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Effect of Drying Pretreatment on Cellulolytic Enzymatic Hydrolysis of Lignin from Napier Grass

Syazmi Zul Arif Hakimi Saadon [1] and Noridah Binti Osman [1,2,*]

[1] Department of Chemical Engineering, Universiti Teknologi PETRONAS, Bandar Seri Iskandar 32610, Perak, Malaysia
[2] Higher Education Center of Excellence–Center for Biofuel and Biochemical Research, Institute of Self-Sustainable Building, Universiti Teknologi PETRONAS, Bandar Seri Iskandar 32610, Perak, Malaysia
* Correspondence: noridah.osman@utp.edu.my

Abstract: Biomass can be a viable supplement and alternative to non-renewable sources of fuel and chemicals. Lignin is an important part of biomass sources which can be used in various chemical and fuel industries. This study explores the pretreatment of lignin from Napier grass using thermal and physical means, as well as extraction of lignin via cellulolytic enzymatic hydrolysis to determine the optimum condition for feedstock pretreatment. Napier grass parts under various drying conditions and particle sizes were treated with enzymes. Moisture analysis, FTIR spectroscopy, UV–Vis analysis, and Klason lignin were carried out to analyze the moisture, functional group, and yield of lignin. Moisture content of the samples were inversely proportional to the drying conditions. The FTIR result showed lower peak intensity for higher drying conditions, while ball-milling showed less reduction in peak intensity. More Klason lignin was extracted under higher drying conditions. The yield of cellulolytic enzymatic lignin (CEL) was found to be more than actual lignin content, suggesting cellulose was not fully degraded. The FTIR spectra of CEL was found to be closer to that of lignin, but purification was still needed. Optimization was carried out by evaluating the statistical significance of each pretreatment effect of the pretreatments.

Keywords: cellulase; enzymatic hydrolysis; lignin; Napier grass; pretreatment

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1. Introduction

Biomass is considered to be a potential source of renewable and sustainable energy going forward. Its usage is gaining momentum because of its wide availability and eco-friendly nature. In Malaysia, Energy Statistics published in 2020 show that fossil fuel is still heavily relied on while biomass-based energy only accommodated 0.2% of the primary fuel production for the year 2018, retaining the same percentage as three years prior [1,2]. Biomass can be categorized into six main sources: (1) dedicated energy crops; (2) agricultural crop residue; (3) forestry residues; (4) algae; (5) wood processing residues; (6) sorted municipal waste; and (7) wet waste [3–5]. Each of these biomass sources can be turned into value-added products for energy consumption, chemical manufacturing, or valuable material production. Napier grass is currently classed as agricultural crop residue, but it has the potential to become an energy crop since it can be turned into solid fuel [6], bioethanol [7–9] and also biogas [10,11].

These valuable products are derived from the lignocellulosic material, which comprises mostly cellulose, lignin hemicellulose and extractives. Lignin is the second most abundant naturally occurring polymer and the most abundant aromatic compound in nature. Its purposes in woody biomass are to provide biological and chemical protection from degradation, as well as structural strength and rigidity to the plant [12]. It can be found in all types of plant, including hardwood, softwood, and herbaceous plants as a major constituent in the structural cell wall. Lignin is the only non-carbohydrate polymer of the three components, and it comprises about one-third of the mass of the lignocellulose. As

it is commonly found in biomass products, it is needless to mention that it can be exploited and applied in various sectors. The research on lignin has increased over the years. As interest in cellulose rises, it has resulted in an incremental increase in lignin research since lignin is considered a by-product of cellulose extraction. According to Web of Science, the number of research publications of cellulose and lignin has increased steadily by an average of 11% each year. Due to the low reproducibility of woody biomass, much research is focusing on herbaceous plants to supplement lignin production from biomass. Although lignin content in herbaceous plants is lower than hardwood and softwood, herbaceous plants have high annual renewability and the largest annual biomass stock [13].

Pretreatment of feedstock is an important step in all biochemical conversions. It is a necessary step to prepare the feedstock for further processes, and functions to enhance the digestibility of lignocellulosic components, increase accessibility to the targeted component, and also to ease extraction. As mentioned by Kumar and Sharma [14], the goal of all pretreatments is to avoid a reduction in size, preserve the saccharide fractions, limit formation of degradation products, and minimize energy and cost. During the pre-treatment process, the recalcitrance of the lignocellulose structure is disrupted when the lignin sheath is broken down, degradation of hemicellulose occurs, and there is a reduction in both crystallinity and degree of cellulose polymerization [15,16].

The aim of this study is to optimize the drying pretreatment process to maximize the production of lignin from the Klason method and cellulolytic enzymatic hydrolysis. In this study, we compared different parts of Napier grass (NG), which were then pretreated with variations in drying time, drying temperature, and milling process. Subsequent enzymatic hydrolysis was performed on the pretreated samples with additional variables of incubation temperature and day. The effect of the variables was analyzed using moisture content analysis, Fourier Transformed Infrared (FTIR) Spectroscopy Analysis and Ultraviolet–visible (UV-Vis) spectrophotometry, as well as by measuring the lignin yield upon pretreatment and enzymatic hydrolysis incubation.

2. Materials and Methods

2.1. Materials

The Napier grass was collected from Lambor Kanan, Perak, Malaysia, where it had been cultivated for about 3 months. The sample was then separated into stems and leaves and cleaned using tap water to remove any dirt or impurities. The Napier grass was left to sun-dry for 5 h. The sample was then placed in a cold room at a temperature of −4 °C to minimize rotting or degradation. Prior to pretreatment, the samples were thoroughly checked for degradation.

Acetic acid, ethanol, sodium bisulfite, sodium hydroxide pellets, nitric acid, sodium chlorite, toluene, and cellulase from Aspergillus Niger were of analytical grade and purchased from Avantis Laboratory Supply, Malaysia. Pure microcrystalline cellulose (MCC) and Kraft lignin (KrL) were also obtained from the same supplier to compare with extracted lignin. Crystalline nanocellulose (CNC) was obtained from PowerNano Malaysia, also to compare with extracted lignin.

2.2. Pretreatments

For thermal pretreatment, the feedstock was dried in the oven at 45, 75, 105 and 135 °C. The drying process was done for 5, 15 and 25 h. In each batch, the Napier grass was weighed to be around 30g, and its weight was recorded again after the drying process.

For physical pretreatment, the feedstock was then shredded using a laboratory blender and was sieved to ensure a uniform size of 250 µm using a sieve shaker. Some of the particles larger than 250 µm were then further milled using a planetary ball mill for 10 min at 500 rpm. The grinded feedstock was kept in an air-tight container to prevent moisture absorption. The pretreatment parameter is listed in Table 1 below.

Table 1. Pretreatment parameters.

Sample	Thermal Pretreatment	Physical Pretreatment
Type • Leaf • Stem	Drying temperature • 45 °C • 75 °C • 105 °C • 135 °C Drying time • 5 h • 15 h • 25 h	Particle size • 250 µm • Ball-milled

2.3. Klason Lignin

Klason lignin is the insoluble residue of acid hydrolysis. This method employs the ASTM D1105 Standard Test Method for Preparation of Extractive-Free Wood [17], the ASTM D1107 Standard Test Method for Ethanol-Toluene Solubility of Wood [18], and the ASTM D1106 Standard Test Method for Acid-Insoluble Lignin in Wood [19]. In general, 1 g of prepared sample was extracted for 4 h with 95% ethanol in a Soxhlet extraction apparatus. It was then extracted with ethanol–toluene mixture for another 8 h. After extraction, the sample was dried in the oven at 105 °C for 1 h and then was left to cool in a desiccator overnight.

The sample was digested in a hot water bath at approximately 100 °C for 3 h. The sample was filtered, washed with hot water and ethanol, and left to dry in air overnight. The air-dried sample was mixed with 15 mL of cold 72% sulfuric acid for 2 h at a temperature between 18 and 20 °C with frequent stirring. After that, the mixture was diluted to 3% concentration sulfuric acid and left to boil for 4 h. The sample was then filtered under suction and washed with hot water. The insoluble residue was dried in the oven for 2 h at 105 °C and left to cool in a desiccator overnight before it was weighed. The dilute acid solvent was collected and stored for analysis. The acid-insoluble lignin was calculated using Equation (1) as follows:

$$\% \, IL = \frac{W_{solid \; extract}}{W_{sample}} \times 100\% \quad (1)$$

2.4. Cellulolytic Enzymatic Hydrolysis

For the enzymatic hydrolysis, the samples were treated with cellulase from Aspergillus Niger. The treatment was based on the procedure described by NREL (ABBREVIATION) [20] and Chang et al. [21] and modified according to Rencoret et al. [22]. In brief, 0.02 M of pH 4.0 acetate buffer was prepared by dissolving sodium acetate trihydrate in acetic acid and water. Then, 1.5 g of sample was left to suspend in acetate buffer and 60 mg of cellulase was added to the suspension to maintain a 1:40 cellulose-to-sample ratio. The suspension was incubated at 250 rpm in an incubator shaker at varying temperatures and number of days. The incubation parameters are listed in Table 2 below. After the incubation, the solid was separated from the solution, where the solution contained soluble lignin. The solid was extracted with 96% dioxane for 4 h and subsequently extracted with 50% dioxane. After the extraction, the solid was washed with acetic acid and water. The solid was dried, weighed, and stored in an air-tight container. The CEL is calculated using Equation (1).

Table 2. Enzymatic incubation parameters.

Incubation Temperature	Incubation Time
• 30 °C • 40 °C • 50 °C	• 1 day • 3 days • 5 days

2.5. Moisture Analysis

Each sample was then evaluated after thermal and physical pretreatment using PRECISA XM60/XM60-HR moisture analyzing equipment. Each sample was weighed to around 0.5 g and placed on a heating plate. The test was repeated three times and the results were averaged. Moisture content was calculated using Equation (2).

$$Moisture\ content\ (\%) = \frac{Initial\ weight - Final\ weight}{Initial\ weight} \times 100\% \quad (2)$$

2.6. Fourier Transformed Infrared (FTIR) Spectroscopy Analysis

FTIR with Attenuated Total Reflectance (ATR) was used to evaluate the pretreated sample. Changes in the intensity of the functional group of the pretreated sample were determined and analyzed based on the FTIR spectrum. The spectra were recorded in the frequency range of 4000–400 cm^{-1} with a resolution of 1 cm^{-1}. The conversion from generated percent transmittance (%T) to absorbance (A) was performed using Equation (3).

$$A = 2 - log(\%T) \quad (3)$$

2.7. Ultraviolet–Visible (UV–Vis) Spectrophotometry

After incubation, the dilute acid solvent and enzymatic solutions were assessed with UV–Vis for soluble lignin content. Acid-soluble lignin content was determined using the UV absorbance measurement of the pretreated samples at 280 nm and 320 nm according to the NREL procedure. Acid-soluble lignin content was calculated according to the following Equation (5):

$$\%\ ASL = \frac{UV_{abs} \times Vol_{filtrate} \times Dilution}{\varepsilon \times Weight_{sample} \times Pathlength} \times 100\% \quad (4)$$

where UVabs is the mean UV–Vis absorbance at 205 and 280 nm, Volfiltrate represents the volume of filtrate, ε refers to the molar absorptivity of biomass, Wsample represents the sample weight in milligrams, and Pathlength is the UV–Vis cell pathlength in cm. Dilution was set to 1 since no other diluting solvent was used.

2.8. Statistical Analysis

SAS statistical software was used to carry out statistical analysis of moisture content, FTIR spectrum peak intensity, and extracted lignin using Analysis of Variance (ANOVA). A significance level (denoted as α) of 0.05 indicated that a significant difference exists. If the p-value is less than or equal to the significance level, the null hypothesis can be rejected which means there is a significant difference between the changes in the set of data. However, when the p-value is greater than the significance level, it indicates that there is not enough evidence to reject the null hypothesis which means that the results do not have a significant difference between them.

3. Results and Discussion

3.1. Moisture Analysis

The moisture content of each datapoint was plotted as shown in Figure 1. As expected, a declining trend was observed with increasing time and temperature. Drying at 135 °C after 15 and 25 h resulted in the lowest moisture content, while a drying temperature of 45 °C showed the lowest loss of moisture content from the sample. A sharp decrease can be seen between 45 °C and 75 °C. At temperatures of 75 °C and above, it can be observed that the moisture content reached below 10%. This is in agreement with Houghton et al., who suggested that higher temperature is used to reduce moisture content [23]. It is also seen that the difference between stems and leaves was very significant, with stems containing and retaining more moisture. Higher moisture content results in less lignocellulose content per weight of sample, since much of the weight is contributed to by the weight of water molecules. In general, a moisture content of lower than 10 % is desired in all extractions so

that the presence of water molecules does not interrupt or hinder the process of extraction and isolation.

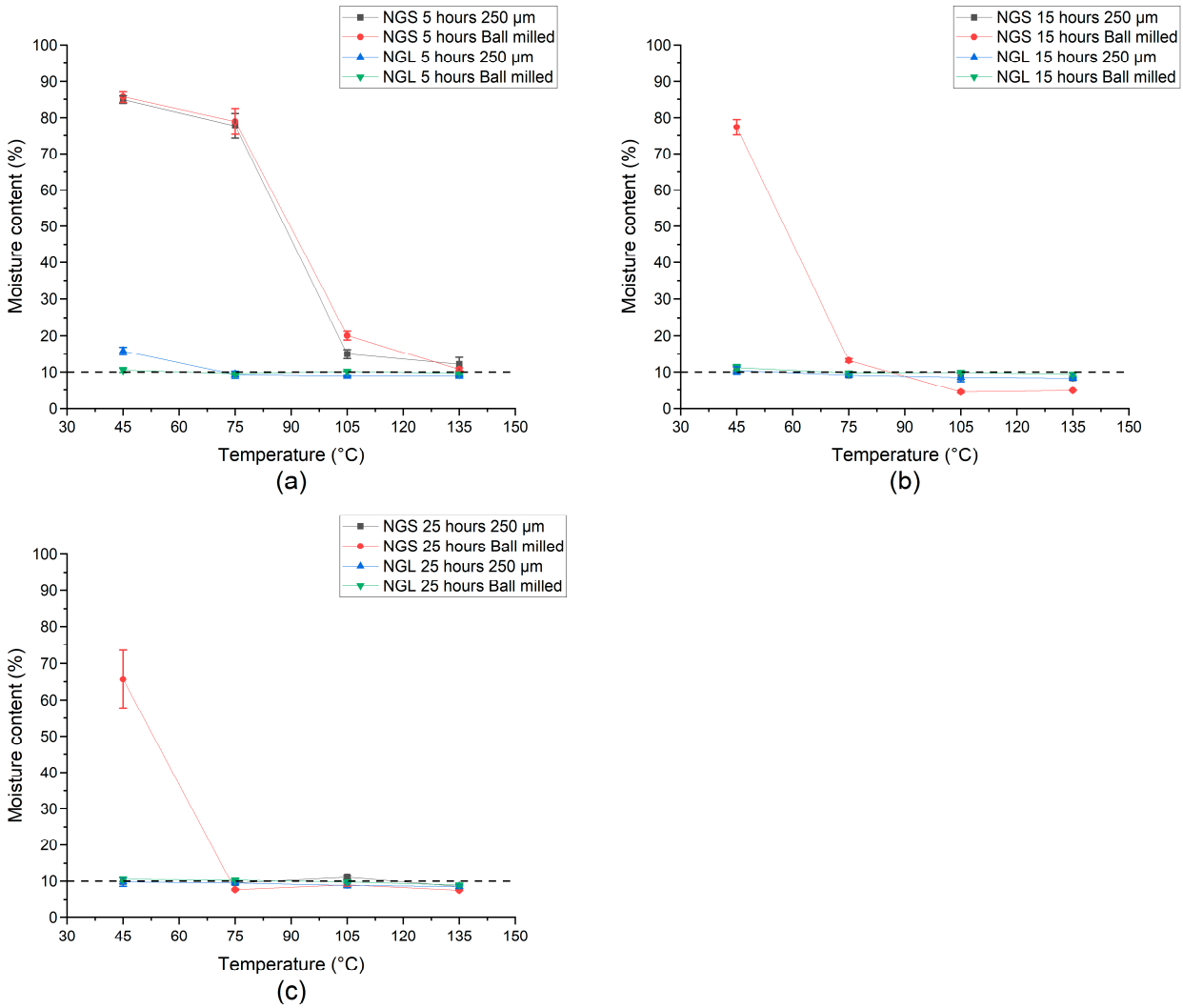

Figure 1. Moisture content against temperature (**a**) 5 h, (**b**) 15 h and (**c**) 25 h.

After statistical analysis, it was found that all effects had a significant impact on the moisture content as shown in Tables 3 and 4. Since both *p*-values for both effects as well as their interactions were less than the α-value (p-value < α), they were considered to be statistically significant. When further analyzed using the least mean square method, any increment above 15 h and 75 °C was found to not have a significant effect on the moisture content. The changes between 105 and 135 °C and 15 and 25 h were very insignificant, since the interaction between the two points had a *p*-value greater than 5% and the changes were not noticeable. The sample type was also significant, until 105 °C when the moisture started to congregate, rendering similar moisture content for both types. The size effect was significant, especially towards stem samples where ball-milled stems had a higher moisture content as compared to 250 µm. Since one of the main objectives of pretreatment is to reduce energy, it can be concluded that pretreatment at 15 h, 105 °C and ball-milled particle onto leaf samples are the optimal conditions, as opposed to the 103 °C for 24 h recommended by ASAE Standards.

Table 3. ANOVA table for moisture content.

ANOVA				Alpha	0.05
Source	**DF**	**Sum of Squares**	**Mean Square**	**F-Value**	**Pr > F**
Model	47	75,479.11	1605.938	675.03	<0.0001
Error	96	228.3904	2.37907		
Total	143	75,707.5			

Table 4. ANOVA table of each effect for moisture content.

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Type	1	10,758.53	10,758.53	4522.16	<0.0001
Time	2	8408.365	4204.182	1767.16	<0.0001
Temp	3	14,117.24	4705.748	1977.98	<0.0001
Size	1	958.4184	958.4184	402.85	<0.0001
Type*Time	2	7571.9	3785.95	1591.36	<0.0001
Type*Temp	3	11,730.29	3910.095	1643.54	<0.0001
Type*Size	1	854.2955	854.2955	359.09	<0.0001
Time*Temp	6	5682.803	947.1338	398.11	<0.0001
Time*Size	2	438.0065	219.0032	92.05	<0.0001
Temp*Size	3	2648.561	882.8538	371.09	<0.0001
Type*Time*Temp	6	5616.94	936.1566	393.5	<0.0001
Type*Time*Size	2	268.1544	134.0772	56.36	<0.0001
Type*Temp*Size	3	3228.422	1076.141	452.34	<0.0001
Time*Temp*Size	6	1875.295	312.5491	131.37	<0.0001
Type*Time*Temp* Size	6	1321.887	220.3145	92.61	<0.0001

3.2. Pretreatment FTIR Analysis

Functional groups and chemical composition were characterized using FTIR analysis. For lignin, there are several functional groups that should be observed, such as hydroxyl, carbonyl, methoxyl, carboxyl, aromatic and phenolic. Assignment of functional groups to FTIR spectrum wavenumbers are listed in Table 5 below.

Table 5. Assignment of functional groups to FTIR spectrum wavenumbers [24,25].

Wavenumber (cm^{-1})	Assignments	Band Assignment
3600–3100	O-H	Stretching vibration of alcoholic and phenolic OH groups involved in hydrogen bonds
2960–2820	C-H	$-CH_2$, $-CH_3$
1770–1685 1680–1650	C=O	Conjugated p-substituent carbonyl and carboxyl
1600–1500, 1430–1420	Aromatic skeletal	Aromatic ring vibrations
1515–1511	C=C	Aromatic skeletal breathing with C-O stretching
1470–1450, 1370–1360	C-H	C–H deformations methyl and methylene
1427–1423	C-H	Aromatic skeletal vibrations combined with C–H in-plane deformation
1375–1397	O-H C-H	Phenolic OH and aliphatic C-H in methyl groups
1170–1150	C-H	Aromatic C-H in-plane deformation in the guaiacyl ring
1145–1140	C-H	Aromatic C-H in-plane deformation in the syringyl ring
1035–1025	C-O, C-H	Aromatic ring and primary alcohol

The spectra at the fingerprint area are shown in Figures 2 and 3. All FTIR spectra of samples after thermal and physical pretreatments showed a similar pattern to the untreated samples. Some differences in terms of absorption intensity were detected between samples in comparison with each other. When varying the 250 µm sample, not much change could be seen except for that dried at 105 °C for 15 h. The sample dried at 75 °C for 5 h seemed to retain most of its bonds, most evidently from its high absorbance at 1098 cm^{-1}. For ball-milled particles, the effect of temperature and time was more noticeable. Higher temperatures reduced the intensity of absorbance, suggesting that higher temperatures broke some of the chemical bonds. Reductions in intensity of 3700–3000 cm^{-1} and 1640 cm^{-1} suggested a loss of water molecules in terms of water and moisture content. According to the literature, the bands at 1240 cm^{-1} (asymmetric stretching vibrations of C–O–C bonds in G-lignin), 1510 cm^{-1} (phenyl ring skeletal vibrations) and 1732 cm^{-1} (carbonyl) were indicative of delignification [25]. When comparing between 250 µm and ball-milled particles, the ball-milled particles had lower intensity at peaks associated with 3314, 1732, 1485, 1244 and 1159 cm^{-1}, and higher peaks at 2920, 2853, 1633, 1316 and 1035 cm^{-1}. This means that ball-milling causes partial delignification to occur. Most of the changes in intensity were not very significant, since most of the changes were of less than 50%.

ANOVA analyses were carried out on peaks at 3314, 2920, 2853, 1732, 1633, 1517, 1485, 1316, 1244, 1159, 1142 and 1035 cm^{-1}. These wavenumbers were chosen since they represent the lignin fingerprint wavenumbers, changes in moisture presence, and also a significant reduction or improvement in intensity compared to the pretreated sample. A summary of the statistical significance is tabulated in Tables 6 and 7. It was found that the interaction between temperature and time with size is not significant for any of the spectra wavenumbers.

Table 6. Summary of F-value significance based on ANOVA for physical and thermal pretreatment at 3314, 2920, 2853, 1732, 1633 and 1517 cm^{-1}.

Source	O-H 3323	C-H 2923	C-H 2855	C=O 1730	Aromatics 1628	Aromatics 1518
Type	<0.0001	0.6167	0.1123	0.2045	0.1351	0.0028
Size	0.4327	0.5907	0.9691	0.1388	0.1830	0.0966
Time	<0.0001	0.0014	0.0142	0.5556	0.001	0.2233
Temp	0.2076	0.1796	0.2215	0.3114	0.7933	0.8583
Type*Size	0.4952	0.1576	0.3258	0.3725	0.2543	0.816
Type*Time	<0.0001	0.2055	0.9864	0.4693	0.002	0.2516
Type*Temp	0.0682	0.1164	0.1051	0.0898	0.1776	0.4277
Size*Time	0.8083	0.0791	0.0723	0.3141	0.3674	0.2864
Size*Temp	0.5814	0.3147	0.3846	0.7167	0.4108	0.3785
Time*Temp	0.1882	0.0378	0.0680	0.1713	0.3216	0.4365
Type*Size*Time	0.8950	0.3432	0.3269	0.5186	0.2168	0.2085
Type*Size*Temp	0.4862	0.2653	0.3086	0.3193	0.341	0.6868
Type*Time*Temp	0.1151	0.0268	0.0392	0.1582	0.2182	0.4483
Size*Time*Temp	0.1882	0.0275	0.0486	0.1402	0.0709	0.1140

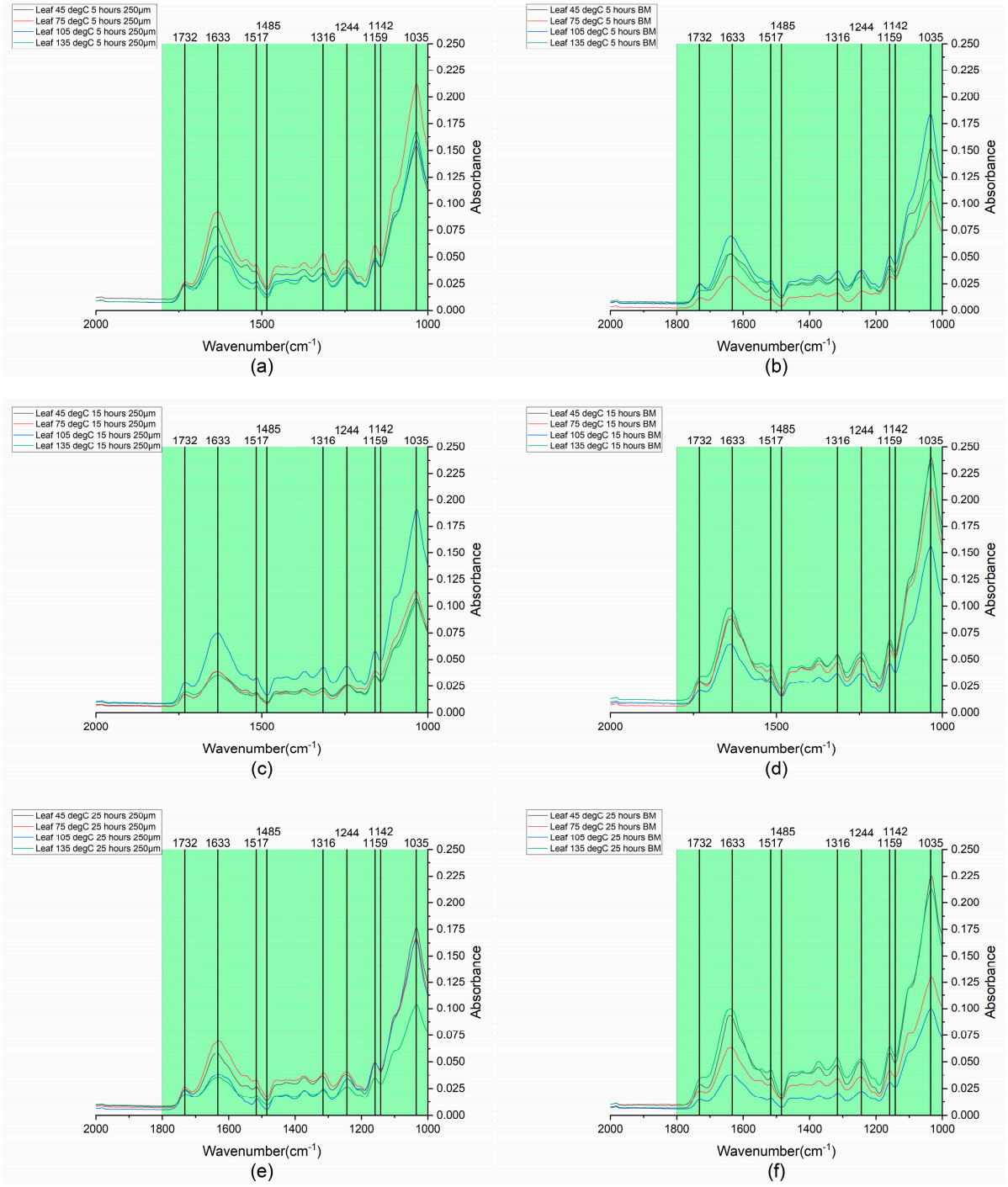

Figure 2. FTIR spectra for leaf particles at varying sizes, times, and temperatures; (**a**) 250 µm for 5 h, (**b**) 250 µm for 15 h, (**c**) 250 µm for 25 h, (**d**) ball-milled for 5 h, € ball-milled for 15 h and (**f**) ball-milled for 25 h.

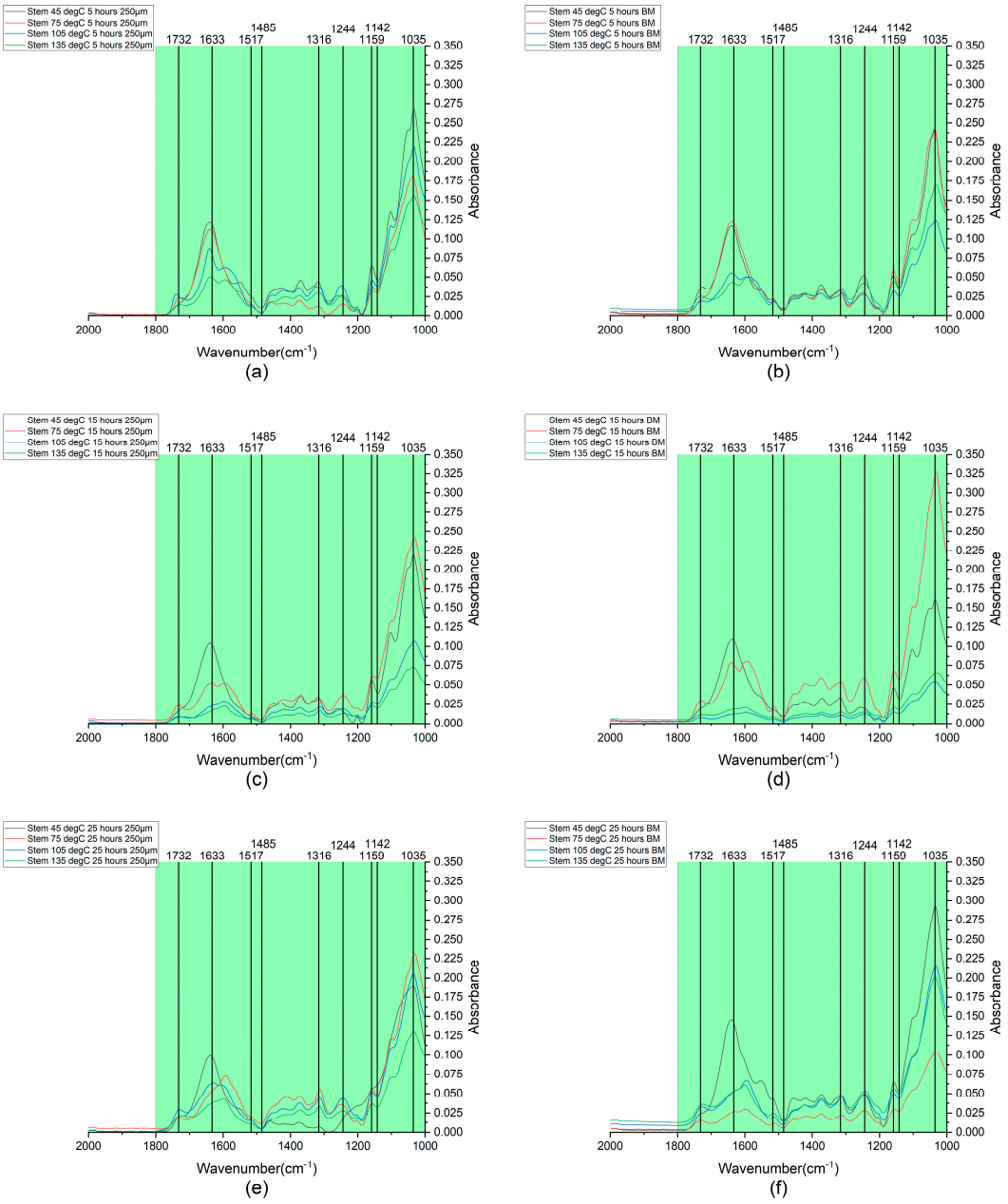

Figure 3. FTIR spectra for stem particles at varying sizes, times and temperatures; (**a**) 250 µm for 5 h, (**b**) 250 µm for 15 h, (**c**) 250 µm for 25 h, (**d**) ball-milled for 5 €, (**e**) ball-milled for 15 h and (**f**) ball-milled for 25 h.

Table 7. Summary of F-value significance based on ANOVA for physical and thermal pretreatment at 1485, 1316, 1244, 1159, 1142 and 1035 cm^{-1}.

Source	C-H 1485	Phenolic 1315	G-Ring 1244	Aromatic G-Ring 1157	Aromatic S-Ring 1142	C-O 1034
Type	0.0007	0.4052	0.1179	0.4774	0.2684	0.2514
Size	0.0866	0.3663	0.0807	0.8448	0.8170	0.6590
Time	0.6398	0.9845	0.9770	0.3051	0.5018	0.1559
Temp	0.8719	0.8548	0.4087	0.6521	0.4464	0.3790
Type*Size	0.2950	0.8364	0.4806	0.6376	0.3361	0.5732
Type*Time	0.5154	0.8491	0.8255	0.3666	0.6479	0.3530

Table 7. *Cont.*

Source	C-H 1485	Phenolic 1315	G-Ring 1244	Aromatic G-Ring 1157	Aromatic S-Ring 1142	C-O 1034
Type*Temp	0.5402	0.1898	0.1133	0.1619	0.1444	0.0994
Size*Time	0.1268	0.6180	0.5144	0.5403	0.3775	0.5616
Size*Temp	0.4583	0.6259	0.7821	0.8479	0.8115	0.6584
Time*Temp	0.5468	0.7195	0.2461	0.4519	0.1508	0.4285
Type*Size*Time	0.2789	0.4263	0.5465	0.7546	0.7144	0.6649
Type*Size*Temp	0.5929	0.5914	0.3127	0.4369	0.3259	0.4431
Type*Time*Temp	0.5134	0.7660	0.3202	0.6511	0.2778	0.5776
Size*Time*Temp	0.1943	0.2606	0.2301	0.4669	0.3054	0.3291

3.3. Klason Lignin from Pretreatment

The total lignin content of a sample consists of Acid-Insoluble Lignin (AIL) and Acid-Soluble Lignin (ASL), where acid-insoluble lignin is obtained from the residue of the lignin recovered from Klason method after filtration, while acid-soluble lignin is obtained from filtrate. From the thermal and physical pretreatment, it is seen that there is a general increase in AIL with increasing temperature, as shown in Figures 4 and 5. The change in sample type and incubation time also showed a significant change in the production of AIL. It was found that acid-insoluble lignin was more easily extracted when the sample was sufficiently dry. The presence of moisture hinders the efficiency of acid hydrolysis since the water molecules reduce the concentration of acid. This result agrees with previous studies by Tucker et al., which found that the wetter the input feedstock, the lower the yield of soluble hemicellulose due to slower heating [26]. It also can be said that the weight of the sample is mostly contributed to by the moisture, therefore the amount of lignocellulosic material is much less since the same weight of sample is used for every run. In comparison, Mardawati et al. found that higher moisture content at pretreatment level would improve lignin degradation [27]. In comparison between stems and leaves, it was found that the stems had a more prominent effect when the drying condition increases. This is due to the high content of moisture present initially when it is obtained. The ASL after thermal and physical pretreatment was found to be only affected significantly by the type of sample. In general, it was found that the leaves had more ASL lignin as compared to the stems, in agreement with findings by Brinkmann et al. [28].

When comparing the total lignin content, it can be said that sample type, time and temperature all play a significant role in the ability to extract lignin from the feedstock, while the size of particle is not as important. With increasing drying time, better lignin extraction was produced. This is due to the decrease in hindrance from the water molecule and more consistent structure of the lignocellulose complex. At low temperatures, the extraction of lignin from the leaf was found to be adequate since more than 20% of lignin can be extracted. For stems however, the amount extracted was very poor and only became better when the drying temperature and time increased. It is worth noting that although the leaf had a greater amount of total lignin extracted, the maximum can be achieved when the stem is exposed to higher drying time. This is fascinating because, conventionally, the stem is known to have more lignin due to the its greater structural rigidity when compared to the leaf. These findings are similar to the data obtained by Mohammed et al. [29], but it was found that the stem had higher lignin content than the leaf. ANOVA data for each effect is tabulated in Table 8.

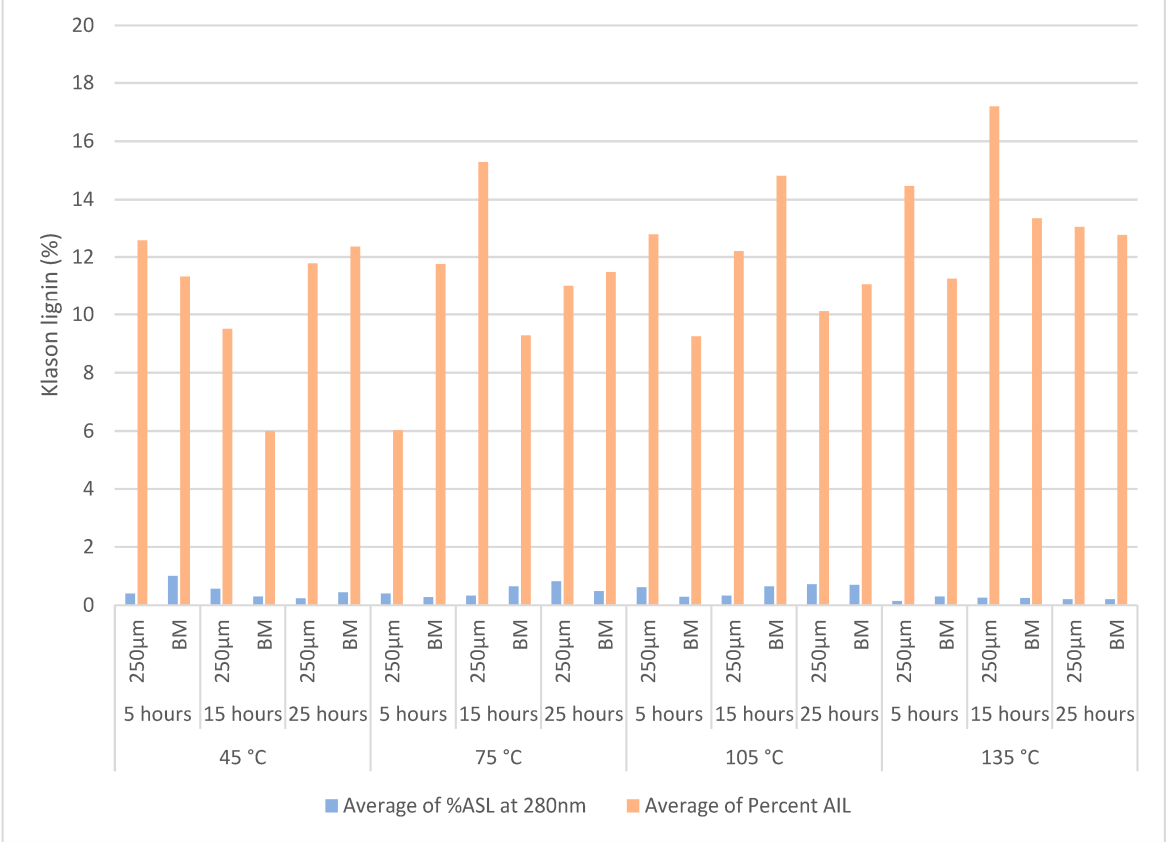

Figure 4. Klason lignin for leaf samples after pretreatment.

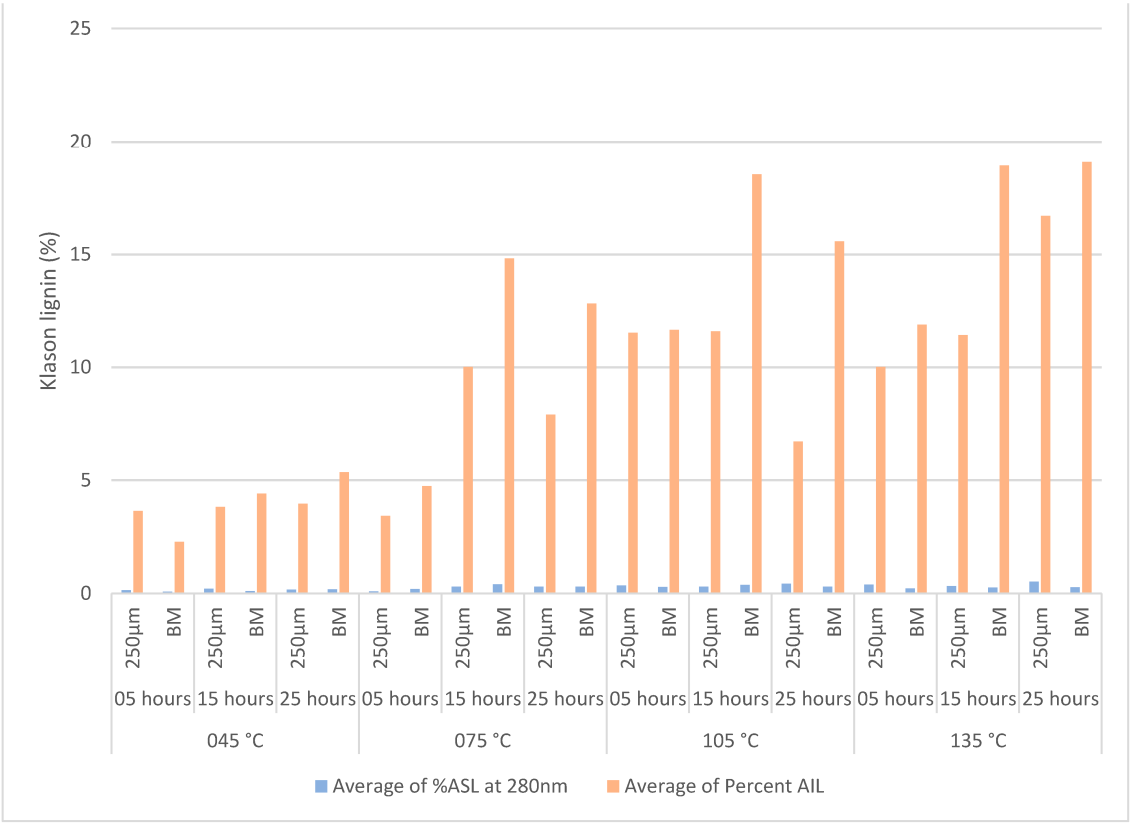

Figure 5. Klason lignin for stem samples after pretreatment.

Table 8. ANOVA table of acid-insoluble and acid-soluble lignin for each effect after physical and thermal pretreatment.

	Acid-Insoluble Lignin					Acid-Soluble Lignin				
Source	**DF**	**Type I SS**	**Mean Square**	**F Value**	**Pr > F**	**DF**	**Type I SS**	**Mean Square**	**F Value**	**Pr > F**
Type	1	32.847	32.847	8.760	0.025	1	0.351	0.351	12.550	0.012
Time	3	320.572	106.857	28.510	0.001	3	0.185	0.062	2.200	0.188
Temp	2	62.566	31.283	8.350	0.019	2	0.036	0.018	0.650	0.557
Size	1	16.253	16.253	4.340	0.083	1	0.000	0.000	0.000	0.988
Type*Time	3	116.797	38.932	10.390	0.009	3	0.331	0.110	3.930	0.072
Type*Temp	2	27.170	13.585	3.620	0.093	2	0.012	0.006	0.210	0.819
Type*Size	1	53.075	53.075	14.160	0.009	1	0.022	0.022	0.770	0.414
Time*Temp	6	82.059	13.677	3.650	0.070	6	0.190	0.032	1.130	0.442
Time*Size	3	18.129	6.043	1.610	0.283	3	0.025	0.008	0.300	0.825
Temp*Size	2	11.966	5.983	1.600	0.278	2	0.026	0.013	0.460	0.654
Type*Time*Temp	6	28.075	4.679	1.250	0.397	6	0.151	0.025	0.900	0.550
Type*Time*Size	3	9.742	3.247	0.870	0.508	3	0.061	0.020	0.730	0.572
Type*Temp*Size	2	21.853	10.927	2.920	0.131	2	0.002	0.001	0.040	0.958
Time*Temp*Size	6	33.178	5.530	1.480	0.324	6	0.244	0.041	1.450	0.331

3.4. Cellulolytic Enzymatic Lignin (CEL) Yield

Based on the results, the pretreatment parameters were reduced to only 75 and 105 °C for the temperature while for time, it was reduced to 15 and 25 h only. The yields of CEL with regard to the pretreatment and incubation parameters are shown in Figures 6 and 7. From all the CEL, it was found that the yield was much more than the Klason lignin, which suggests that the solids obtained are not entirely lignin. Cellulose was not fully removed; instead it is inferred that the cellulose was broken down into nanocellulose. Although the cellulase is selective towards the cellulose component, the reaction might not be as severe and the cellulose might not be completely disintegrated into simple sugars such as glucose. This is also because of the presence of xylan within the lignocellulose complex, which acts as the limiting factor in enzymatic hydrolysis [30].

A further look into the statistical analysis of the pretreatment conditions reveals that the changes in the factors did not all have a significant effect on the final yield of CEL. The summary of the ANOVA and the *p*-values of each factor interaction are shown in Tables 9 and 10. It is seen that NGL produced a more consistent CEL when compared to the stem samples. For the leaf samples, the CEL extracted after ball-milling was slightly less, whilst more CEL was obtained from the stem after ball-milling. The sample type provided the most significance when interacting with other factors. Leaf samples seemed to produce a higher amount and more consistent yield of CEL as compared to stem, which is more susceptible to pretreatment conditions.

Table 9. ANOVA table for CEL.

ANOVA				**Alpha**	**0.05**
Source	**DF**	**Sum of Squares**	**Mean Square**	**F-Value**	**Pr > F**
Model	139	7603.004	54.698	2.58	0.183
Error	4	84.931	21.233		
Total	143	7687.935			

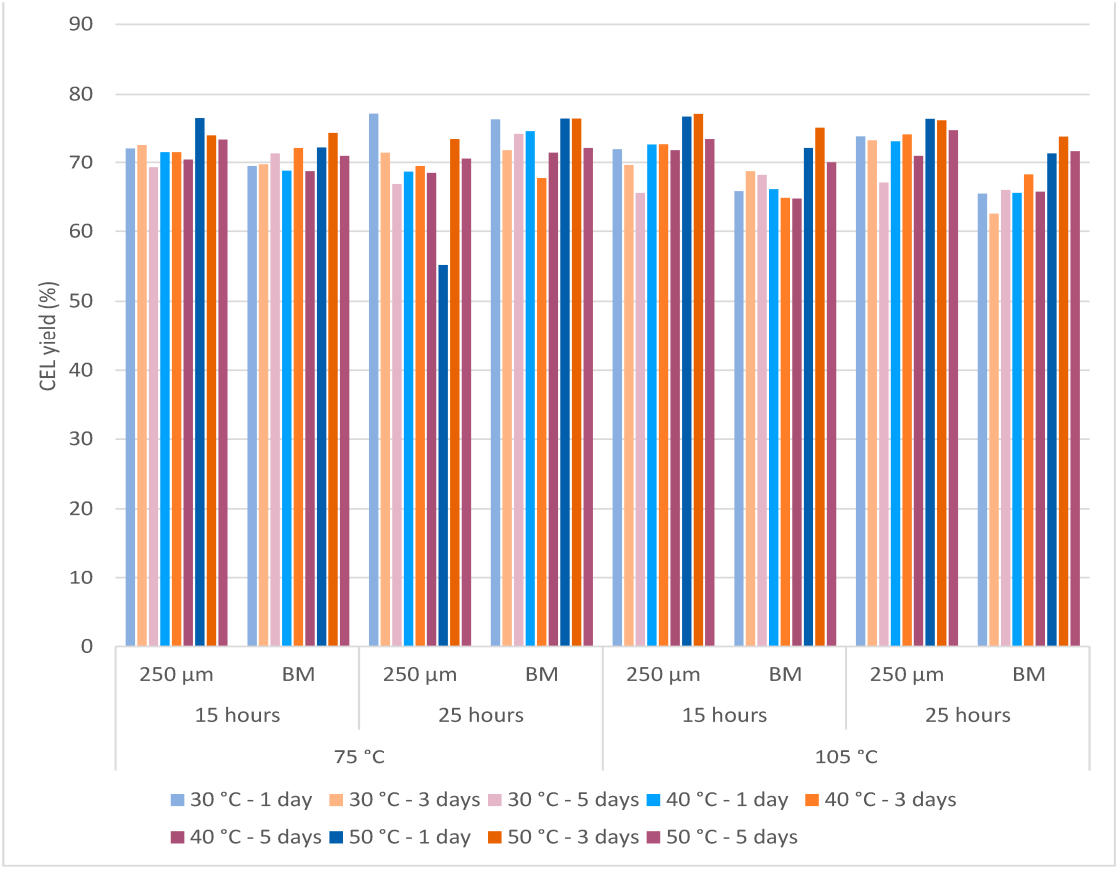

Figure 6. CEL yield for leaf samples.

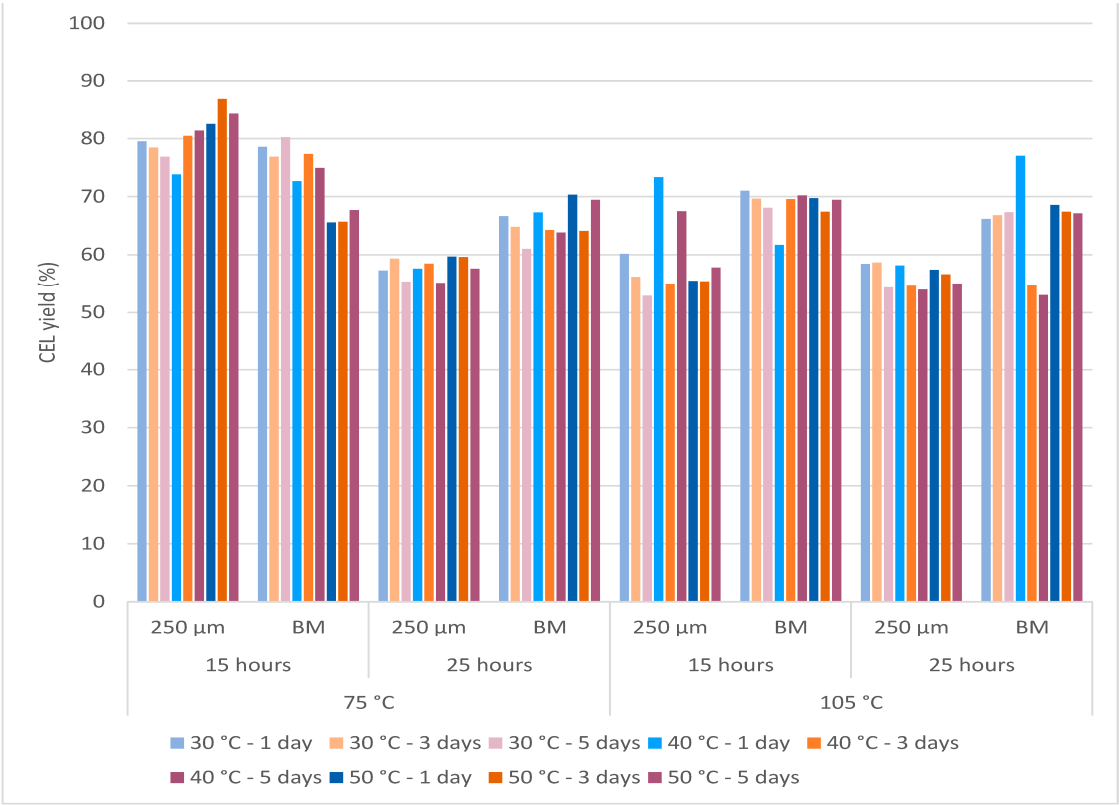

Figure 7. CEL yield for stem samples.

Table 10. *p*-value summary for CEL yield ANOVA.

Source	Pr > F	Source	Pr > F	Source	Pr > F
Type	0.0026	Type*Pretreatment Temperature *Pretreatment Time	0.0199	Type*Pretreatment Temperature*Pretreatment Time*Incubation Temperature	0.3135
Pretreatment Temperature	0.0068	Type*Pretreatment Temperature*Size	0.0084	Type*Pretreatment Temperature*Pretreatment Time*Incubation Day	0.9583
Pretreatment Time	0.0039	Type*Pretreatment Temperature*Incubation Temperature	0.4052	Type*Pretreatment Temperature*Size*Incubation Temperature	0.2545
Size	0.1086	Type*Pretreatment Temperature*Incubation Day	0.5976	Type*Pretreatment Temperature*Size*Incubation Day	0.8537
Incubation Temperature	0.2495	Type*Pretreatment Time*Size	0.1639	Type*Pretreatment Temperature*Incubation Temperature*Incubation Day	0.6087
Incubation Day	0.3419	Type*Pretreatment Time*Incubation Temperature	0.2597	Type*Pretreatment Time*Size*Incubation Temperature	0.7927
Type*Pretreatment Temperature	0.0175	Type*Pretreatment Time*Incubation Day	0.4462	Type*Pretreatment Time*Size*Incubation Day	0.9339
Type*Pretreatment Time	0.0043	Type*Size*Incubation Temperature	0.4247	Type*Pretreatment Time*Incubation Temperature*Incubation Day	0.5310
Type*Size	0.014	Type*Size*Incubation Day	0.9383	Type*Size*Incubation Temperature*Incubation Day	0.8234
Type*Incubation Temperature	0.266	Type*Incubation Temperature*Incubation Day	0.8406	Pretreatment Temperature*Pretreatment Time*Size*Incubation Temperature	0.2566
Type*Incubation Day	0.552	Pretreatment Temperature*Pretreatment Time*Size	0.0209	Pretreatment Temperature*Pretreatment Time*Size*Incubation Day	0.9723
Pretreatment Temperature*Pretreatment Time	0.0147	Pretreatment Temperature*Pretreatment Time*Incubation Temperature	0.7661	Pretreatment Temperature*Pretreatment Time*Incubation Temperature*Incubation Day	0.7745
Pretreatment Temperature*Size	0.4488	Pretreatment Temperature*Pretreatment Time*Incubation Day	0.9394	Pretreatment Temperature*Size*Incubation Temperature*Incubation Day	0.9792
Pretreatment Temperature*Incubation Temperature	0.4221	Pretreatment Temperature*Size*Incubation Temperature	0.2330	Pretreatment Time*Size*Incubation Temperature*Incubation Day	0.8115

Table 10. *Cont.*

Source	Pr > F	Source	Pr > F	Source	Pr > F
Pretreatment Temperature*Incubation Day	0.5145	Pretreatment Temperature*Size*Incubation Day	0.5347	Type *Pretreatment Temperature*Pretreatment Time*Size*Incubation Temperature	0.5699
Pretreatment Time*Size	0.0343	Pretreatment Temperature*Incubation Temperature*Incubation Day	0.8158	Type*Pretreatment Temperature*Pretreatment Time*Size*Incubation Day	0.3964
Pretreatment Time*Incubation Temperature	0.6025	Pretreatment Time*Size*Incubation Temperature	0.1792	Type*Pretreatment Temperature*Pretreatment Time*Incubation Temperature*Incubation Day	0.7400
Pretreatment Time*Incubation Day	0.5302	Pretreatment Time*Size*Incubation Day	0.2509	Type*Pretreatment Temperature*Size*Incubation Temperature*Incubation Day	0.9281
Size*Incubation Temperature	0.393	Pretreatment Time*Incubation Temperature*Incubation Day	0.6616	Type*Pretreatment Time*Size*Incubation Temperature*Incubation Day	0.5420
Size*Incubation Day	0.7657	Size*Incubation Temperature*Incubation Day	0.5843	Pretreatment Temperature*Pretreatment Time*Size*Incubation Temperature*Incubation Day	0.5709
Incubation Temperature*Incubation Day	0.6866	Type*Pretreatment Temperature*Pretreatment Time*Size	0.2655		

An increase in the pretreatment temperature and time, as well as their interaction with other factors, decreased the overall solid yield. The pretreatment helps to remove the unwanted component from the materials, exposing the lignocellulosic component to be extracted. This is aligned with previous studies which indicated that exposing the sample at higher temperatures for a prolonged period of time could degrade the phenol content, decrease water content and may retard the extraction [31]. For the single effect of pretreatment the size effect was not significant, while both of the enzymatic hydrolysis parameters did not affect the CEL content. When looking at the incubation parameters, the changes are not obvious. This shows that the incubation effect did not have a significant effect. The change in incubation temperature is not prominent but shows the expected trend. At 40 °C, the production of CEL was lowest when compared to the results at 30 and 50 °C. This shows that the cellulase is most effective at breaking down the cellulose content, which is in agreement with the literature [32]. Above the optimum, the enzyme would be denatured, and less cellulose would be degraded, as is shown in the results. For incubation time, the longer the incubation takes, the more cellulose is degraded, and this translates into a lower solid yield.

3.5. CEL UV–Vis Analysis

The soluble lignin was tested at 205 and 280 nm, and the results are shown in Figures 8 and 9. It was found that the percent of soluble lignin obtained from the buffer solution after enzymatic hydrolysis was extremely small. Lignin was not dissolved in the solution and remained as a solid. This is good for the whole process since no separation of lignin from the solution is needed.

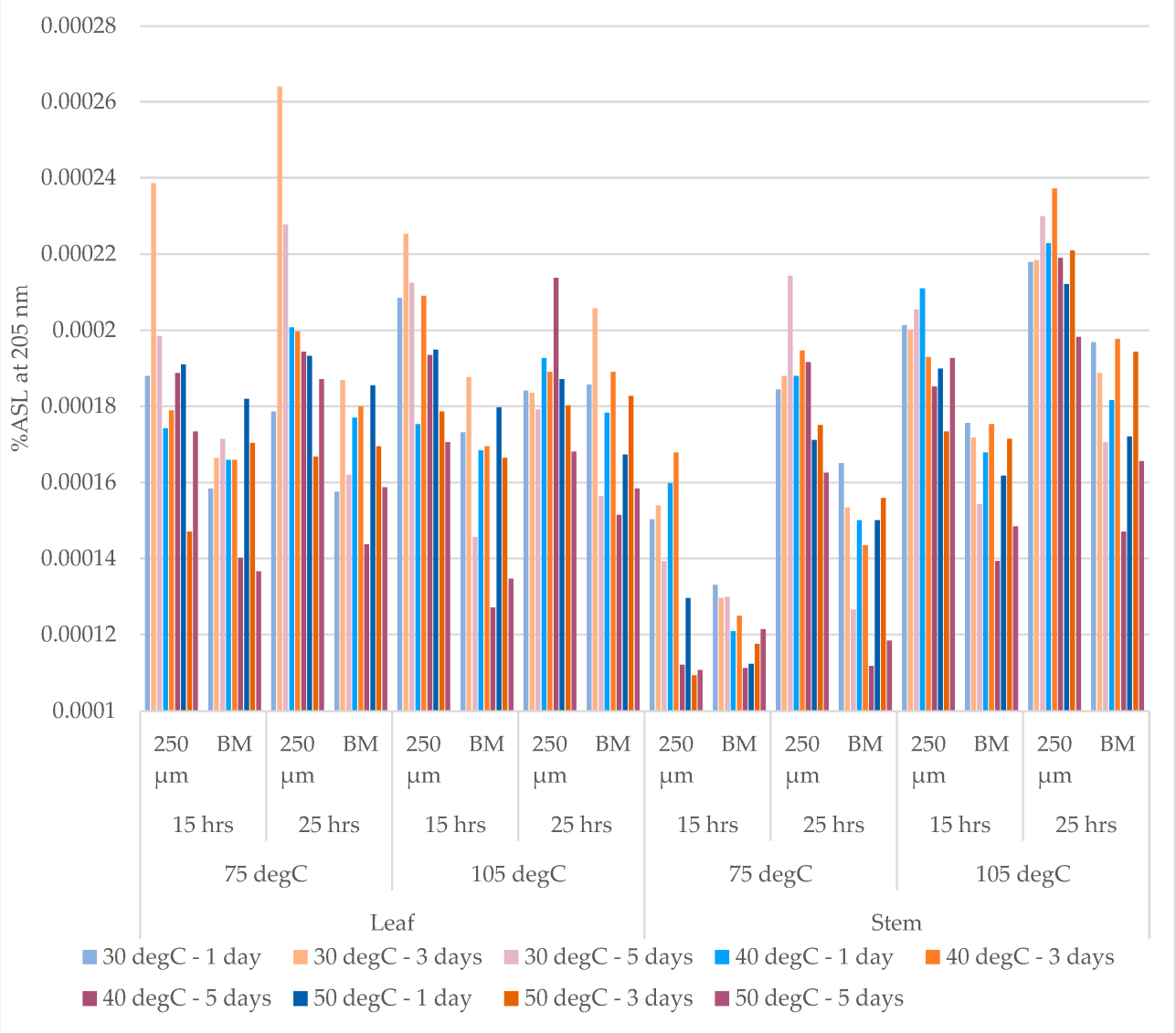

Figure 8. Soluble lignin content after enzymatic hydrolysis at 205 nm.

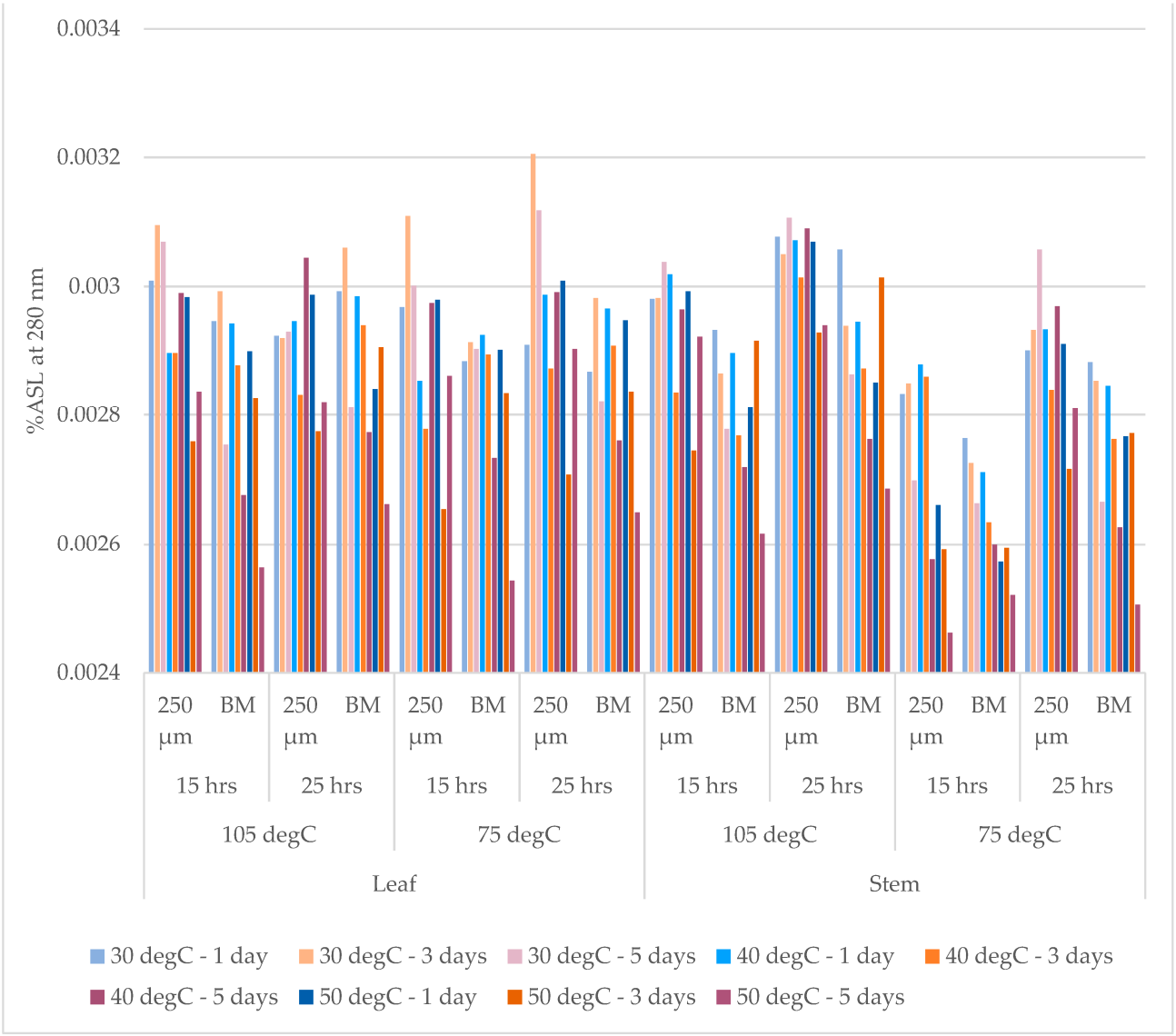

Figure 9. Soluble lignin content after enzymatic hydrolysis at 280 nm.

The statistical analyses for soluble lignin indicated that most factors had a significant effect, as shown in the summary in Table 11. Leaf samples and higher drying conditions produced higher soluble lignin content while ball-milling only decreased the soluble lignin content. Higher incubation temperatures and longer times also produced lower amounts of soluble lignin. Overall, since the percentage of soluble lignin was very little, it is of low concern which means that lignin does not have to be separated from the solution and can be disregarded from the total lignin extracted.

Table 11. *p*-value summary for soluble lignin yield ANOVA.

Factors	205 nm	280 nm	Factors	205 nm	280 nm
Type	<0.0001	<0.0001	Pretreatment Temperature*Pretreatment Time*Size	0.0005	0.0012
Pretreatment Temperature	<0.0001	<0.0001	Pretreatment Temperature*Pretreatment Time*Incubation Temperature	0.0054	0.0119
Pretreatment Time	<0.0001	<0.0001	Pretreatment Temperature*Pretreatment Time*Incubation Day	0.0241	0.0032
Size	<0.0001	<0.0001	Pretreatment Temperature*Size*Incubation Temperature	0.003	0.004
Incubation Temperature	<0.0001	<0.0001	Pretreatment Temperature*Size*Incubation Day	0.2397	0.406
Incubation Day	0.0118	<0.0001	Pretreatment Temperature*Incubation Temperature*Incubation Day	0.0077	0.0094
Type*Pretreatment Temperature	<0.0001	<0.0001	Pretreatment Time*Size*Incubation Temperature	0.0254	0.0184
Type*Pretreatment Time	<0.0001	0.0001	Pretreatment Time*Size*Incubation Day	0.0661	0.0367
Type*Size	0.0059	0.0015	Pretreatment Time*Incubation Temperature*Incubation Day	0.0776	0.2712
Type*Incubation Temperature	0.0318	0.0426	Size*Incubation Temperature*Incubation Day	0.0235	0.0017
Type*Incubation Day	0.0291	0.0546	Type*Pretreatment Temperature*Pretreatment Time*Size	0.1019	0.0562
Pretreatment Temperature*Pretreatment Time	0.0009	0.0018	Type*Pretreatment Temperature*Pretreatment Time*Incubation Temperature	0.3378	0.1633
Pretreatment Temperature*Size	0.585	0.4558	Type*Pretreatment Temperature*Pretreatment Time*Incubation Day	0.0183	0.0057
Pretreatment Temperature*Incubation Temperature	0.008	0.0389	Type*Pretreatment Temperature*Size*Incubation Temperature	0.0457	0.0961
Pretreatment Temperature*Incubation Day	0.1851	0.0119	Type*Pretreatment Temperature*Size*Incubation Day	0.578	0.1493
Pretreatment Time*Size	0.0128	0.1254	Type*Pretreatment Temperature*Incubation Temperature*Incubation Day	0.0122	0.0172
Pretreatment Time*Incubation Temperature	0.0109	0.0234	Type*Pretreatment Time*Size*Incubation Temperature	0.0351	0.018
Pretreatment Time*Incubation Day	0.0299	0.013	Type*Pretreatment Time*Size*Incubation Day	0.0649	0.0233
Size*Incubation Temperature	0.003	0.001	Type*Pretreatment Time*Incubation Temperature*Incubation Day	0.0137	0.0223
Size*Incubation Day	0.0029	0.0002	Type*Size*Incubation Temperature*Incubation Day	0.0023	0.002
Incubation Temperature*Incubation Day	0.0014	0.0002	Pretreatment Temperature*Pretreatment Time*Size*Incubation Temperature	0.0505	0.038
Type*Pretreatment Temperature*Pretreatment Time	0.1902	0.1306	Pretreatment Temperature*Pretreatment Time*Size*Incubation Day	0.2941	0.0353
Type*Pretreatment Temperature*Size	0.009	0.0153	Pretreatment Temperature*Pretreatment Time*Incubation Temperature*Incubation Day	0.0752	0.0261

Table 11. *Cont.*

Factors	205 nm	280 nm	Factors	205 nm	280 nm
Type*Pretreatment Temperature*Incubation Temperature	0.0514	0.0208	Pretreatment Temperature*Size*Incubation Temperature*Incubation Day	0.0206	0.0828
Type*Pretreatment Temperature*Incubation Day	0.0311	0.0041	Pretreatment Time*Size*Incubation Temperature*Incubation Day	0.0197	0.0672
Type*Pretreatment Time*Size	0.0005	0.0023	Type*Pretreatment Temperature*Pretreatment Time*Size*Incubation Temperature	0.0358	0.0205
Type*Pretreatment Time*Incubation Temperature	0.0103	0.024	Type*Pretreatment Temperature*Pretreatment Time*Size*Incubation Day	0.016	0.0032
Type*Pretreatment Time*Incubation Day	0.1095	0.0284	Type*Pretreatment Temperature*Pretreatment Time*Incubation Temperature*Incubation Day	0.0349	0.0693
Type*Size*Incubation Temperature	0.0183	0.0062	Type*Pretreatment Temperature*Size*Incubation Temperature*Incubation Day	0.0178	0.0176
Type*Size*Incubation Day	0.1845	0.0065	Type*Pretreatment Time*Size*Incubation Temperature*Incubation Day	0.3709	0.4726
Type*Incubation Temperature*Incubation Day	0.0037	0.0045	Pretreatment Temperature*Pretreatment Time*Size*Incubation Temperature*Incubation Day	0.8026	0.1518

3.6. CEL FTIR Analysis

The FTIR peaks obtained from each solid yield were compared with those from pure MCC and KrL. The spectra for full FTIR fingerprint wavenumber area for leaf and stem samples are shown in Figures 10 and 11 and Figures 12 and 13, respectively. All spectra lie around the same absorbance, indicating that the pretreatment and incubation parameters do not change the chemical bonding. The spectra also follow the same trend as each other. This means that the process is not selective towards any chemical bonding and removal of bonding happens only in the form of degradation of the sample. When compared to pure MCC and KrL, it was found that the spectra of extracted CEL were lower than MCC but higher than KrL. This suggests that the CEL is not entirely lignin and that microcellulose is present. Much of the literature suggests that nanocellulose can be produced when cellulase breaks down the lignocellulosic complex, but as indicated from the FTIR spectra of samples and pure CNC, this is not evident from the sample. Nanocellulose might also be produced in smaller amounts, but this cannot be determined from FTIR and requires other analyses to confirm. Ting et al. reported that nanocellulose spectra were lower than MCC, similar to our results which support this inference [33]. It can be said that lignin is partly extracted since all the spectra stayed close to those of pure KrL, even though some cellulose peaks can still be observed. Further purification is needed to remove the cellulose component still present in the sample.

Looking into the statistical analysis of the FTIR spectra, the significance of each factor and its interactions can be examined. The summary of *p*-values of each interaction is shown in Table 12. Characteristic peaks at 1639 and 1516 cm^{-1} indicating conjugated carbonyl groups and aromatic skeletal vibrations, respectively, showed significant changes only when type and size were varied. This confirms that lignin is present much more in the stem, while the ball-milling process improves the lignin aromatic skeletal bonding within the sample. The non-conjugated carbonyl groups peak at 1729 cm^{-1}, G ring breathing with carbonyl stretching peak at 1251 cm^{-1}, aromatic C-H in-plane deformation in the guaiacyl ring peak at 1165 cm^{-1}, and the aromatic C-H in-plane deformation in the Syringyl ring peak at 1146 cm^{-1}, also show the same trend with only type, size and several of their interactions. The 1059 cm^{-1} peak, which only present in cellulose and is absent in lignin, is

seen to be greatly reduced in lignin. When comparing between leaf and stem samples, it is seen that stem samples produce a steadier result very close to pure KrL spectra, suggesting that the lignin extracted from stem samples is less affected by the variables and was able to be extracted more consistently.

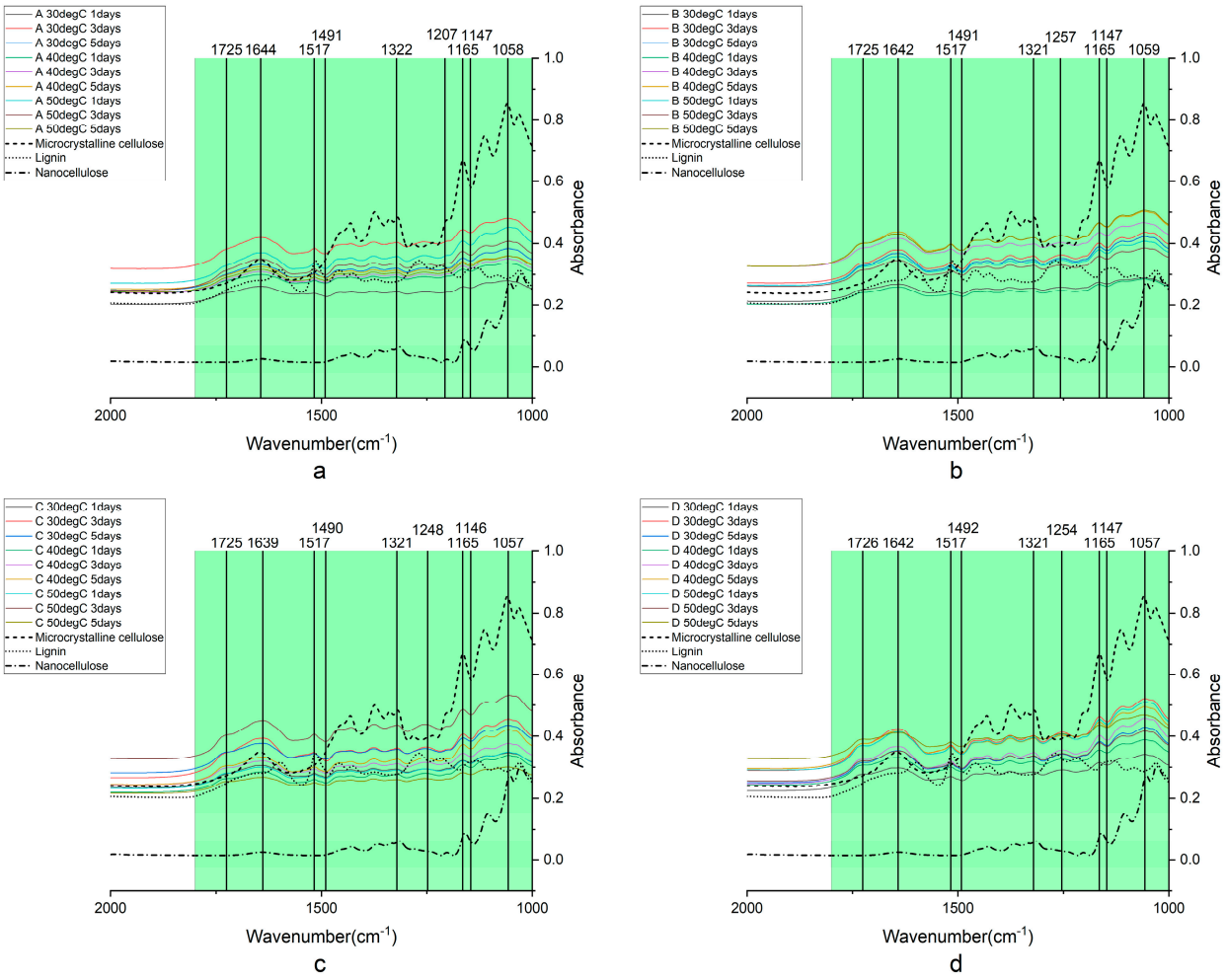

Figure 10. FTIR spectra of leaf samples pretreated at 75 °C at varying incubation temperatures and times; (**a**) 15 h 250 µm, (**b**) 15 h ball-milled, (**c**) 25 h 250 µm, (**d**) 25 h ball-milled.

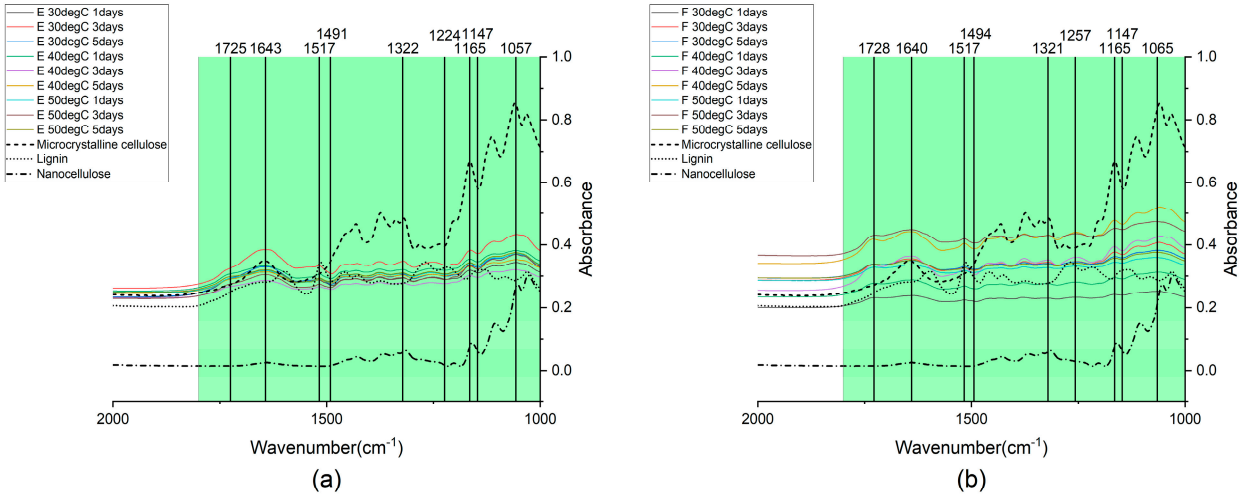

Figure 11. *Cont.*

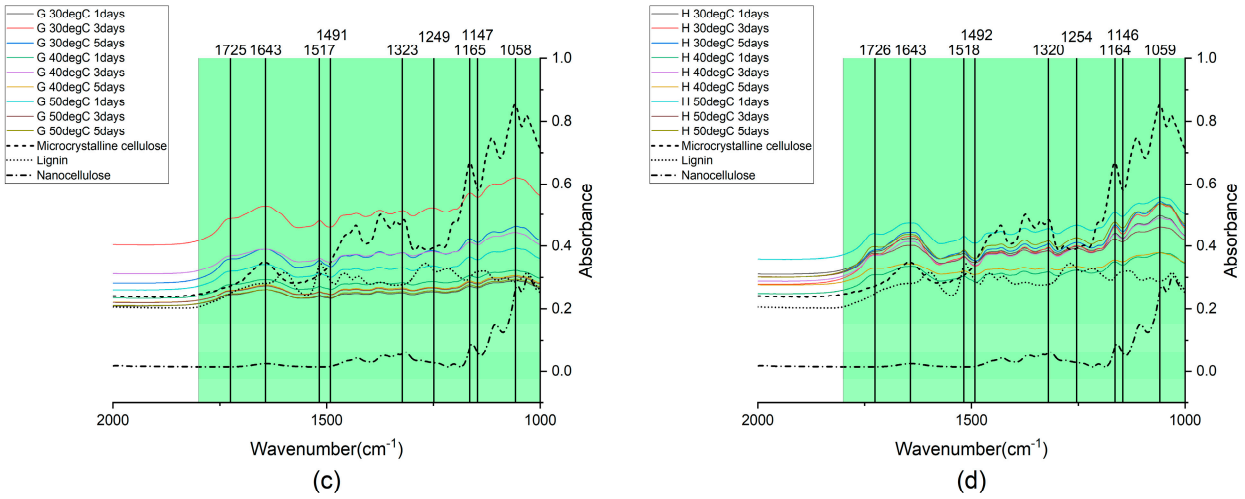

Figure 11. FTIR spectra of leaf samples pretreated at 105 °C at varying incubation temperatures and times; (**a**) 15 h 250 µm, (**b**) 15 h ball-milled, (**c**) 25 h 250 µm, (**d**) 25 h ball-milled.

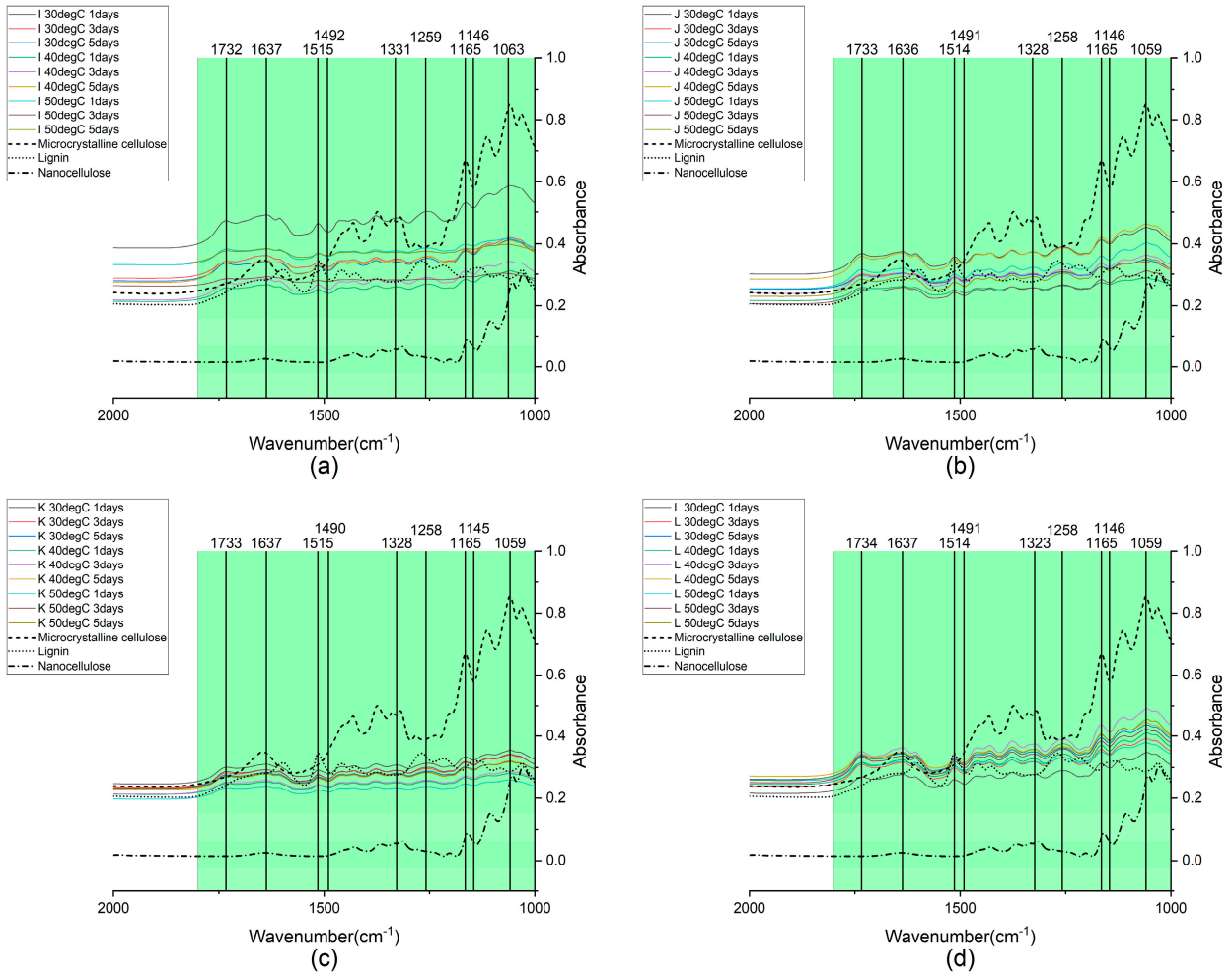

Figure 12. FTIR spectra of stem samples pretreated at 75 °C at varying incubation temperatures and times; (**a**) 15 h 250 µm, (**b**) 15 h ball-milled, (**c**) 25 h 250 µm, (**d**) 25 h ball-milled.

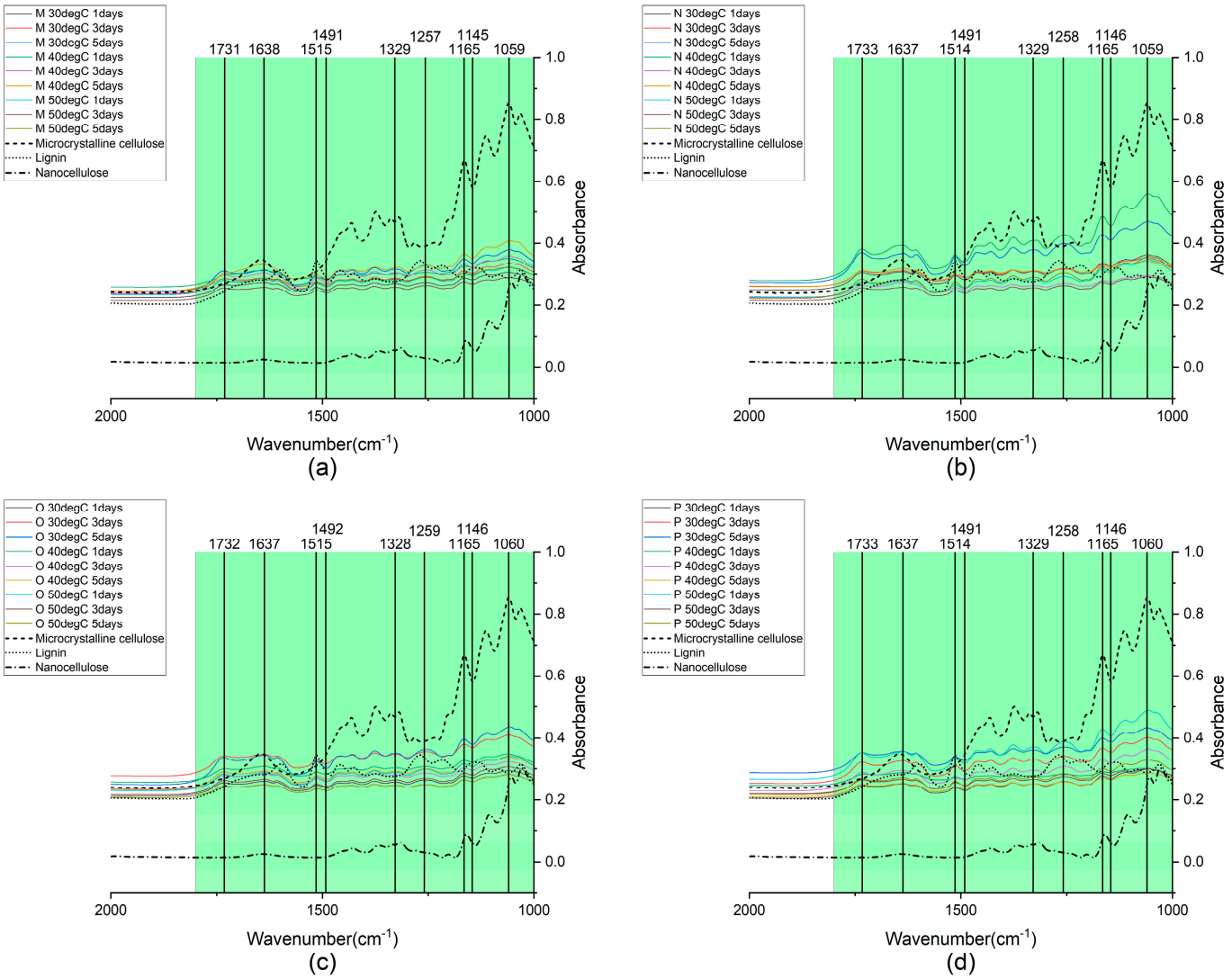

Figure 13. FTIR spectra of stem samples pretreated at 105 °C at varying incubation temperatures and times; (**a**) 15 h 250 µm, (**b**) 15 h ball-milled, (**c**) 25 h 250 µm, (**d**) 25 h ball-milled.

Table 12. *p*-value summary for CEL FTIR ANOVA.

Factor	3600–3100	2960–2820	2860–2840	1720	1670	1510	1490	1330	1270	1140	1125	1030
Type	0.0149	0.0061	0.0068	0.0104	0.0014	0.0050	0.0060	0.0068	0.0159	0.0103	0.0073	0.0073
Pretreatment Temperature	0.3148	0.3647	0.3967	0.5300	0.2863	0.3101	0.4058	0.3328	0.3267	0.3092	0.3149	0.3149
Pretreatment Time	0.3499	0.9165	0.8464	0.8614	0.6569	0.9195	0.6818	0.6488	0.5888	0.3735	0.4603	0.4603
Size	0.0164	0.0169	0.0203	0.0081	0.0104	0.0129	0.0201	0.0130	0.0108	0.0141	0.0142	0.0142
Incubation Temperature	0.2093	0.2258	0.2370	0.1658	0.1179	0.1524	0.2162	0.2197	0.2425	0.2769	0.2672	0.2672
Incubation Day	0.0958	0.0617	0.0670	0.0576	0.0462	0.0566	0.0720	0.0666	0.0822	0.0963	0.0862	0.0862
Type*Pretreatment Temperature	0.3134	0.1327	0.1172	0.0752	0.1223	0.1042	0.0949	0.1770	0.1759	0.3243	0.2755	0.2755
Type*Pretreatment Time	0.0674	0.0594	0.0648	0.0309	0.0256	0.0385	0.0507	0.0502	0.0496	0.0634	0.0555	0.0555
Type*Size	0.2402	0.0801	0.0707	0.0401	0.0347	0.0560	0.0517	0.1002	0.1321	0.2239	0.1614	0.1614
Type*Incubation Temperature	0.3131	0.1588	0.1518	0.1455	0.1793	0.1559	0.1508	0.1745	0.1837	0.2432	0.2121	0.2121
Type*Incubation Day	0.0448	0.0287	0.0304	0.0248	0.0186	0.0245	0.0294	0.0293	0.0355	0.0424	0.0381	0.0381
Pretreatment Temperature*Pretreatment Time	0.3632	0.1509	0.1269	0.1279	0.1137	0.1139	0.1019	0.1982	0.2306	0.3140	0.2615	0.2615
Pretreatment Temperature*Size	0.7040	0.3898	0.3275	0.2888	0.3823	0.3006	0.2624	0.5150	0.5801	0.7830	0.7174	0.7174
Pretreatment Temperature*Incubation Temperature	0.6288	0.4359	0.4276	0.3925	0.3374	0.3998	0.4125	0.4399	0.5098	0.5474	0.5217	0.5217
Pretreatment Temperature*Incubation Day	0.7882	0.5684	0.5376	0.5795	0.6252	0.5745	0.5105	0.6161	0.6776	0.7045	0.6776	0.6776
Pretreatment Time*Size	0.0779	0.0975	0.1315	0.0858	0.0430	0.0739	0.1140	0.0757	0.0798	0.0680	0.0694	0.0694
Pretreatment Time*Incubation Temperature	0.4411	0.5064	0.5368	0.5409	0.4226	0.5380	0.6242	0.4904	0.4915	0.4569	0.4704	0.4704
Pretreatment Time*Incubation Day	0.2547	0.1866	0.2011	0.1305	0.1455	0.1542	0.1931	0.1741	0.1705	0.2061	0.1953	0.1953
Size*Incubation Temperature	0.1391	0.0956	0.1058	0.0684	0.0671	0.0776	0.0975	0.0952	0.1008	0.1449	0.1283	0.1283
Size*Incubation Day	0.3959	0.2001	0.1909	0.1814	0.1880	0.1766	0.1776	0.2383	0.2662	0.3353	0.2926	0.2926
Incubation Temperature*Incubation Day	0.1247	0.0885	0.0931	0.0755	0.0619	0.0757	0.0888	0.0853	0.0958	0.1073	0.0954	0.0954
Type*Pretreatment Temperature*Pretreatment Time	0.7798	0.7982	0.8453	0.9269	0.7199	0.8160	0.9868	0.6935	0.7314	0.6118	0.6874	0.6874
Type*Pretreatment Temperature*Size	0.5101	0.5020	0.5412	0.4435	0.5121	0.5039	0.5588	0.4885	0.5194	0.4916	0.5226	0.5226
Type*Pretreatment Temperature*Incubation Temperature	0.5891	0.5675	0.5568	0.4613	0.3656	0.4870	0.4140	0.5713	0.6367	0.6728	0.6316	0.6316
Type*Pretreatment Temperature*Incubation Day	0.9441	0.7779	0.7469	0.6570	0.7195	0.6531	0.7011	0.8108	0.7930	0.8918	0.8733	0.8733
Type*Pretreatment Time*Size	0.6590	0.3323	0.2636	0.2415	0.5562	0.2968	0.2175	0.3986	0.4043	0.6322	0.5707	0.5707
Type*Pretreatment Time*Incubation Temperature	0.2765	0.1883	0.1848	0.1526	0.1472	0.1533	0.1736	0.1944	0.2012	0.2438	0.2267	0.2267

Table 12. *Cont.*

Factor	3600–3100	2960–2820	2860–2840	1720	1670	1510	1490	1330	1270	1140	1125	1030
Type*Pretreatment Time*Incubation Day	0.3320	0.2020	0.2021	0.1860	0.2090	0.2000	0.2112	0.2164	0.2228	0.2731	0.2512	0.2512
Type*Size*Incubation Temperature	0.7287	0.2545	0.2116	0.1679	0.2307	0.1846	0.1500	0.3383	0.4046	0.6613	0.5099	0.5099
Type*Size*Incubation Day	0.4087	0.2862	0.2824	0.2485	0.2371	0.2556	0.2587	0.3074	0.3297	0.3806	0.3510	0.3510
Type*Incubation Temperature*Incubation Day	0.4449	0.4082	0.4286	0.4108	0.2644	0.3524	0.4342	0.3935	0.4169	0.4464	0.4252	0.4252
Pretreatment Temperature*Pretreatment Time*Size	0.2590	0.1225	0.1155	0.0932	0.2163	0.1297	0.1076	0.1461	0.1254	0.2208	0.1819	0.1819
Pretreatment Temperature*Pretreatment Time*Incubation Temperature	0.1337	0.1393	0.1547	0.1352	0.1083	0.1418	0.1657	0.1332	0.1416	0.1372	0.1345	0.1345
Pretreatment Temperature*Pretreatment Time*Incubation Day	0.7608	0.6672	0.6672	0.5691	0.6209	0.5972	0.6062	0.6946	0.6836	0.7862	0.7457	0.7457
Pretreatment Temperature*Size*Incubation Temperature	0.3439	0.1629	0.1527	0.1358	0.1480	0.1309	0.1296	0.1904	0.2310	0.2905	0.2480	0.2480
Pretreatment Temperature*Size*Incubation Day	0.5921	0.5322	0.5805	0.5831	0.5441	0.5821	0.6229	0.5243	0.5458	0.5219	0.5224	0.5224
Pretreatment Temperature*Incubation Temperature*Incubation Day	0.2043	0.1306	0.1393	0.1037	0.1196	0.1177	0.1403	0.1346	0.1389	0.1763	0.1634	0.1634
Pretreatment Time*Size*Incubation Temperature	0.3410	0.2253	0.2083	0.1768	0.2158	0.1976	0.1895	0.2412	0.2626	0.3459	0.3008	0.3008
Pretreatment Time*Size*Incubation Day	0.4295	0.1718	0.1495	0.1283	0.1398	0.1274	0.1252	0.2106	0.2383	0.3577	0.2948	0.2948
Pretreatment Time*Incubation Temperature*Incubation Day	0.4761	0.3539	0.3611	0.2930	0.2855	0.3073	0.3505	0.3863	0.4162	0.5004	0.4688	0.4688
Size*Incubation Temperature*Incubation Day	0.6888	0.7602	0.7888	0.7867	0.6536	0.7530	0.7674	0.7628	0.7924	0.7536	0.7516	0.7516
Type*Pretreatment Temperature*Pretreatment Time*Size	0.0472	0.0505	0.0594	0.0414	0.0304	0.0399	0.0668	0.0507	0.0581	0.0583	0.0616	0.0616
Type*Pretreatment Temperature*Pretreatment Time*Incubation Temperature	0.7992	0.6296	0.5763	0.6068	0.6550	0.6235	0.5131	0.7188	0.7930	0.8737	0.8299	0.8299
Type*Pretreatment Temperature*Pretreatment Time*Incubation Day	0.8352	0.7252	0.6731	0.6132	0.7497	0.6397	0.6187	0.7365	0.7180	0.8291	0.7976	0.7976
Type*Pretreatment Temperature*Size*Incubation Temperature	0.4940	0.3678	0.3863	0.3452	0.3420	0.3473	0.4063	0.3581	0.3604	0.4311	0.4076	0.4076
Type*Pretreatment Temperature*Size*Incubation Day	0.5379	0.5154	0.5600	0.4484	0.5164	0.5152	0.6201	0.4991	0.4947	0.5041	0.5286	0.5286
Type*Pretreatment Temperature*Incubation Temperature*Incubation Day	0.2936	0.2801	0.2985	0.2269	0.2644	0.2607	0.3012	0.2749	0.2733	0.3071	0.3034	0.3034
Type*Pretreatment Time*Size*Incubation Temperature	0.7681	0.8461	0.8103	0.7199	0.7454	0.7357	0.7482	0.8756	0.9080	0.8715	0.8959	0.8959

Table 12. *Cont.*

Factor	3600–3100	2960–2820	2860–2840	1720	1670	1510	1490	1330	1270	1140	1125	1030
Type*Pretreatment Time*Size*Incubation Day	0.2386	0.1419	0.1457	0.1208	0.1336	0.1263	0.1455	0.1422	0.1571	0.1835	0.1738	0.1738
Type*Pretreatment Time*Incubation Temperature*Incubation Day	0.9611	0.8023	0.7656	0.7042	0.7416	0.7105	0.6797	0.8285	0.8539	0.9362	0.8973	0.8973
Type*Size*Incubation Temperature*Incubation Day	0.5135	0.2975	0.3011	0.2390	0.2595	0.2644	0.2743	0.3185	0.3473	0.4278	0.3844	0.3844
Pretreatment Temperature*Pretreatment Time*Size*Incubation Temperature	0.6578	0.8091	0.8689	0.8346	0.8268	0.8946	0.9130	0.7462	0.6808	0.6363	0.6765	0.6765
Pretreatment Temperature*Pretreatment Time*Size*Incubation Day	0.9728	0.8484	0.8316	0.8280	0.8802	0.8666	0.8176	0.9083	0.9289	0.9528	0.9218	0.9218
Pretreatment Temperature*Pretreatment Time*Incubation Temperature*Incubation Day	0.2361	0.1348	0.1367	0.1047	0.1096	0.1114	0.1288	0.1292	0.1355	0.1694	0.1558	0.1558
Pretreatment Temperature*Size*Incubation Temperature*Incubation Day	0.6415	0.3512	0.3321	0.2964	0.3098	0.2959	0.2977	0.3928	0.4350	0.5786	0.5108	0.5108
Pretreatment Time*Size*Incubation Temperature*Incubation Day	0.6547	0.6076	0.6409	0.5564	0.5275	0.5725	0.6431	0.5922	0.6066	0.6390	0.6352	0.6352
Type*Pretreatment Temperature*Pretreatment Time*Size*Incubation Temperature	0.8285	0.8642	0.8943	0.7894	0.6337	0.7647	0.8315	0.8701	0.8982	0.8964	0.9016	0.9016
Type*Pretreatment Temperature*Pretreatment Time*Size*Incubation Day	0.6309	0.4455	0.4491	0.3646	0.4826	0.4089	0.4296	0.4408	0.4193	0.4989	0.4799	0.4799
Type*Pretreatment Temperature*Pretreatment Time*Incubation Temperature*Incubation Day	0.4897	0.4257	0.4426	0.3800	0.3826	0.3904	0.4677	0.3953	0.3760	0.4089	0.4138	0.4138
Type*Pretreatment Temperature*Size*Incubation Temperature*Incubation Day	0.6127	0.3346	0.3324	0.3002	0.3201	0.2974	0.3028	0.3700	0.4123	0.4989	0.4491	0.4491
Type*Pretreatment Time*Size*Incubation Temperature*Incubation Day	0.8582	0.7399	0.7522	0.6423	0.7510	0.7169	0.7383	0.7135	0.7213	0.7494	0.7523	0.7523
Pretreatment Temperature*Pretreatment Time*Size*Incubation Temperature*Incubation Day	0.7106	0.4441	0.4048	0.3571	0.4479	0.3932	0.3515	0.4392	0.4553	0.5412	0.4977	0.4977

3.7. Optimal Condition

The optimal condition for pretreatment was also applied to produce the most favorable CEL. As mentioned above, the main objectives for pretreatment are to avoid size reduction, preserve the saccharide fractions, limit formation of degradation products and minimize energy and cost. According to the moisture reduction, there were no significant decreases above 15 h and 75 °C. Ball-milling, longer incubation time and higher temperature used much more energy, and this translated into a higher cost of operation. When comparing the FTIR spectra, it is evident that higher temperature and time reduced the intensity of peaks. Ball-milling also reduced the intensity, but the changes were not too prominent within the lignin range. When comparing the overall time, the ball-milling process took a shorter time to prepare since it already can produce consistent size reduction without the need of a sieve, which could increase the energy consumption. Overall, it was found that the best conditions in which to pretreat the feedstock were at 15 h, 75 °C and using the ball mill on a leaf sample.

Stem sample FTIR spectra of CEL were much closer to KrL, indicating that more lignin was present. Increasing the temperature and the drying time caused the amount of CEL produced to be more consistent and closer to pure lignin spectra. Little change was seen with increasing incubation time and temperature but 40 °C appeared to obtain the lowest cellulose content, which is ideal. Longer incubation only improved cellulose breakdown by a tiny margin, such that choosing the middle point would be ideal to save energy and time while also producing a commendable amount of lignin. Therefore, using a stem sample, pretreated at 105 °C for 25 h and incubated at 40 °C for 3 days would be the optimal parameter to obtain lignin from Napier grass.

A comparison of Klason lignin and CEL with other studies is shown in Table 13.

Table 13. Comparison of lignin content from previous and current studies.

Reference	Lignin Content
Manokhoon and Rangseesuriyachai [34]	Untreated: 16.7% NaOH treated: 6.9–8.1%
Mohammed et al. [29]	Napier stem: 26.99 ± 1.29% Napier leaf: 30.09 ± 1.30%
Phitsuwan et al. [35]	Untreated: 29.8% NaOH treated: 9.1% $CaOH_2$ treated: 20.1% NH_3 treated: 12.0% aH_2O_2 treated: 15.4%
Phitsuwan et al. [36]	29.8–12.3%
Song et al. [37]	5.7–6.2%
This study	Klason lignin: 4.48–38.2% CEL: 52.9–86.9%

4. Conclusions

Pretreatment has been successfully carried out on Napier grass leaf and stem samples through physical and thermal methods, and the pretreated samples were then incubated for cellulolytic enzymatic hydrolysis. The moisture content is directly affected by the drying temperature as seen in the result presented. A higher drying temperature and longer drying time will lead to higher moisture loss from the sample. From the observation of FTIR spectra after physical and thermal pretreatment, drying temperature does affect the composition of functional groups in pretreated samples, but it is evident that partial delignification occurs due to a reduction in the lignin fingerprint band. From the Klason method, ASL was found to differ significantly between the two types of samples. CEL was extracted from the pretreated sample after varying incubation parameters. The extracted CEL showed a higher solid yield than the actual lignin content, indicating that impure lignin was obtained and that cellulose was not fully disintegrated from the sample. Soluble lignin was detected in a very small amount, negating the need of separation from the

solution. The FTIR results obtained for CEL were slightly higher than pure Kraft lignin which means that cellulose was still present in the sample. Optimization of parameters was carried out to ensure that an easier process can be performed while producing a better lignin product. The optimized conditions for pretreatment were found to be 75 °C, 15 h and balling onto a leaf sample. For cellulolytic enzymatic hydrolysis, incubation at 30 °C for 3 days is the optimum. These obtained data can be a reliable precursor for other studies on the extraction of lignin from Napier grass, as well as other grass-type biomass which optimize and improve the process of valorizing the biomass sources. The data also can be a good starting point for research on other pretreatment and extraction methods.

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