

# The Effect of Ultrasonication and Delignification Treatment on the Sugar Released Value of Wood

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## Abstract

The objective of these research was to study the impact of ultrasonication and delignification pretreatments in several wood species on their easiness for enzymatic hydrolysis, having the potential to be developed as the feedstocks for bioethanol production. Four different wood species from three botanical gardens in Indonesia have been selected, i.e., *Gymnostoma sumatranum*, *Firmiana malayana*, *Pterocarpus indicus*, and *Alstonia scholaris*, due to their higher sugar released values than a fast-grown tree, Sengon (*Paraserianthes falcataria*), when all were directly enzymatically hydrolyzed without pretreatment. The sugar released values after ultrasonication and enzymatic hydrolysis were between 3 – 5.5 mg/100 mg wood meal. When delignification pretreatment was performed, the sugar released values were higher than those with ultrasonication, ranging between 4 – 10.2 mg/100 mg wood meal. All the sugar released values after pretreatment were higher than those without pretreatment. *Gymnostoma sumatranum* was selected as the most potential wood species in this study due to its consistency among the species producing highest sugar released across different treatments. The SEM results showed that there was no significant changes in the morphological structure of the untreated fiber before and after enzymatic saccharification since it still had a complex structure due to the high lignin content. However, after the delignification treatment, the surface morphology of the fiber showed a decrease in the number of pits of the fiber, the surface residual of pits were reduced, parallel lines were more clearly visible, and the fiber structure was damaged with more small holes presented. The surface morphology of the fiber from the wood powder having delignification pretreatment supports the sugar released values which shows that the these values were higher than other treatments due to more recalcitrant substances were degraded, making it was easier for enzymes to break down cellulose. After ultrasonication, the SEM result showed less disrupted cell wall compared to after delignification which confirmed the higher sugar released data with delignification.

**Keywords:** bioethanol, delignification, enzymatic saccharification, pretreatment, sugar released, ultrasonication

## Introduction

Biofuels, unlike fossil fuels, are potential alternative energy solutions due to their feedstocks' abundant availability, renewability, and sustainability, enhancing energy security. Biofuels have many advantages over fossil fuels especially due to their more environmentally friendly characteristic in reducing greenhouse gas emissions, which is much lower than the emissions released from burning fossil fuels. The use of bioethanol, one form of biofuels, can reduce greenhouse gas emissions by 30~85% when compared to the use of fossil fuels (Fulton *et al.* 2004 in Sainz 2009). Global production and energy use of biofuels increased from 18.2 billion liters in 2000 to 60.6 billion liters in 2007, of which bioethanol is the main supplier of biofuels, accounting for 85% (Coyle 2007 in Sainz 2009). Bioethanol is still widely used as an additive in fossil fuels, but it is possible that bioethanol will replace the function of fossil fuels in the future.

Currently, the raw materials for making bioethanol are still dominated by food crops (first generation bioethanol), including corn, sugar cane, and starch which can threaten world food security. Therefore, alternative sources of raw materials that do not interfere with food security are needed, namely lignocellulosic materials (second generation bioethanol). Lignocellulosic biomass, which can come from

forest resources, agricultural and agro-industrial waste, is one of the feedstocks for biofuels and is available in abundance, renewable, and inexpensive. Making bioethanol from lignocellulosic biomass that is financially feasible is a national priority and a scientific challenge.

Many studies on the developments of bioethanol from lignocellulosic materials have been carried out, generally using lignocellulosic waste, such as molasses waste, agricultural residues, and food processing waste. The use of waste can increase its added value and reduce pollution, but on the other hand, there will be a dependency of raw materials for bioethanol industry to other industries, which can disrupt the sustainability of the production process. Lignocellulose from wood can be a solution to this issue. Wood has some advantages over other lignocellulosic materials, such as high cellulose content (up to 80% holocellulose) and it can grow on marginal lands where agricultural crops cannot grow (Kaida *et al.* 2009). Mixing 85% wood-based bioethanol (E85) with fuel can reduce carbon emissions by 65% compared to starch-based bioethanol which only reduces emissions by 17~23% (Watanabe 2008). Bioethanol raw material from wood does not need a storage area as required by other lignocellulosic materials. Timber can also be supplied from industrial forest plantations (*Hutan Tanaman Industri/HTI*) so that the

continuous supply of materials can be guaranteed. Cellulose produced from forest plantations reaches  $5.0 \times 10^9$  tons per year which can be converted into  $2.6 \times 10^{12}$  L bioethanol (Hayashi 2009). Cultivation of wood on HTI land will be in line with the Kyoto Protocol because the planted trees can increase the amount of carbon stock on earth which in turn will reduce the effect of greenhouse gas emissions in the atmosphere.

Wood cellulose microfibrils consist of a large amount of para-crystal 1,4- $\beta$ -glucan in the form of nanofibers with a width and thickness of 3 and 4 nm, respectively. The glucan surface of the nanofibers overlaps the hemicellulose, so that each nanofiber is in each glucan layer (O'Sullivan 1997). Nanofibers form a collection of rigid and hydrophobic bundles, resulting in a crystalline region (Hackney *et al.* 1994), while the combination of several para-crystal glucans forms a non-crystalline (amorphous) region.

The conversion of wood into bioethanol is highly dependent on the biomass content. The more biomass content, the greater the possibility of bioethanol produced. Therefore, wood with high biomass content with fast growth is needed, such as fast-grown wood species, while it is also possible that other wood species can also produce high bioethanol yield. Several wood species that have been studied, such as *Acacia* (*Acacia mangium*) and *Sengon* (*Paraserianthes falcataria*) have shown different characteristics in producing bioethanol. *Acacia* has a higher cellulose content than *Sengon*, but its conversion to bioethanol is lower. The existence of different characteristics of each wood species is an opportunity to find the most suitable wood species to be converted into bioethanol. Since Indonesia has a high diversity of wood species, it can become a major bioethanol producer in the future by exploring many wood species, especially from less commercial and lesser-known species. Research on the possibility of using these various wood species as raw materials for bioethanol is very necessary to provide information about their potential as raw materials for bioethanol production.

The main steps in the process of making bioethanol from lignocellulosic biomass generally start with thermochemical pretreatment, including dilute acid, alkali, ammonia expansion process, and steam explosion process. This aims to free cellulose microfibrils from the lignin matrix and increase their surface area, as well as dissolve hemicellulose, thereby making cellulose more open and easily broken down from polysaccharides into simple sugars in the subsequent enzymatic hydrolysis process (Sanchez and Cardona 2008). Even though many physical and chemical pretreatments to increase the bioconversion of cellulose to glucose have been widely reported, the problems are still revolving around how to improve the conversion of cellulose to glucose units with the help of enzymes, due to the strong crystalline structure of cellulose and its resistance to enzyme attack. The most important thing in fast and thorough enzymatic hydrolysis is the pretreatment of cellulose which can open the cellulose structure and

eliminate interactions between glucose chains. Hayashi (2009) stated that wood is very resistant to enzymatic degradation which makes it difficult to degrade into fermentable sugars. Furthermore, the high price of enzymes causes the manufacture of ethanol from wood cellulose to be un-economical.

Delignification and ultrasonication are among many pretreatments that can be used to improve enzymatic saccharification of lignocellulosic biomass. Delignification is required prior to hydrolysis in order to release cellulose and hemicellulose (Sarkar *et al.* 2012). Chlorite delignification for black spruce wood has been reported back in 1970 by Ahlgren and Goring (1971) that showed this treatment could selectively remove lignin during 60% of delignification which afterward would cost the undesired removal of glucomannan and galactan. Lignin is linked to cellulose and hemicellulose in a way that makes the biomass become highly recalcitrant to enzymes and acidified sodium chlorite pretreatment (Wise method) was proven effective in softwood delignification (Kumar *et al.* 2013).

On the other hand, ultrasonication is a possible pretreatment method because it is safe for the environment. Ultrasound creates a hydrodynamic shear force in the aqueous phase that makes it easier for the coarse particles in a slurry to break down into finer particles. This makes more surface area available for enzyme activity (Nitayavardhana *et al.* 2010). Furthermore, the coarser lignocellulosic materials can be broken down into finer particles by using ultrasonication, which significantly increases the surface area for enzymatic attack for bioethanol production. Through the reduction of lignocellulose's structural rigidity and the elimination of mass-transfer resistances, ultrasound can be used to optimize the hydrolysis process and increase product yield while using less time to process and enzyme (Subhedar & Gogate, 2013). When cellulose fibers are subjected to ultrasonication, the fibrils on the surface of the fibers peel off, causing partial fibrillation and subsequent separation of the fibers. In addition, lignin and hemicellulose are released through homolysis of lignin-carbohydrate linkages brought on by sonication (Rehman *et al.* 2013).

## Materials and Methods

### Materials

There were four wood species used in this study as a result from screening many wood species that have the potential as raw materials for bioethanol, carried out on the collections of woody plants in Cibodas Botanical Gardens (49 species), Purwodadi (32 species), and Eka Karya Bali (20 species). These four wood species were *Gymnostoma sumatranum*, *Firmiana malayana*, *Pterocarpus indicus*, and *Alstonia scholaris*. The raw materials used in this study were collected from the first branches of these wood species. The bark was removed, then crushed using a Hammer mill and Disk mill into wood meal in the size of 40–60 mesh and 115–170 mesh, then dried until it reached air dry conditions.

The chemicals used were ethanol absolute, benzene, sulfuric acid 95~98%, sodium chlorite (NaClO<sub>2</sub>) 25%, acetic acid glacial 100%, acetone, sodium hydroxide (NaOH) pellet, silica gel, Tween 20, yeast extract (*Saccharomyces cerevisiae*), cellulase, aquades, sodium hydrogen carbonate, ammonium acetate, glucose, di-sodium hydrogen phosphate anhydrous (Na<sub>2</sub>HPO<sub>4</sub>), sodium arsenate dibasic heptahydrate, copper (II) sulfate pentahydrate, copper (II) chloride dihydrate, potassium sodium tartrate (K-Na Tartrate), citric acid monohydrate, potassium sulfate, ammonium molybdate, calcium chloride dehydrate, sodium carbonate anhydrous, and sodium Sulfate.

### Equipments

The main equipments used in this research were: (a) Wood sampling equipments: hand saw, circular saw, hammer mill, and disk mill; (b) dimensional measuring equipment (caliper) and weight (digital weigh); (c) laboratory equipments: drying oven, autoclave, centrifugation, incubation, ultrasonication and rotary shaker; and (d) testing equipments: Gas Chromatography (GS) and Scanning Electron Microscopy (SEM).

### Methods

**Ultrasonic Pretreatment.** The wood meal was autoclaved at 120°C for 3 minutes so that it became impregnated with water, washed once with water by centrifugation, then precipitated with 2 ml of water and ultrasonicated for 5 minutes with Branson Sonifer 250. The radiation intensity was adjusted at the microtype limit 7 and duty cycle 50%. The ultrasonicated wood meal would then be used for enzymatic hydrolysis.

**Delignification Pretreatment.** Delignification of wood meal (100mg) which had been autoclaved at 120°C for 3 minutes was carried out in 3 ml of 8% sodium chlorite solution containing 1.5% acetic acid with stirring at 50 rpm at 35°C for 40 hours, with fresh acid-chlorite solution was added every 8 h. The samples obtained were determined as nondetectable lignin by the Klason method, and then used for enzymatic hydrolysis.

Table 1. Chemical compositions of the wood species

Botanical Garden	Wood species	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Eka Karya Bali	<i>Alstonia scholaris</i>	42.9	15.5	26.8
Purwodadi	<i>Firmiana malayana</i>	43.2	20.5	25.9
Purwodadi	<i>Pterocarpus indicus</i>	47.6	13.1	25.6
Cibodas	<i>Gymnostoma sumatranum</i>	43.8	13.5	24.1

### Sugar Released after Enzymatic saccharification of Wood Species with and without Pretreatment

**Enzymatic Hydrolysis without Pretreatment.** Figure 1 shows the comparison of sugar released without pretreatment from several wood species based on the size of wood meal. The highest sugar released was from *P. indicus*

**Enzymatic Hydrolysis.** A hundred (100) mg of wood meal was autoclaved at 120°C for 3 minutes, so that it was impregnated with water and washed once with water by centrifugation. Commercial cellulase enzymes (Meicelase, Meiji Seika Co., Tokyo, Japan) derived from *Trichoderma viridae* were used to decompose the wood meal. These enzymes contain endocellulases, exocellulases (CBHI and CBHII), xyloglucanase, xylanase, galactanase, and polygalacturonase. Enzymatic hydrolysis of sawdust was carried out in a mixture of 2 ml of 50 mM sodium acetate buffer pH 4.8; 0.02% Tween 20; and 0.4 units of cellulase filter paper (2.0 mg). One filter paper unit was defined as 1 µL glucose per minute released from 50 mg Whatman filter paper at 45°C in a rotary shaker at 135 rpm. Approximately, 100 µL of the supernatant was collected at 24 h after hydrolysis was initiated and used for sugar analysis. The released sugar was measured as reduced sugar using the Nelson-Somogyi method. Furthermore, the released sugar was analyzed directly as alditol acetate using Gas Chromatography (GC).

**Scanning Electron Microscope (SEM).** The dried xylem was immersed in water under reduced pressure and autoclaved at 120°C for 2 minutes. The wet xylem is then cut by hand using a razor in cross section with a length of about 0.5~1.0 mm. Each part was treated with cellulase preparations used for saccharification, then washed in water three times and dried at 40°C for one night. Each section was observed under a field emission scanning electron microscope (FE-SEM).

## Results and Discussion

### Chemical Composition of The Wood Species

The chemical components of the four wood species can be seen in Table 1 with their respective botanical gardens' growth location. It can be seen that *P. indicus* has the highest cellulose content, followed by *G. sumatranum*, *F. malayana*, and *A. scholaris*. On the other hand, *G. sumatranum* has the lowest lignin content followed by *P. indicus*, *F. malayana*, and *A. scholaris*.

for coarse wood meal (40~60 mesh) and *G. sumatranum* for fine wood meal (115~ 170 mesh). The size of the wood meal could affect the conversion of cellulose into sugar released. The finer the wood meal, the higher the conversion. Generally, the enzyme will attack the finer powder first (Zhu *et al.* 2009), while the larger powder will only be hydrolyzed after a few hours of the saccharification process.

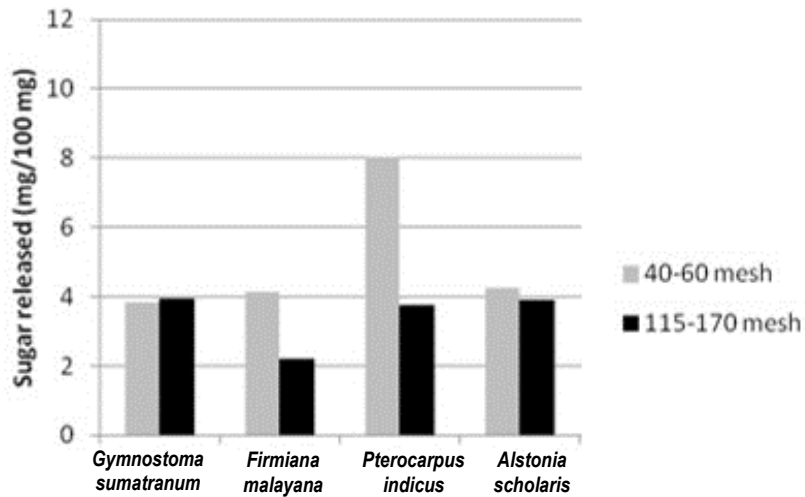


Figure 1. Sugar released without pretreatment.

**Enzymatic Hydrolysis after Ultrasonication and Delignification Treatment.** The results of sugar released after ultrasonication and delignification pretreatments of the four wood species based on the size of wood meal are shown in Figure 2 and 3. Delignification treatment was carried out to remove lignin from lignocellulosic materials. Lignin is an inhibitor for the action of the cellulase enzyme and is a binder of fibers. From the figures, it can be seen that the powder size has no significant effect on the delignification pretreatment. This was because the impact of lignin removal as an inhibitor of the cellulase enzyme was greater than the powder size during enzymatic hydrolysis. These samples were generally relatively pure cellulose, both in fine and coarse powders, so

that the sugar released during the hydrolysis process tended to have not been affected by the particle sizes used in this study.

In Figure 4, it is known that the ultrasonication and delignification pretreatments resulted in higher sugar released than without pretreatment. This was because the cellulose proportions of the pretreated samples were higher than those in the samples without pretreatment due to decreased lignin content. Since lignin can inhibit the enzymatic hydrolysis process, the lower lignin content in the samples after the pretreatments leads to a better process of breaking down cellulose into sugar by enzymes.

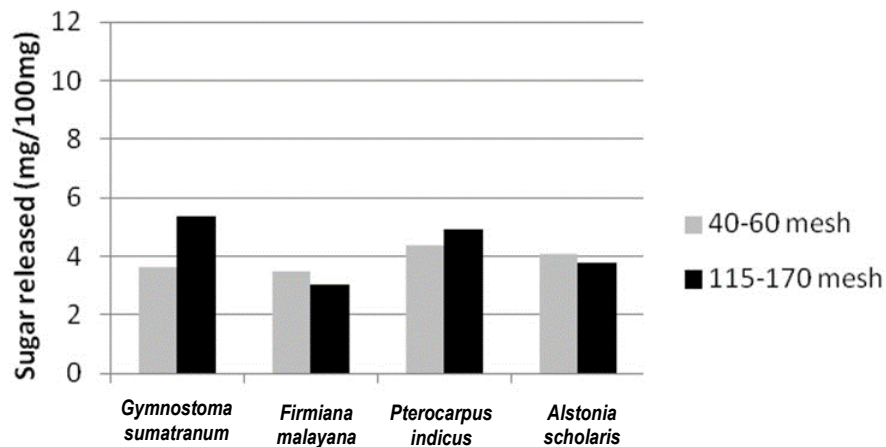


Figure 2. Sugar released after ultrasonication treatment.

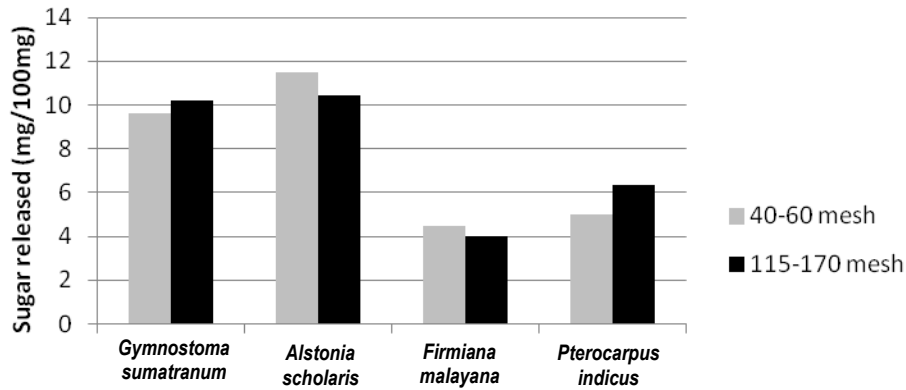


Figure 3. Sugar released after delignification treatment.

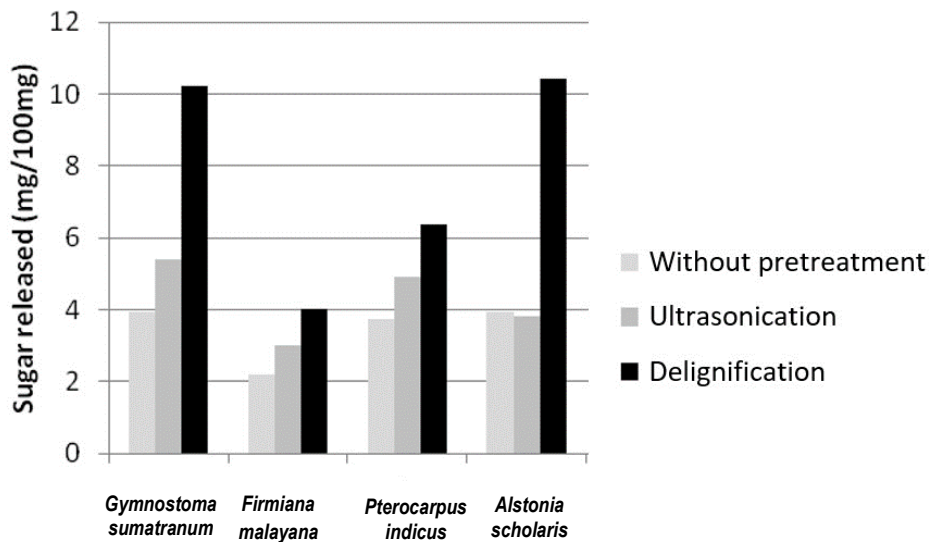


Figure 4. Comparison of sugar released without pretreatment and with ultrasonication and delignification pretreatments (wood meal size 115~170mesh).

### Scanning Electron Microscope (SEM)

Based on the results of sugar released with and without pretreatment, *G. sumatranum* wood from Cibodas Botanical Garden was selected as the most potential bioethanol feedstock among the four wood species. Therefore, SEM analyses before and after the ultrasonication and delignification process were performed on this wood to study the morphological changes of the wood fibers and how it affected the subsequent enzymatic saccharification.

**Raw Fiber Surface Morphology.** In this experiment, two sizes of powder were used, namely 40~60 mesh and 115~170 mesh. The image obtained from the SEM results shows that the fiber surface of the initial test sample consists of 2 main morphologies, namely fiber and pits structure, as shown by arrows F and P in Figure 5. There is no significant morphological difference between the 40~60 mesh and 115~170 mesh size powders. The fiber surface consists of parallel stripes partially covered by residue. Meanwhile, the pits section has a more brittle and fragmented structure, and there are small pores (Figures 5a and 5c).

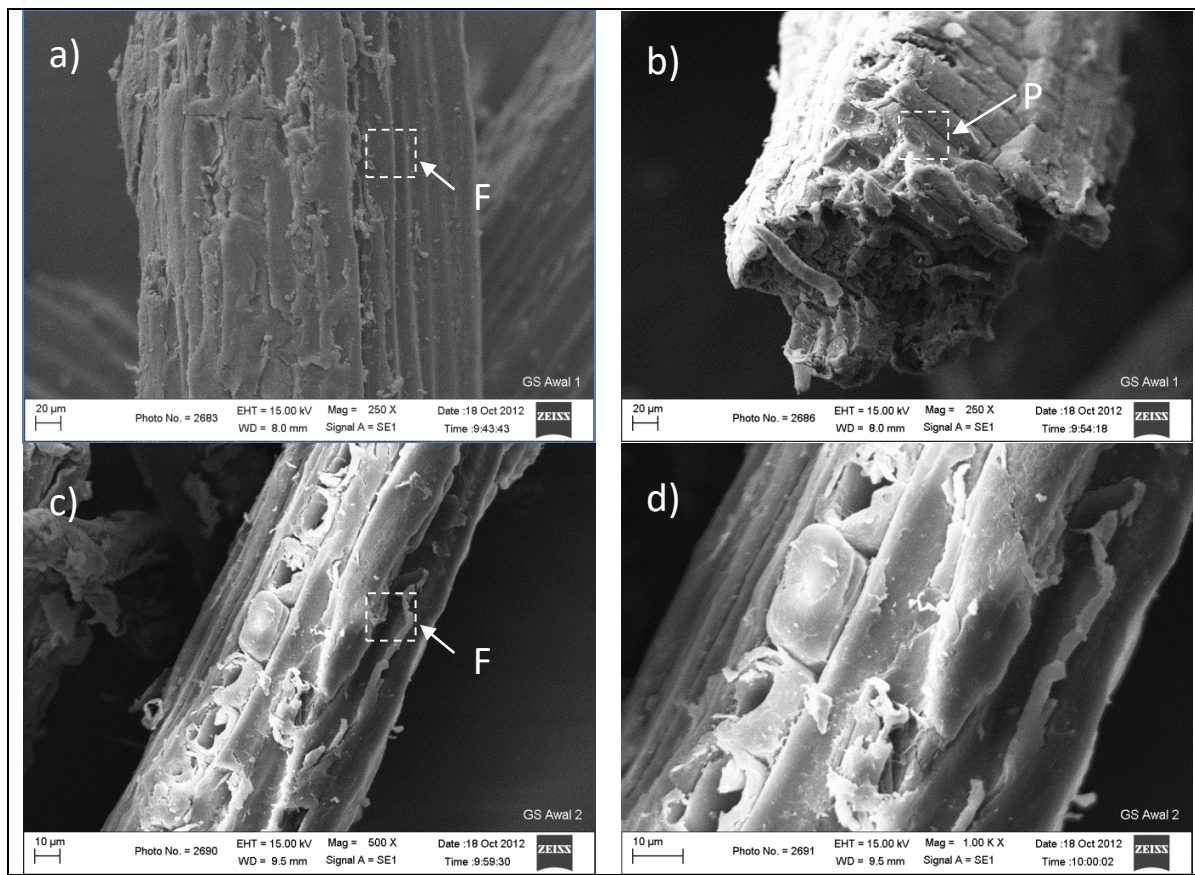


Figure 5. Initial surface morphology of *Gymnostoma sumatranum* (a) Fiber surface section showing parallel lines with residues for 40~60 mesh powder; (b) 40~60 mesh powder pits section; (c) Fiber section showing parallel lines with residues for 115~170 mesh powder; (d) reinforcement of fiber sections.

**Fiber Surface Morphology After Enzymatic Hydrolysis without Pretreatment.** The SEM results showed that there was no significant changes in the morphological structure of the fibers in the test samples that were not pretreated and just

directly hydrolyzed by enzymes (Figure 6) with the raw fibers in Figure 5. This indicates that the untreated wood meal still had a complex structure due to the high levels of lignin, making it is difficult for enzymes to hydrolyze cellulose.

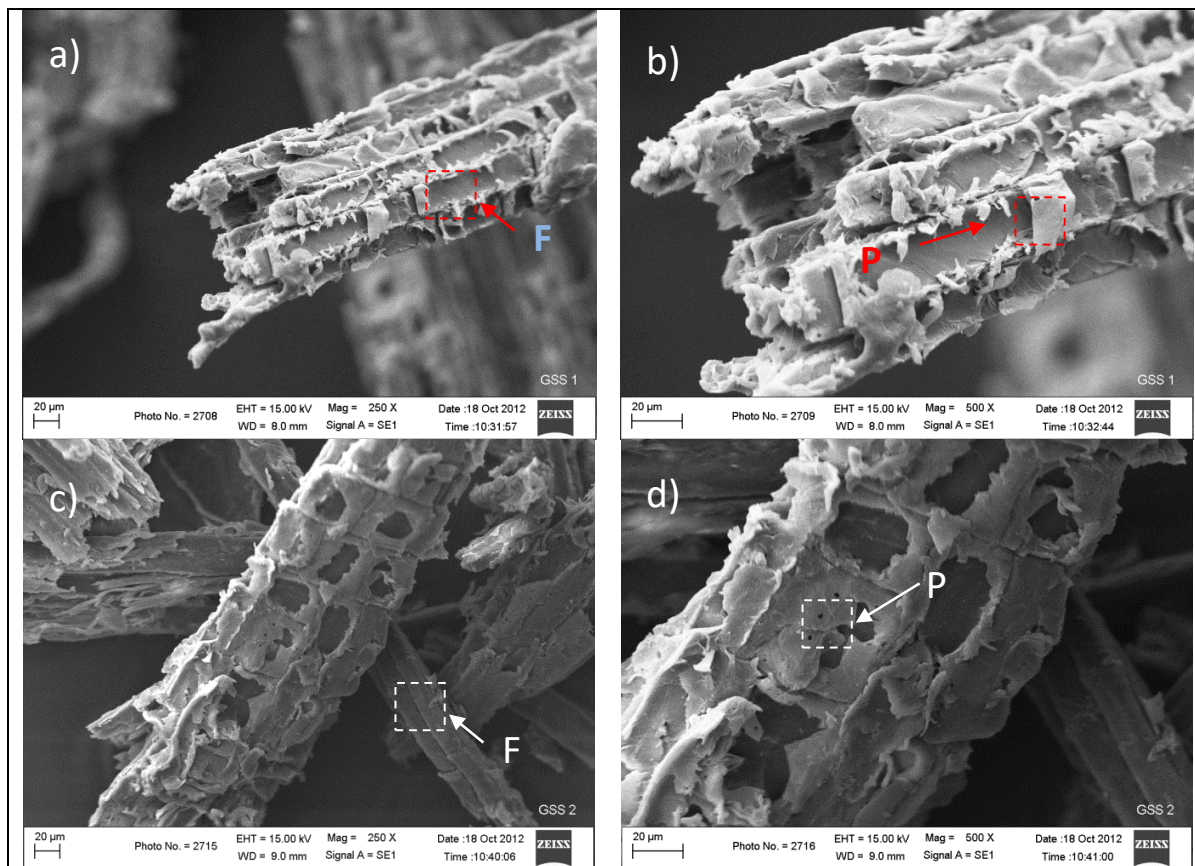


Figure 6. Fiber surface morphology of directly saccharified *Gymnostoma sumatranum* (a) 40~60 mesh powder fiber section; (b) 40~60 mesh powder pits section; (c) 115~170 mesh powder fiber section; (d) Pits powder section 115~170 mesh.

**Surface Morphology after Ultrasonication and Delignification Pretreatment.** Surface morphology after ultrasonication and delignification treatment can be seen in Figures 7 and 8. After ultrasonication (Figure 7), cell ruptures can be clearly seen (Figs. 7a and 7c) and a decrease in pits sections (Figs. 7b and 7c) as reported by other study (Subhedar & Gogate, 2013) that sonication increase surface area due to the cell disintegration. Delignification of sawdust was carried out using 8% NaClO<sub>2</sub> solution which has the ability to degrade lignin in wood. After delignification pretreatment, the surface morphology of the fibers showed that there was a decrease in the number of pits sections in the fibers of both powder sizes (Figs. 8a and 8c). This indicates that the pits section is more susceptible to degradation than the fiber structure section. Figures 8b and

8d show that the surface of the fiber section also changes due to the delignification process. Surface residual pits reduced and parallel lines are more clearly visible. In addition, the fiber structure was also damaged (small holes) due to some chemical components being degraded by NaClO<sub>2</sub>. To find out the chemical components that are degraded, it is necessary to carry out further analysis of the chemical components on the powder that has been given delignification treatment. The surface morphology of the fiber from the wood powder that was treated with delignification supported the sugar released data in Figure 4 which shows that the value of sugar released in the powder that was treated with delignification is higher than other treatments, because the more recalcitrant that is degraded, the easier the enzymes break down cellulose.

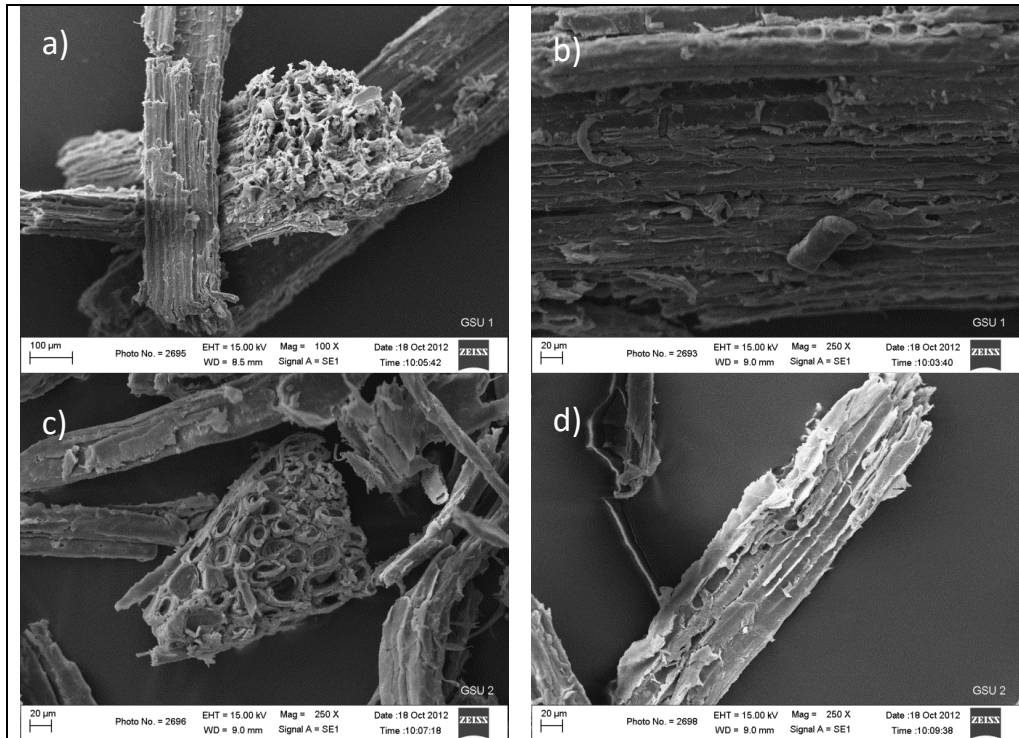


Figure 7. Fiber surface morphology of *Gymnostoma sumatranum* treated with ultrasonication (a) General appearance of 40~60 mesh powder fiber; (b) Reinforcement of 40~60 mesh powder fiber section; (c) General appearance of 115~170 mesh powder; (d) Reinforcement of 115~170 mesh powder fiber section.

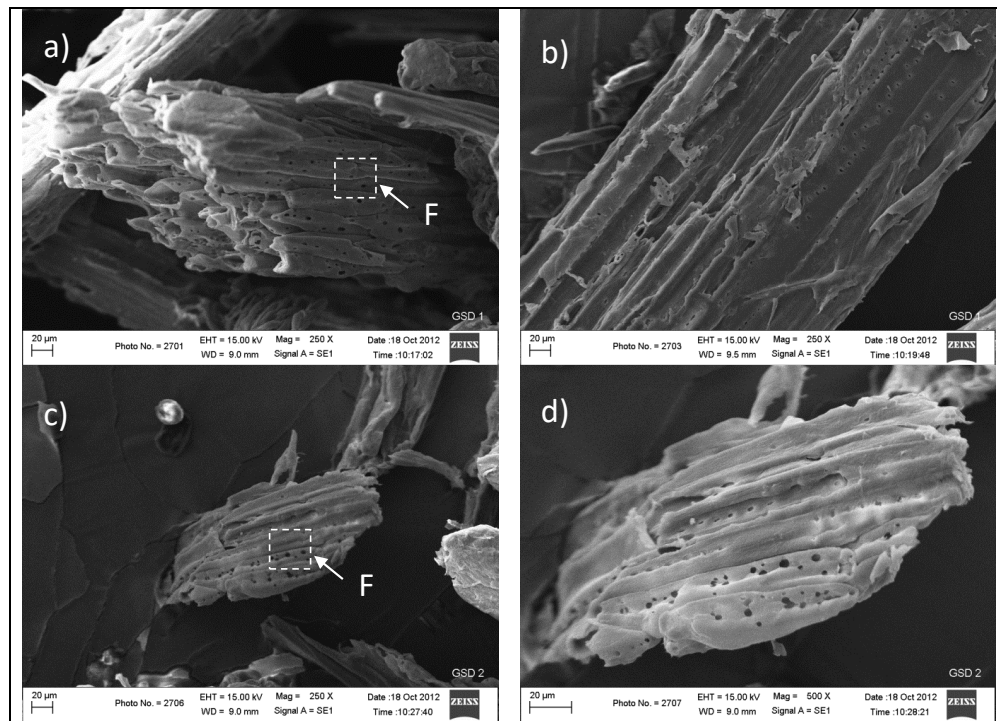


Figure 8. Fiber surface morphology of delignified *Gymnostoma sumatranum* (a) General appearance of 40~60 mesh powder fiber; (b) Reinforcement of 40~60 mesh powder fiber section; (c) General appearance of 115~170 mesh powder fiber; (d) Reinforcement of 115~170 mesh powder fiber section.

## Conclusions

Four less commercial and lesser-known wood species from three botanical gardens in Indonesia have been studied for their potential as bioethanol feedstocks. Ultrasonication and delignification pretreatments resulted in higher sugar released after enzymatic hydrolysis than those without pretreatment. Enzymatic saccharification after delignification gave higher sugar released than after ultrasonication, ranging between 3 – 5.5 mg/100 mg wood meal with ultrasonication and between 4 – 10.2 mg/100 mg wood meal after delignification. All the sugar released after pretreatment was higher than those without pretreatment. *G. sumatranum* was selected as the most potential wood species in this study due to its constantly among the species producing highest sugar released among treatments. The SEM results showed that there was no significant change in the morphological structure of the fiber before and after enzymatic hydrolysis as it still had a complex structure due to the high lignin content. However, after the ultrasonication and delignification treatment, the surface morphology of the fiber showed significant changes due to cell disruption, more recalcitrant components were degraded, facilitating easier enzyme penetration to the cellulose. These morphological changes supported the sugar released values where it was higher with pretreatment than without pretreatment.

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