treated with 75%, 80%, and 85% H_3PO_4 had no significant differences ($p < 0.05$). The optimal pretreatment conditions are determined by an exceptionally high glucose recovery value that approaches the initial glucan content of the biomass [14,80]. Consequently, 75% H_3PO_4 was the most appropriate pretreatment condition for *T. latifolia* feedstock because it maximized both the HE (86.9 ± 0.7%) and GR (32.8 ± 0.3%) yields, which were enhanced roughly 2.9 and 2.7 times, respectively, compared with the raw material, as both the solid (55.8 ± 0.6%) and glucan (93.0 ± 0.3%) recovery levels were found to be relatively high. Under these conditions, the 33.9 ± 0.2% lignin retained in the treated biomass demonstrated that removing complete lignin from the biomass is unnecessary. In addition, increasing the percentage of CrI values (61.2%) in the treated sample did not impact the hydrolysis yield. Modifications in the chemical composition, morphological structure, and crystallinity of the treated biomass were correlated with the enhancement of HE and GR yields, and these observed events are similar to those described for other feedstocks, including *A. aspera*, *S. acuta* [29], *D. zibethinus* Murr [24], *H. cannabinus* [26], *V. pusilla* [14], and *G. glabra* [57].

4.6. Bioethanol Fermentation

Compared with the CT medium, the proliferation of yeast cells was considerably affected by the non-detoxified TBH medium. Furthermore, the ethanol yield in the CT medium was slightly greater than that in the TBH medium. The findings suggest that the non-detoxified TBH medium could contain inhibitory compounds, such as furfural, 5-hydroxymethyl furfural, levulinic acid, and other aromatic compounds, that were generated during the acid pretreatment of the raw feedstock [4,23,81]. These inhibitors adversely affect microbial growth, leading to a reduction in the rate of sugar uptake, which might subsequently decrease ethanol production [7,23]. Hydrolysate detoxification is considered imperative to prevent the inhibitory effects on fermenting organisms before its use as a substrate in bioethanol fermentation [82,83]. Despite these challenges, cellulosic ethanol was successfully produced using *S. cerevisiae* TISTR 5339 in the TBH medium derived from enzymatic saccharification without a detoxification process. These findings suggest that TBH could potentially be utilized as a sustainable carbon source for cellulosic ethanol production.

By employing the most effective H_3PO_4 pretreatment strategy for producing cellulosic ethanol, the material balance of *T. latifolia* feedstock was assessed, as shown in Figure 8. The 1000 g of *T. latifolia* feedstock pretreated with 75% H_3PO_4 resulted in the recovery of approximately 558 g of the treated material. Subsequently, both the untreated and treated samples were subjected to enzymatic hydrolysis to investigate the influence of pretreatment on enzymatic saccharification, which substantially improved both the HE and GR yields. The treated samples exhibited the highest HE (86.9%) and GR (328 g) yields after 72 h of saccharification. In contrast, untreated samples exhibited the lowest production of HE (29.5%) and GR (120 g). Compared with the untreated material, the HE and GR yields increased by approximately 2.9 and 2.7-fold, respectively. Next, a non-detoxified TBH medium containing 20 g/L glucose from the treated biomass was fermented using *S. cerevisiae* TISTR 5339 for ethanol production. The glucose from the untreated and treated feedstocks yielded an

estimated 53 and 145 g of cellulosic ethanol, respectively. The ethanol yield from the treated feedstock demonstrated an enhancement of approximately 2.7-fold compared with that of the untreated samples. The results of the current study emphasize the potential utilization of a non-detoxified TBH medium produced from *T. latifolia* feedstock as a feasible carbon source for the manufacture of cellulosic ethanol by *S. cerevisiae* TISTR 5339.
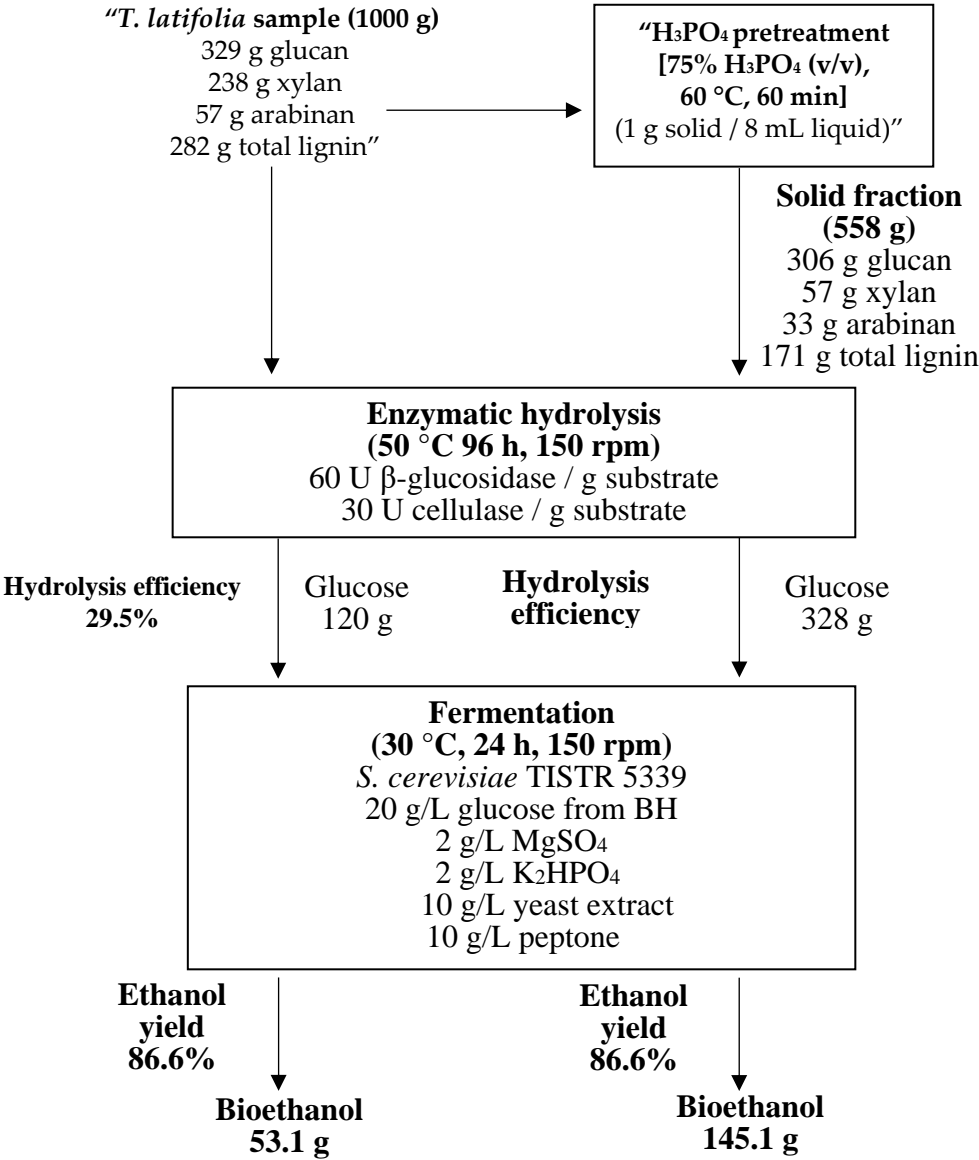
"T. latifolia sample (1000 g)
329 g glucan
238 g xylan
57 g arabinan
282 g total lignin"

"H₃PO₄ pretreatment
[75% H₃PO₄ (v/v),
60 °C, 60 min]
(1 g solid / 8 mL liquid)"

Solid fraction
(558 g)
306 g glucan
57 g xylan
33 g arabinan
171 g total lignin

Enzymatic hydrolysis
(50 °C 96 h, 150 rpm)
60 U β-glucosidase / g substrate
30 U cellulase / g substrate

Hydrolysis efficiency 29.5%   Glucose 120 g   **Hydrolysis efficiency**   Glucose 328 g

Fermentation
(30 °C, 24 h, 150 rpm)
S. cerevisiae TISTR 5339
20 g/L glucose from BH
2 g/L MgSO₄
2 g/L K₂HPO₄
10 g/L yeast extract
10 g/L peptone

Ethanol yield 86.6%   **Ethanol yield 86.6%**

Bioethanol 53.1 g   **Bioethanol 145.1 g**

Figure 8. Material balance of *T. latifolia* biomass utilized for bioethanol fermentation.

Overall, our findings provide crucial information and suggest that treatment of *T. latifolia* biomass with H₃PO₄ enhances the efficiency of enzymatic hydrolysis and ethanol production. In the future, research should focus on response surface methodology and other similar techniques to analyze the complexities of pretreatment to increase yield and pretreatment efficiency. These experimental designs help assess the effects of critical pretreatment variables, such as acid concentration, temperature, duration, solid and glucan recovery, lignin removal, solid loading in the enzymatic hydrolysis process, and HE and GR yield. Moreover, modification of the genome of the microbial strain is required to enhance its fermentation efficiency and increase its resistance to inhibitors.

5. Conclusions

This study explored the use of *T. latifolia* feedstock for biochemical conversion, highlighting its potential as a cost-effective alternative energy source. The results showed that *T. latifolia* feedstock could be utilized as a viable source of lignocellulosic biomass for the production of environmentally friendly biofuels and other bio-based chemicals without affecting global food security. The pretreatment of *T. latifolia* feedstock with H_3PO_4 provided substantial advantages as it accelerated the decomposition of hemicellulose and lignin while preserving cellulose. Such modifications could enhance the cellulose surface area, resulting in an increased efficacy of enzymatic hydrolysis and optimal utilization of this biomass. The maximum GR and HE yields were obtained by pretreatment of *T. latifolia* feedstock with 75% H_3PO_4 at 60 °C for 60 min. The observed delignification rate of approximately 66% suggests that complete lignin removal may not be necessary. Furthermore, this study demonstrated the potential use of glucose in the TBH medium as a sustainable carbon source for cellulosic ethanol production without additional detoxification, resulting in a considerably higher ethanol yield than that of untreated biomass. Therefore, utilizing *T. latifolia* feedstock provides an opportunity to enhance the sustainability of bioenergy production and mitigate adverse environmental consequences associated with improper biomass disposal practices. In future studies, the analysis of the effects of pretreatment parameters using response surface methodology will be crucial. Furthermore, bioethanol yields can be increased by enhancing fermentation efficiency through the modification of the microbial strain to improve its efficiency and tolerance to inhibitors.

References

1. Afraz, M.; Muhammad, F.; Nisar, J.; Shah, A.; Munir, S.; Ali, G.; Ahmad, A. Production of value added products from biomass waste by pyrolysis: An updated review. *Waste Management Bulletin.* **2024**, *1*, 30–40. DOI:10.1016/j.wmb.2023.08.004.

2. Mankar, A.R.; Pandey, A.; Modak, A.; Pant, K.K. Pretreatment of lignocellulosic biomass: A review on recent advances. *Bioresour. Technol.* **2021**, *334*, 125235. DOI:10.1016/j.biortech.2021.125235.

3. Zadeh, Z.E.; Abdulkhani, A.; Aboelazayem, O.; Saha, B. Recent insights into lignocellulosic biomass pyrolysis: A critical review on pretreatment, characterization, and products upgrading. *Processes.* **2020**, *8*. DOI:10.3390/pr8070799.

4. Zhao, L.; Sun, Z.F.; Zhang, C.C.; Nan, J.; Ren, N.Q.; Lee, D.J.; Chen, C. Advances in pretreatment of lignocellulosic biomass for bioenergy production: Challenges and perspectives. *Bioresour. Technol.* **2022**, *343*, 126123. DOI:10.1016/j.biortech.2021.126123.

5. Shukla, A.; Kumar, D.; Girdhar, M.; Kumar, A.; Goyal, A.; Malik, T.; Mohan, A. Strategies of pretreatment of feedstocks for optimized bioethanol production: Distinct and integrated approaches. *Biotechnol. Biofuels Bioprod.* **2023**, *16*, 44. DOI:10.1186/s13068-023-02295-2.

6. Toor, M.; Kumar, S.S.; Malyan, S.K.; Bishnoi, N.R.; Mathimani, T.; Rajendran, K.; Pugazhendhi, A. An overview on bioethanol production from lignocellulosic feedstocks. *Chemosphere.* **2020**, *242*, 125080. DOI:10.1016/j.chemosphere.2019.125080.

7. Bhatia, S.K.J.; Jagtap, S.S.; Bedekar, A.A.; Bhatia, R.K.; Patel, A.K.; Pant, D.; Rajesh Banu, J.; Rao, C.V.; Kim, Y.G.; Yang, Y.H. Recent developments in pretreatment technologies on lignocellulosic biomass: Effect of key parameters, technological improvements, and challenges. *Bioresour. Technol.* **2020**, *300*, 122724. DOI:10.1016/j.biortech.2019.122724.

8. Sharma, S.; Tsai, M.-L.; Sharma, V.; Sun, P.-P.; Nargotra, P.; Bajaj, B.K.; Chen, C.-W.; Dong, C.-D. Environment friendly pretreatment approaches for the bioconversion of lignocellulosic biomass into biofuels and value-added products. *Environments.* **2023**, *10*. DOI:10.3390/environments10010006.

9. Hoang, A.T.; Nizetic, S.; Ong, H.C.; Chong, C.T.; Atabani, A.E.; Pham, V.V. Acid-based lignocellulosic biomass biorefinery for bioenergy production: Advantages, application constraints, and perspectives. *J. Environ. Manage.* **2021**, *296*, 113194. DOI:10.1016/j.jenvman.2021.113194.

10. Sun, W.; Li, X.; Zhao, J.; Qin, Y. Pretreatment strategies to enhance enzymatic hydrolysis and cellulosic ethanol production for biorefinery of corn stover. *Int. J. Mol. Sci.* **2022**, *23*. DOI:10.3390/ijms232113163.

11. Xiao, K.; Li, H.; Liu, L.; Liu, X.; Lian, Y. Quantitative comparison of the delignification performance of lignocellulosic biomass pretreatment technologies for enzymatic saccharification. *Environ. Sci. Pollut. Res. Int.* **2023**, *30*, 22929–22940. DOI:10.1007/s11356-022-23817-9.

12. Guo, Z.; Zhang, Q.; You, T.; Ji, Z.; Zhang, X.; Qin, Y.; Xu, F. Heteropoly acids enhanced neutral deep eutectic solvent pretreatment for enzymatic hydrolysis and ethanol fermentation of *Miscanthus x giganteus* under mild conditions. *Bioresour. Technol.* **2019**, *293*, 122036. DOI:10.1016/j.biortech.2019.122036.

13. Larnaudie, V.; Ferrari, M.D.; Lareo, C. Switchgrass as an alternative biomass for ethanol production in a biorefinery: Perspectives on technology, economics and environmental sustainability. *Renew. Sustain. Energy Rev.* **2022**, *158*, 112115. DOI:10.1016/j.rser.2022.112115.

14. Wongleang, S.; Premjet, D.; Premjet, S. Cellulosic ethanol production from weed biomass hydrolysate of *Vietnamosasa pusilla*. *Polymers.* **2023**, *15*, 1103. DOI:10.3390/polym15051103.

15. Sespene, J.; Fetalvero, E.; Faminial, T. Tiger grass industry in Marigondon Norte, San Andres, Romblon: Implications for research and development. *Romblon State University Research Journal.* **2011**, *1*, 81–95. Available online: https://ojs.rsu.edu.ph/index.php/rsurj/article/view/24.

16. Shrestha, S.; Park, J.H.; Cho, J.G.; Lee, D.Y.; Jeong, R.H.; Song, M.C.; Cho, S.K.; Lee, D.S.; Baek, N.I. Phytochemical constituents from the florets of tiger grass *Thysanolaena latifolia* from Nepal. *J. Asian Nat. Prod. Res.* **2016**, *18*, 206–213. DOI:10.1080/10286020.2015.1062757.

17. Pandiyan, K.; Singh, A.; Singh, S.; Saxena, A.K.; Nain, L. Technological interventions for utilization of crop residues and weedy biomass for second generation bio-ethanol production. *Renew. Energy.* **2019**, *132*, 723–741. DOI:10.1016/j.renene.2018.08.049.

18. Saikia, D.C.; Goswami, T.; Chaliha, B.P. Paper from *Thysanolaena maxima*. *Bioresour. Technol.* **1992**, *40*, 245–248. DOI:10.1016/0960-8524(92)90150-V.

19. Flora & fauna, *Thysanolaena latifolia*. Available online: https://www.nparks.gov.sg/florafaunaweb/flora/2/5/2512# (accessed on 20 Jan 2024).

20. Komolwanich, T.; Prasertwasu, S.; Khumsupan, D.; Tatijarern, P.; Chaisuwan, T.; Luengnaruemitchai, A.; Wongkasemjit, S. Evaluation of highly efficient monomeric sugar yield from Thai Tiger grass (*Thysanolaena maxima*). *Mater. Res. Innov.* **2016**, *20*, 259–267. DOI:10.1080/14328917.2015.1105573.

21. Kumar, B.; Bhardwaj, N.; Agrawal, K.; Chaturvedi, V.; Verma, P. Current perspective on pretreatment technologies using lignocellulosic biomass: An emerging biorefinery concept. *Fuel Process. Technol.* **2020**, *199*, 106244. DOI:10.1016/j.fuproc.2019.106244.

22. Rollin, J.A.; Zhu, Z.; Sathitsuksanoh, N.; Zhang, Y.H.P. Increasing cellulose accessibility is more important than removing lignin: A comparison of cellulose solvent-based lignocellulose fractionation and soaking in aqueous ammonia. *Biotechnol. Bioeng.* **2011**, *108*, 22–30. DOI:10.1002/bit.22919.

23. Satari, B.; Karimi, K.; Kumar, R. Cellulose solvent-based pretreatment for enhanced second-generation biofuel production: A review. *Sustainable Energy Fuels.* **2019**, *3*, 11–62. DOI:10.1039/C8SE00287H.

24. Obeng, A.; Premjet, D.; Premjet, S. Fermentable sugar production from the peels of two durian (*Durio zibethinus* Murr.) cultivars by phosphoric acid pretreatment. *Resources.* **2018**, *7*, 60. DOI:10.3390/resources7040060.

25. Premjet, S.; Dana, S.; Obeng, A.K.; Premjet, D. Enzymatic response to structural and chemical transformations in *Hibiscus sabdariffa* var. altissima bark and core during phosphoric acid pretreatment. *BioRes.* **2018**, *13*, 6778–6789. DOI:10.15376/biores.13.3.6778-6789.

26. Premjet, D.; Wongleang, S.; Premjet, S. Enhancing glucose recovery from *Hibiscus cannabinus* L. through phosphoric acid pretreatment. *Energies.* **2022**, *15*, 7573. DOI:10.3390/en15207573.

27. Yu, H.; Xiao, W.; Han, L.; Huang, G. Characterization of mechanical pulverization/phosphoric acid pretreatment of corn stover for enzymatic hydrolysis. *Bioresour. Technol.* **2019**, *282*, 69–74. DOI:10.1016/j.biortech.2019.02.104.

28. Jin, Y.; Liu, J.; Yang, H.; Shi, Z.; Zhao, P.; Yang, J. Improving enzymatic saccharification and ethanol production of bamboo residues with sulfomethylation-aided phosphoric acid pretreatment. *Ind. Crops Prod.* **2021**, *170*, 113733. DOI:10.1016/j.indcrop.2021.113733.

29. Siripong, P.; Duangporn, P.; Takata, E.; Tsutsumi, Y. Phosphoric acid pretreatment of *Achyranthes aspera* and *Sida acuta* weed biomass to improve enzymatic hydrolysis. *Bioresour. Technol.* **2016**, *203*, 303–308. DOI:10.1016/j.biortech.2015.12.037.

30. Chen, D.; Tang, W.; Wang, H.; Sheng, Y.; Tan, X.; Shi, Y.; Fan, W.; Ge, S. Phosphoric acid pretreatment of poplar to optimize fermentable sugars production based on orthogonal experimental design. *Front. Chem.* **2023**, *11*, 1119215. DOI:10.3389/fchem.2023.1119215.

31. Mund, N.K.; Dash, D.; Barik, C.R.; Goud, V.V.; Sahoo, L.; Mishra, P.; Nayak, N.R. Evaluation of efficient glucose release using sodium hydroxide and phosphoric acid as pretreating agents from the biomass of *Sesbania grandiflora* (L.) pers.: A fast growing tree legume. *Bioresour. Technol.* **2017**, *236*, 97–105. DOI:10.1016/j.biortech.2017.03.177.

32. Sluiter, A.; Hames, B.; Ruiz, R.; Scarlata, C.; Sluiter, J.; Templeton, D. Determination of ash in biomass. In *Technical Report NREL/TP-510-42622;* National Renewable Energy Laboratory (National Renewable Energy Laboratory, Office of Energy Efficiency and Renewable Energy): Golden, CO, USA, 2008; pp. 1–8.

33. Sluiter, A.; Hames, B.; Ruiz, R.; Scarlata, C.; Sluiter, J.; Templeton, D.; Crocker, D. Determination of structural carbohydrates and lignin in biomass. In *Technical Report NREL/TP-510-42618;* National Renewable Energy Laboratory (National Renewable Energy Laboratory, Office of Energy Efficiency and Renewable Energy): Golden, CO, USA, 2012; pp. 1–15.

34. Sluiter, A.; Ruiz, R.; Scarlata, C.; Sluiter, J.; Templeton, D. Determination of extractives in biomass. In *Technical Report NREL/TP-510-42619*; National Renewable Energy Laboratory (National Renewable Energy Laboratory, Office of Energy Efficiency and Renewable Energy): Golden, CO, USA, 2008; pp. 1–12.

35. Segal, L.; Creely, J.J.; Martin, A.E.; Conrad, C.M. An empirical method for estimating the degree of crystallinity of native cellulose using the X-ray diffractometer. *Text. Res. J.* **1959**, *29*, 786–794. DOI:10.1177/004051755902901003.

36. Siripong, P.; Doungporn, P.; Yoo, H.Y.; Kim, S.W. Improvement of sugar recovery from *Sida acuta* (Thailand weed) by NaOH pretreatment and application to bioethanol production. *Korean J. Chem. Eng.* **2018**, *35*, 2413–2420. DOI:10.1007/s11814-018-0170-1.

37. Wongleang, S.; Premjet, D.; Premjet, S. Physicochemical pretreatment of *Vietnamosasa pusilla* for bioethanol and xylitol production. *Polymers.* **2023**, *15*, 3990. DOI:10.3390/polym15193990.

38. Binod, P.; Sindhu, R.; Singhania, R.R.; Vikram, S.; Devi, L.; Nagalakshmi, S.; Kurien, N.; Sukumaran, R.K.; Pandey, A. Bioethanol production from rice straw: An overview. *Bioresour. Technol.* **2010**, *101*, 4767–4774. DOI:10.1016/j.biortech.2009.10.079.

39. Khan, M.A.; Dharmalingam, B.; Chuetor, S.; Cheng, Y.-S.; Sriariyanun, M. Comprehensive review on effective conversion of lignocellulosic biomass to levulinic acid. *Biomass Convers. Biorefin.* **2023**. DOI:10.1007/s13399-023-04663-2.

40. Manyi-Loh, C.E.; Lues, R. Anaerobic digestion of lignocellulosic biomass: Substrate characteristics (challenge) and innovation. *Fermentation.* **2023**, *9*. DOI:10.3390/fermentation9080755.

41. Li, H.Y.; Chen, X.; Wang, C.Z.; Sun, S.N.; Sun, R.C. Evaluation of the two-step treatment with ionic liquids and alkali for enhancing enzymatic hydrolysis of Eucalyptus: Chemical and anatomical changes. *Biotechnol. Biofuels.* **2016**, *9*, 166. DOI:10.1186/s13068-016-0578-y.

42. Pan, S.; Chi, Y.; Zhou, L.; Li, Z.; Du, L.; Wei, Y. Evaluation of squeezing pretreatment for improving methane production from fresh banana pseudo-stems. *Waste Manag.* **2020**, *102*, 900–908. DOI:10.1016/j.wasman.2019.12.011.

43. Montoya-Rosales, J.J.; Peces, M.; González-Rodríguez, L.M.; Alatriste-Mondragón, F.; Villa-Gómez, D.K. A broad overview comparing a fungal, thermal and acid pre-treatment of bean straw in terms of substrate and anaerobic digestion effect. *Biomass Bioenergy.* **2020**, *142*, 105775. DOI:10.1016/j.biombioe.2020.105775.

44. Bonfiglio, F.; Cagno, M.; Yamakawa, C.K.; Mussatto, S.I. Production of xylitol and carotenoids from Switchgrass and *Eucalyptus globulus* hydrolysates obtained by intensified steam explosion pretreatment. *Ind. Crops Prod.* **2021**, *170*, 113800. DOI:10.1016/j.indcrop.2021.113800.

45. Fonseca, B.G.; Mateo, S.; Roberto, I.C.; Sánchez, S.; Moya, A.J. Bioconversion in batch bioreactor of olive-tree pruning biomass optimizing treatments for ethanol production. *Biochem. Eng. J.* **2020**, *164*, 107793. DOI:10.1016/j.bej.2020.107793.

46. Liu, Y.; Zheng, X.; Tao, S.; Hu, L.; Zhang, X.; Lin, X. Process optimization for deep eutectic solvent pretreatment and enzymatic hydrolysis of sugar cane bagasse for cellulosic ethanol fermentation. *Renew. Energy.* **2021**, *177*, 259–267. DOI:10.1016/j.renene.2021.05.131.

47. Broda, M.; Yelle, D.J.; Serwańska, K. Bioethanol production from lignocellulosic biomass—Challenges and solutions. *Molecules.* **2022**, *27*. DOI:10.3390/molecules27248717.

48. Li, T.; Fang, Q.; Chen, H.; Qi, F.; Ou, X.; Zhao, X.; Liu, D. Solvent-based delignification and decrystallization of wheat straw for efficient enzymatic hydrolysis of cellulose and ethanol production with low cellulase loadings. *RSC Adv.* **2017**, *7*, 10609–10617. DOI:10.1039/C6RA28509K.

49. Rahmati, S.; Doherty, W.; Dubal, D.; Atanda, L.; Moghaddam, L.; Sonar, P.; Hessel, V.; Ostrikov, K. Pretreatment and fermentation of lignocellulosic biomass: Reaction mechanisms and process engineering. *React. Chem. Eng.* **2020**, *5*, 2017–2047. DOI:10.1039/D0RE00241K.

50. Arora, A.; Nandal, P.; Singh, J.; Verma, M.L. Nanobiotechnological advancements in lignocellulosic biomass pretreatment. *Mater. Sci. Energy Technol.* **2020**, *3*, 308–318. DOI:10.1016/j.mset.2019.12.003.

51. Yoo, C.G.; Meng, X.; Pu, Y.; Ragauskas, A.J. The critical role of lignin in lignocellulosic biomass conversion and recent pretreatment strategies: A comprehensive review. *Bioresour. Technol.* **2020**, *301*, 122784. DOI:10.1016/j.biortech.2020.122784.

52. Kundu, C.; Samudrala, S.P.; Kibria, M.A.; Bhattacharya, S. One-step peracetic acid pretreatment of hardwood and softwood biomass for platform chemicals production. *Sci. Rep.* **2021**, *11*, 11183. DOI:10.1038/s41598-021-90667-9.

53. Lorenci Woiciechowski, A.; Dalmas Neto, C.J.; Porto de Souza Vandenberghe, L.; de Carvalho Neto, D.P.; Novak Sydney, A.C.; Letti, L.A.J.; Karp, S.G.; Zevallos Torres, L.A.; Soccol, C.R. Lignocellulosic biomass: Acid and alkaline pretreatments and their effects on biomass recalcitrance—Conventional processing and recent advances. *Bioresour. Technol.* **2020**, *304*, 122848. DOI:10.1016/j.biortech.2020.122848.

54. Zhang, Y.H.; Ding, S.Y.; Mielenz, J.R.; Cui, J.B.; Elander, R.T.; Laser, M.; Himmel, M.E.; McMillan, J.R.; Lynd, L.R. Fractionating recalcitrant lignocellulose at modest reaction conditions. *Biotechnol. Bioeng.* **2007**, *97*, 214–223. DOI:10.1002/bit.21386.

55. Prasad, S.; Malav, M.K.; Kumar, S.; Singh, A.; Pant, D.; Radhakrishnan, S. Enhancement of bio-ethanol production potential of wheat straw by reducing furfural and 5-hydroxymethylfurfural (HMF). *Bioresour. Technol. Rep.* **2018**, *4*, 50–56. DOI:10.1016/j.biteb.2018.09.007.

56. Sathitsuksanoh, N.; Zhu, Z.; Zhang, Y.H.P. Cellulose solvent- and organic solvent-based lignocellulose fractionation enabled efficient sugar release from a variety of lignocellulosic feedstocks. *Bioresour. Technol.* **2012**, *117*, 228–233. DOI:10.1016/j.biortech.2012.04.088.

57. Zhang, Y.; Guo, Y.; Xie, X.; Chernyshev, V.M.; Liu, Y.; Qi, W. Effects of phosphoric acid/hydrogen peroxide, ammonia/hydrogen peroxide and deep eutectic solvent pretreatments on component separation and enzymatic saccharification of *Glycyrrhiza residue*. *Ind. Crops Prod.* **2023**, *196*, 116525. DOI:10.1016/j.indcrop.2023.116525.

58. Wongleang, S.; Dana, S.; Premjet, D.; Premjet, S. Phosphoric acid pretreatment of *Corchorus capsularis* L. biomass for enhancing glucose recovery. *NU. Int. J. Sci.* **2023**, *20*, 1–13.

59. Wang, Q.; Shen, F.; Yang, G.; Zhang, Y.; Deng, S.; Hu, Y.; Zhang, J.; Song, C.; Zeng, Y. Pretreating Luffa sponge (*Luffa cylindrica* L.) with concentrated phosphoric acid and subsequent enzymatic saccharification. *BioResources.* **2016**, *11*, 14. DOI:10.15376/biores.11.1.899-912.

60. Ling, Z.; Chen, S.; Zhang, X.; Xu, F. Exploring crystalline-structural variations of cellulose during alkaline pretreatment for enhanced enzymatic hydrolysis. *Bioresour. Technol.* **2017**, *224*, 611–617. DOI:10.1016/j.biortech.2016.10.064.

61. Zoghlami, A.; Paës, G. Lignocellulosic biomass: Understanding recalcitrance and predicting hydrolysis. *Front. Chem.* **2019**, *7*, 874. DOI:10.3389/fchem.2019.00874.

62. Hossain, A.; Rahaman, M.S.; Lee, D.; Phung, T.K.; Canlas, C.G.; Simmons, B.A.; Renneckar, S.; Reynolds, W.; George, A.; Tulaphol, S.; et al. Enhanced softwood cellulose accessibility by H_3PO_4 pretreatment: High

sugar yield without compromising lignin integrity. *Ind. Eng. Chem. Res.* **2020**, *59*, 1010–1024. DOI:10.1021/acs.iecr.9b05873.

63. Wang, Q.; Tian, D.; Hu, J.; Shen, F.; Yang, G.; Zhang, Y.; Deng, S.; Zhang, J.; Zeng, Y.; Hu, Y. Fates of hemicellulose, lignin and cellulose in concentrated phosphoric acid with hydrogen peroxide (PHP) pretreatment. *RSC Adv.* **2018**, *8*, 12714–12723. DOI:10.1039/C8RA00764K.

64. Cai, D.; Li, P.; Chen, C.; Wang, Y.; Hu, S.; Cui, C.; Qin, P.; Tan, T. Effect of chemical pretreatments on corn stalk bagasse as immobilizing carrier of *Clostridium acetobutylicum* in the performance of a fermentation-pervaporation coupled system. *Bioresour. Technol.* **2016**, *220*, 68–75. DOI:10.1016/j.biortech.2016.08.049.

65. Obeng, A.K.; Premjet, D.; Premjet, S. Improved glucose recovery from durian peel by alkaline-catalyzed steam pretreatment. *PeerJ.* **2021**, *9*, e12026. DOI:10.7717/peerj.12026.

66. Zheng, Q.; Zhou, T.; Wang, Y.; Cao, X.; Wu, S.; Zhao, M.; Wang, H.; Xu, M.; Zheng, B.; Zheng, J.; et al. Pretreatment of wheat straw leads to structural changes and improved enzymatic hydrolysis. *Sci. Rep.* **2018**, *8*, 1321. DOI:10.1038/s41598-018-19517-5.

67. Du, X.; Lucia, L.A.; Ghiladi, R.A. Development of a highly efficient pretreatment sequence for the enzymatic saccharification of Loblolly pine wood. *ACS Sustainable Chem. Eng.* **2016**, *4*, 3669–3678. DOI:10.1021/acssuschemeng.6b00198.

68. Sathitsuksanoh, N.; Zhu, Z.; Wi, S.; Zhang, Y.H. Cellulose solvent-based biomass pretreatment breaks highly ordered hydrogen bonds in cellulose fibers of switchgrass. *Biotechnol. Bioeng.* **2011**, *108*, 521–529. DOI:10.1002/bit.22964.

69. Zhang, Y.H.; Cui, J.; Lynd, L.R.; Kuang, L.R. A transition from cellulose swelling to cellulose dissolution by o-phosphoric acid: Evidence from enzymatic hydrolysis and supramolecular structure. *Biomacromolecules.* **2006**, *7*, 644–648. DOI:10.1021/bm050799c.

70. Zhang, J.; Zhang, J.; Lin, L.; Chen, T.; Zhang, J.; Liu, S.; Li, Z.; Ouyang, P. Dissolution of microcrystalline cellulose in phosphoric acid—Molecular changes and kinetics. *Molecules.* **2009**, *14*, 5027–5041. DOI:10.3390/molecules14125027.

71. Gabhane, J.; Prince William, S.P.M.; Vaidya, A.N.; Mahapatra, K.; Chakrabarti, T. Influence of heating source on the efficacy of lignocellulosic pretreatment—A cellulosic ethanol perspective. *Biomass Bioenergy.* **2011**, *35*, 96–102. DOI:10.1016/j.biombioe.2010.08.026.

72. Gourlay, K.; Arantes, V.; Saddler, J.N. Use of substructure-specific carbohydrate binding modules to track changes in cellulose accessibility and surface morphology during the amorphogenesis step of enzymatic hydrolysis. *Biotechnol. Biofuels.* **2012**, *5*, 51. DOI:10.1186/1754-6834-5-51.

73. Jackson de Moraes Rocha, G.; Martin, C.; Soares, I.B.; Souto Maior, A.M.; Baudel, H.M.; Moraes de Abreu, C.A. Dilute mixed-acid pretreatment of sugarcane bagasse for ethanol production. *Biomass Bioenergy.* **2011**, *35*, 663–670. DOI:10.1016/j.biombioe.2010.10.018.

74. Tong, D.; Zhan, P.; Zhang, W.; Zhou, Y.; Huang, Y.; Qing, Y.; Chen, J. Surfactant-assisted dilute phosphoric acid plus steam explosion of poplar for fermentable sugar production. *ChemistrySelect.* **2022**, *7*, e202200423. DOI:10.1002/slct.202200423.

75. Tong, W.; Fang, H.; Song, K.; Xie, X.; Wang, J.; Jin, Y.; Wu, S.; Hu, J.; Chu, Q. Modified acid pretreatment to alter physicochemical properties of biomass for full cellulose/hemicellulose utilization. *Carbohydr. Polym.* **2023**, *299*, 120182. DOI:10.1016/j.carbpol.2022.120182.

76. Haldar, D.; Purkait, M.K. Thermochemical pretreatment enhanced bioconversion of Elephant grass (*Pennisetum purpureum*): Insight on the production of sugars and lignin. *Biomass Convers. Biorefin.* **2022**, *12*, 1125–1138. DOI:10.1007/s13399-020-00689-y.

77. Shiva, R.-J., R.M.; Rodríguez-Jasso, R.M.; Rosero-Chasoy, G.; López-Sandin, I.; Morais, A.R.C.; Ruiz, H.A. Enzymatic hydrolysis, kinetic modeling of hemicellulose fraction, and energy efficiency of autohydrolysis pretreatment using agave bagasse. *Bioenergy Res.* **2023**, *16*, 75–87. DOI:10.1007/s12155-022-10442-0.

78. Karimi, K.; Taherzadeh, M.J. A critical review of analytical methods in pretreatment of lignocelluloses: Composition, imaging, and crystallinity. *Bioresour. Technol.* **2016**, *200*, 1008–1018. DOI:10.1016/j.biortech.2015.11.022.

79. Sathitsuksanoh, N.; George, A.; Zhang, Y.-H.P. New lignocellulose pretreatments using cellulose solvents: A review. *J. Chem. Technol. Biotechnol.* **2013**, *88*, 169–180. DOI:10.1002/jctb.3959.

80. Yoo, H.Y.; Lee, J.H.; Kim, D.S.; Lee, J.H.; Lee, S.K.; Lee, S.J.; Park, C.; Kim, S.W. Enhancement of glucose yield from canola agricultural residue by alkali pretreatment based on multi-regression models. *J. Ind. Eng. Chem.* **2017**, *51*, 303–311. DOI:10.1016/j.jiec.2017.03.018.

81. Arisht, S.N.; Abdul, P.M.; Liu, C.-M.; Lin, S.-K.; Maaroff, R.M.; Wu, S.-Y.; Jahim, J.M. Biotoxicity assessment and lignocellulosic structural changes of phosphoric acid pre-treated young coconut husk hydrolysate for biohydrogen production. *Int. J. Hydrog. Energy.* **2019**, *44*, 5830–5843. DOI:10.1016/j.ijhydene.2019.01.116.

82. Lee, J.; Kim, S.; Lee, K.H.; Lee, S.K.; Chun, Y.; Kim, S.W.; Park, C.; Yoo, H.Y. Improvement of bioethanol production from waste chestnut shells via evaluation of mass balance-based pretreatment and glucose recovery process. *Environ. Technol. Innov.* **2022**, *28*, 102955. DOI:10.1016/j.eti.2022.102955.

83. Nandal, P.; Sharma, S.; Arora, A. Bioprospecting non-conventional yeasts for ethanol production from rice straw hydrolysate and their inhibitor tolerance. *Renew. Energy.* **2020**, *147*, 1694–1703. DOI:10.1016/j.renene.2019.09.067.