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Acid-mediated hydrolysis of cellulose from *Gigantochloa levis* (Bolo) leaf and branch

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Abstract

Cellulose from *Gigantochloa levis* leaf and branch were extracted and hydrolyzed in acidic medium to produce reducing sugars at different reaction times (30, 60, 90, 120 minutes). The extraction of cellulose from *G. levis* leaf and branch were carried out by a three-step delignification process. The extracted cellulose from both samples were characterized via FTIR, morphological analysis and test for lignin using photomicrograph. Furthermore, crude cellulose from both samples were hydrolyzed to produce reducing sugars. The produced reducing sugars were qualitatively confirmed using Benedict's test, then quantitatively determined through Lane- Eynon titration. Significant difference on the percentage yield of the produced cellulose and on the amount of reducing sugars produced at various reaction times were also determined. The extracted crude cellulose from *G. levis* leaf and branch were white in appearance, having yields of 19.1897 ± 0.2907% and 19.0183 ± 0.7095%, respectively. FTIR spectra of both cellulose samples do not show a C=C stretching and C=O stretching vibrations while the chemical test for lignin of both samples gave a negative result. Both results imply that lignin and hemicellulose were successfully removed. Meanwhile, the amount of reducing sugars for both samples increased from 30 to 90 minutes but decreases beyond the 90- minute reaction time. There is no significant difference in the percentage yield of both cellulose while there exists a significant difference in the amount of reducing sugars produced at various reaction time. Overall, the results suggested that the *G. levis* is a possible source of cellulose and its corresponding hydrolysates.

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Introduction

Cellulosic biomass is known to be an abundant renewable source, which includes agricultural crops and trees, wood residues, municipal residues, among others. (Aboody, 2013) and (Ayeni, *et al.*, 2015). It is mostly made up of a molecule called cellulose, which is the primary component of plant cell walls. With this, it possesses a huge potential pool of renewable energy if degraded to yield glucose molecules and other degradants for various applications.

Bamboo is one of the fastest-growing, cellulose- rich plant in the world that can grow up to four feet a day. Although bamboo is recognized as a useful resource, such as handicrafts, paper-making, on construction materials as well as its edible shoots, some parts such as leaf and branch of bamboo after the production were only discarded. Thus, their use must be maximized and their full potential as a source of cellulose must be investigated. (Truong and Anh Le, 2014)

Dilute acid hydrolysis process was used to hydrolyze the crude cellulose from bamboo to produce reducing sugars. This process uses an acid catalyst to break the cleavage of the chemical bonding with the addition of water molecules. During hydrolysis, the bonded glucose monomers which forms the cellulose can be broken to form glucose molecules. (Saha, 2005)

Studying the factors affecting the hydrolysis process from naturally- sourced cellulose materials could then lead to the highest possible yield of reducing sugars. Therefore, the main objective of this study is to produce and characterize crude cellulose from a specific bamboo species, *Gigantochloa levis* leaf and branch samples and to characterize when subjected to acid hydrolysis at various reaction times.

Material and methods

Sample Collection and Material Preparation

The aerial part of *Gigantochloa levis* (Bolo bamboo) raw branch and leaves samples were randomly collected along the river bank in Km. 7 Upper Pasonanca Zamboanga City and was identified by the Department of Environment and Natural Resources (DENR) Region IX as *Gigantochloa levis* (Bolo bamboo). All other chemical and reagents used in study were in analytical- grade and was directly used without further purifications.

Extraction of Cellulose using Three- Step Delignification Process

A total amount of 40 grams of the homogenized samples (*G. levis* leaf and branch) were separately weighed and placed in a 1000mL beaker. Then, an amount of 5% nitric acid was added in a ratio (1 g : 15mL) and were heated for 2 hours at 80°C. Treated samples were then placed in a water bath at a room temperature to cool, then filtered and washed thoroughly with distilled water until pH 7. The washed samples were then dried in an oven for 2 hours at 80°C. Dried pretreated samples were separately placed in a beaker and added with 2.0M NaOH (1g : 15mL). The samples were heated for 2 hours at 80°C, placed in a water bath at a room temperature to cool, filtered and washed until the filtrate attained neutrality. Residues were dried in an oven for 2 hours at 80°C, then cooled.

The dried samples (after treating with HNO_3 and NaOH) were placed separately in a 250mL beaker and were treated with 5% NaOCl (1g : 15mL) ratio. The resulting mixtures were heated for 30 minutes at 80°C then placed in a water bath to allow it to cool. The treated samples were filtered and washed thoroughly until no odor was left in the samples. After which, the samples were oven dried for 2 hours at 80°C, then cooled and weighed. Resulting weights of the treated samples were recorded, and were kept for further analysis. (Dai *et al.*, 2014).

Characterization of Cellulose from G. levis Leaf and Branch

The produced crude cellulose samples were first subjected to FTIR analysis (Shimadzu IRAffinity-1S) to determine the different relevant peaks projected by the cellulose. In addition, the surface of the produced cellulose samples was observed using a photomicrograph (Ecoline by Motic) to visualize the separation of the fibers. Furthermore, cellulose samples were also stained with Phloroglucinol -HCl to verify if lignin has still retained from the samples after chemical treatments.

Time-Dependent Hydrolysis of Produced Crude Cellulose from G. levis Leaf and Branch

About two (2) grams of produced cellulose from *G. levis* leaf and branch were separately placed in a clean and dry 250mL round bottom flask. From there, each were added with 6% (w/v) phosphoric acid. The resulting solution was secured in an oil bath and heated at 170°C, where an amount of 30mL of aliquot on the heated samples were acquired on a 30- minute interval (30, 60, 90, 120 minutes) for about 30 seconds each. After the gathering, an amount of 4mL distilled water was poured on the sides of the flask in compensation for the vapor loss. The aliquots were then immediately neutralized using 20% $NaHCO_3$ solution to attain neutrality, with a universal pH paper used to monitor the pH. After neutralization, the aliquots were immediately stored in a properly-labelled vial and chilled in an ice box to be utilized for quantitative analysis. The above procedure was repeated in order to obtain two replicates. (dela Rosa *et al.*, 2014) Benedict's test was then used to confirm the presence of reducing sugars from hydrolyzed samples of *G. levis* leaf and branch.

Quantitative Determination of Reducing Sugar from Hydrolyzed Cellulose from G. levis Leaf and Branch

The reducing sugars from hydrolyzed cellulose samples were quantitatively determined using Lane-Eynon titration method at various reaction times (30, 60, 90, and 120 minutes). In addition, standard glucose solution was prepared for the standardization of Fehling's solution. A 50mL burette was first filled with the hydrolyzed cellulose aliquot. Then, an amount of 5mL Fehling's A and 5mL of Fehling's B were placed in 250mL Erlenmeyer flask, then diluted using 10mL distilled water. The resulting mixture was placed in a hot plate and was heated until 60°C. The heated solution was then added with two drops of methylene blue to serve as an indicator. At this point, the flask containing the methylene blue mixture was titrated against the hydrolyzed cellulose solution, continuing the process while heating until a brick-red color of solution appears. The entire process was repeated twice for all hydrolyzed samples at all acquired reaction times. (Pandiar *et al.*, 2017)

The amount of reducing sugar can be determined using Equation 1.

$$\text{Amount Reducing Sugar} = \frac{\text{Factor} \times 100}{S_2}$$

[Equation 1]

where Factor = factor of Fehling's solution and S_2 = the consumed volume (mL) of hydrolyzed sample.

Results and discussion

Percent Yield of Produced Cellulose from Leaf and Branch

The results of the percent yield of crude cellulose after a three-step pretreatment of *Gigantochloa levis* (Bolo) leaf and branch is shown in Table 1.

Table 1. Percent Yield Determination of Cellulosic Material of *Gigantochloa levis* (Bolo) Leaf and Branch.

Parameters	Sample	
	Branch	Leaves
Mean ± Std Dev	19.0183 ± 0.7095	19.1897 ± 0.2907
t Calculated	0.3852	
P(T<=t) two-tail	0.7257	
t Critical two-tail	3.1824	
Implication	Not significant	

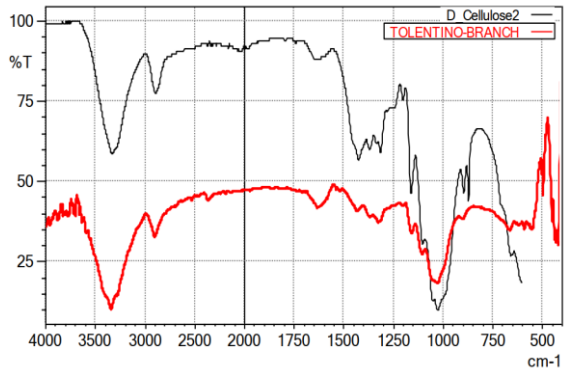
The yield of produced cellulose from *G. levis* branch and leaf were 19.0183% and 19.1897%. In addition, the amount of sample lost after the strong acid and strong base pretreatments were reduced into half. Thus, it implies that the lignin and hemicellulose were removed from both samples as the relative weights after delignification process have significantly reduced. Using *t*- test, there exist a significant difference in percentage yield of cellulose between branch and leaf sample (at α= 0.05).

Characterization of Produced Cellulose from G. levis Branch and Leaf

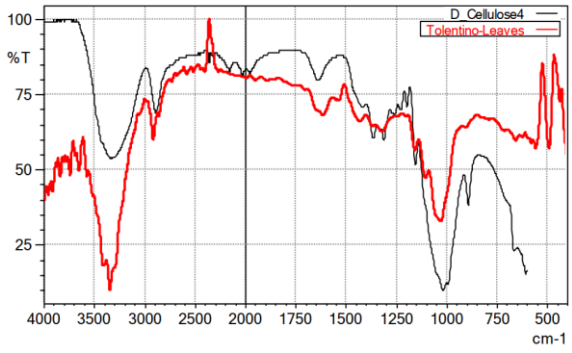
Spectral matching with that from the instrument's library shows that the produced crude cellulose from *Gigantochloa levis* (branch and leaf) and the standard cellulose were similar, as shown in Fig. 1.

Fig. 1 shows the FTIR spectra of the cellulose from *G. levis* branch and leaf. The broad peaks appear from the intense frequency at 3342.70cm^{-1} and 3333.05cm^{-1},

corresponds to the bending of -OH groups. Meanwhile, the frequency located at 2912.56cm^{-1} and 2912.56cm^{-1} were due to the symmetrical C-H stretching of the structure. The located band at the frequency of 1624.09cm^{-1} and 1430.24cm^{-1} were attributed to the absorption of the -OH group, causing the bending of the absorbed water. In connection, the presence of the O-H of water indicates that the remaining water molecules were strongly bonded to the cellulose macromolecules via hydrogen bonding. (Aboody, 2013) Furthermore, the vibration at the range of 899.81cm^{-1} is caused by the C-H bond which corresponds to the β-linkage present in the structure of crude cellulose. The peaks at the range of 1433-1156cm^{-1} corresponds to pyranose ring skeleton, while at 1026.15cm^{-1} and 1156.34cm^{-1}, represent the absorbance peak assigned to C-O-C stretching with a weak intensity. (Moran *et al.*, 2008).



A



B

Fig. 1. Infrared Spectra of Produced Cellulose from *Gigantochloa levis* A) Branch and B) Leaf.

The result shows the absence of aromatic C=C stretch, and C=O asymmetric stretch in both spectra. Thus, this implies that unwanted compounds such as lignin, pectin and hemicellulose were successfully removed, as all of them contains the mentioned functional groups.

Overall, the results based from the IR spectra indicates that the crude cellulose from *G. levis* leaf and branch were successfully extracted. In addition, the IR spectra confirms that the three- step delignification process was an effective method in removing lignin, pectin and hemicellulose for both samples.

Morphological Analysis of Cellulose from G. levis Branch and Leaf

After the three-step delignification process, the extracted cellulose was subjected to morphological analysis for further characterize the samples using a photomicrograph, as shown on Fig. 2.

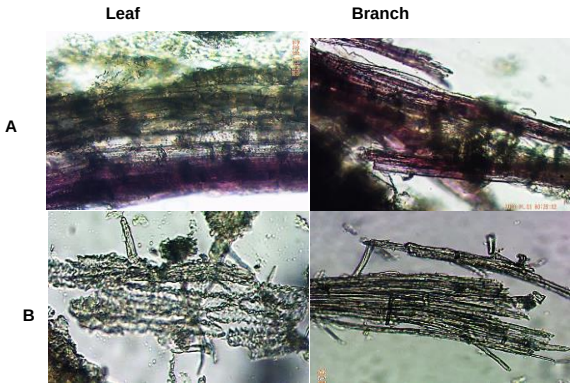


Fig. 2. Photomicrograph Illustration of *Gigantochloa levis* Leaf and Branch A) Raw Samples B) Cellulose after subjected to Staining (400x).

As shown, the raw samples gave a positive result to Phloroglucinol-HCl staining as it imparted a red-violet stain. This means that the raw samples still contain lignin. The aldehyde end groups of lignin appeared to react with phloroglucinol- HCl to impart a red-violet color. On the other hand, the crude cellulose gave a negative response to staining test. Thus, the produced crude cellulose from both samples doesn't have any end groups of lignin that will react with Phloroglucinol- HCl. This suggests that the lignin was successfully removed from the crude cellulose. (Messiry, 2014)

In addition, the surface morphology of raw material from *G. levis* branch and leaf showed that the fibers of

raw samples were not separated before the delignification process. After the delignification and bleaching process, the extracted cellulose of both samples showed the separation of fibers. In addition, its surface was less dense and clearer compared to the raw samples. Thus, this also implies that the lignocellulosic materials such as lignin and hemicellulose were dissolved during the delignification process. (Stange *et al.*, 2002)

Qualitative Determination of Reducing Sugars in the Hydrolyzed Cellulosic Material from G. levis by Benedict's Test

The hydrolyzed samples *G. levis* leaf and branch were subjected to Benedict's test to qualitatively confirm the presence of reducing sugars. Table 2 shows the summary results of the hydrolysates from *G. levis* (Bolo bamboo) at different reaction times.

Table 2. Summary Results in the Determination of the Presence of Reducing Sugars from *G. levis* Hydrolysate using Benedict's Test.

Samples	Time, min	Results	Inference
Leaves	30	+	Presence of reducing sugar
	60	++	
	90	+++	
	120	+	Small amount of reducing sugar present
Branch	30	+	Presence of reducing sugar
	60	++	
	90	+++	
	120	+	Small amount of reducing sugar present
Standard (5% Glucose)	---	+++	Presence of reducing sugars

Legend: (+) green – yellow solution (++) orange ppt (+++) brick- red ppt.

The results show that all the hydrolysates of *G. levis* leaf and branch gave positive results to Benedict's test showing a red precipitate for all replicates at 170°C. at all reaction times. The change in color is due the blue copper(II) ions from copper(II) sulphate which are reduced to red copper(I) ions by the aldehyde groups present in the reducing sugars. The red copper(I) oxide formed is insoluble in water and is precipitated out of the solution.

The hydrolysates for both samples collected at 30-minute reaction time imparted a green to yellow solution. This means that cellulose had degraded into smaller units called erythrodextrins. In addition, as the reaction time progressed at 60 minutes, the hydrolysates for both samples imparted an orange precipitate upon treatment with Benedict's reagent. This implies now that erythrodextrins had even further shortened, forming achrodextrins. Further heating at a 90- minute reaction time renders the full degradation of achrodextrins, forming reducing sugars such as maltose and for glucose, as confirmed by the production of a red precipitate upon treatment with Benedict's reagent. However, as the time progressed at 120 minutes, the amount of sugar decreased. This may be due to the extended reaction time where the reducing sugar may have further undergone dehydration and formed degradation products thus decreasing its amount in the solution by the indication of a blue solution. (Woldu and Tsigie, 2015)

Quantitative Determination of Reducing Sugars in the Hydrolyzed Samples of G. levis Leaf and Branch Samples by Lane-Eynon Method

The amount of reducing sugars present in the cellulose hydrolysates from *G. levis* were quantitatively determined using Lane-Eynon titration method, as shown in Fig. 3.

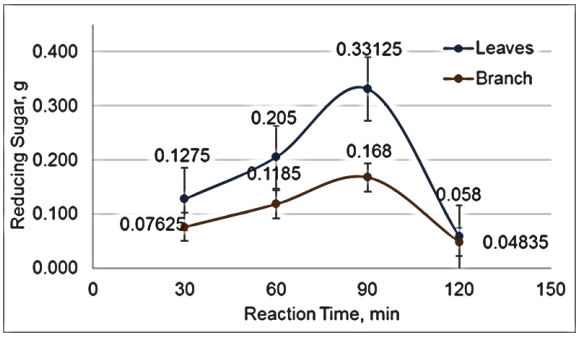


Fig. 3. Reducing Sugars Content of *G. levis* Branch and Leaves Hydrolysates at Various Reaction Times.

The amount of reducing sugars for both leaf and branch hydrolysates increased as the time increased from 30 minutes to 90 minutes. However, as the reaction time increased beyond 90 minutes, the amount of reducing sugars were reduced.

This may be due to the extended period of time and in the presence of acid at higher temperature where reducing sugars further undergone dehydration and loses three water molecules which formed degradation products like furfural, hydroxymethylfurfural (HMF), luvunilic acid and formic acid, thus decreasing its amount in the solution. (Ma *et al.*, 2012) The formation of HMF begins with the glucose condensation reaction with the protonation of C_1–OH, whereas both dehydration and isomerization reactions are initiated by the protonation of C_2–OH to form a common 5-member ring intermediate which is the HMF. (Qian, 2012)

Meanwhile, the hydrolysates from *G. levis* leaf yields a higher amount of reducing sugars than the hydrolyzed samples from *G. levis* branch. Leaves are mainly composed of glucose, in which it was produced through photosynthesis. Therefore, the amount of reducing sugar from the *G. levis* leaves is higher than the amount of reducing sugar from *G. levis* branch. The maximum amount of reducing sugars produced from both samples were achieved at 90 minutes with constant temperature of 170°C and acid concentration of 6% H_3PO_4, then decreases beyond that timeframe.

Statistical Analysis of Amount of Reducing Sugars from Cellulosic Hydrolysates from G. levis Leaf and Branch using One-way ANOVA

The total amount of reducing sugars of the *G. levis* leaf and branch at various reaction times were determined using one- way ANOVA. Table 3 shows the F ratio values for the determination of significant difference of the mean amount of reducing sugars from *G. levis* leaf and branch hydrolysates at various reaction times.

Table 3. Comparison of Means on the Amount of Reducing Sugars from *G. levis* Leaf and Branch Hydrolysates Using One-way ANOVA.

Sample	Source of Variation	Sum of Squares	*df*	Mean of Squares	F Ratio	P-value	F crit
G. levis leaf hydrolysate	Between Groups	0.0822	3	0.0274	4120.54	1.96174E-07	6.5913
	Within Groups	2.66E-05	4	6.65E-06			
	Total	0.0823	7	--			
G. levis branch hydrolysate	Between Groups	0.0163	3	0.0054	2252.25	6.56213E-07	6.5913
	Within Groups	9.67E-06	4	2.41E-06			
	Total	0.0163	7	--			
Implication					Significant		

The table shows the calculated F ratios of the *G. levis* leaf and branch. The calculated F ratio = 4120.54 from leaf and F ratio = 2252.25 from branch are greater than the confidence level at 0.05. Thus it implies that the null hypothesis was rejected and conclude that there is a significant difference in the amount of the produced reducing sugars for both leaf and branch at various reaction times given. Therefore, the amount of reducing sugars produced from both samples are significantly different at different reaction times.

Conclusion

The results show a total yield of 19.0183 ± 0.7095% from *G. levis* branch and 19.1897 ± 0.2907% from *G. levis* leaf. The produced crude cellulose from *Gigantochloa levis* leaf and branch were characterized via FTIR analysis, in which it does not show the peak bond of C=C and C=O stretches, which implies that the removal of lignin and hemicellulose using three-delignification process was successful. In connection, phloroglucinol-HCl staining of both cellulose products does not impart a red- violet stain. This implies the absence of lignin and hemicellulose. Morphological analysis also shows that the removal of lignin and hemicellulose using three-delignification process was successful.

Qualitative as well as quantitative tests confirm that the amount of reducing sugars for both leaf and branch hydrolysates increased as the reaction time progressed from 30 minutes to 90 minutes.

However, as the reaction time extended beyond 90 minutes, the amount of reducing sugars gradually decreased. The comparison on the total amount of reducing sugars at various reaction times using one-way ANOVA indicates that there is a significant difference on the amount of produced reducing sugars at various reaction times. It is then recommended that the hydrolysates present for both samples be identified and quantified to ascertain the extent of hydrolysis that the samples had underwent to.

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