e-ISSN: 2655-8777 https://journals.itb.ac.id/index.php/3bio

# A Comparison of Alkali and Biological Pretreatment Methods in Napier Grass (*Pennisetum purpureum* Scumach.) for Reducing Lignin Content in the Bioethanol Production Process

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Received 5/27/2019
Accepted for publication 4/20/2020

## Abstract

Napier grass is one of lignocellulosic plants that has the potential to be converted as bioethanol due to high productivity and relatively fast harvesting time. However, the problems of processing lignocellulosic plants into bioethanol are the high lignin content and the different lignin structure of each plant. Lignin can inhibit biological agents in accessing cellulose and hemicellulose. Therefore, it is necessary to select and optimize the pretreatment process with the aim of degrading lignin and maintaining the value of the cellulose. This study was conducted to compare the effectiveness of two different pretreatments (biological and alkaline) in degrading lignin. For the alkali pretreatment, lime ($Ca(OH)_2$) was added to the Napier grass substrate using concentrations of 0.05, 0.1, and 0.5 grams/gram, which was then incubated at 23-25 $^oC$ each within 6, 24, and 96 hours period. For biological pretreatment, *Aspergillus niger* spore was used as an agent, which was incubated onto the Napier grass substrate using concentrations of $10^6$, $10^7$, and $10^8$ cells/mL, an optimal temperature of 35$^oC$ and within 1, 3, 5, 7, and 9 days period. As a comparison, *Phanerochaete chrysosporium* was also incubated using a concentration of $10^6$ a temperature of 35 $^oC$ within 28 days period. The extracted Napier grass was then analyzed for lignocellulose content, which included hot water soluble, Hemicellulose, cellulose, lignin, and ash, using Chesson-Datta method and reducing sugar test. Comparison of biological pretreatment between *Aspergillus niger* and *Phanerochaete chrysosporium* showed that *Aspergillus niger* was better at degrading lignin, with a lignin-to-cellulose ratio of 24.3%, smaller than *Phanerochaete chrysosporium* at 30.645%. This ratio was furthermore compared with the ratio resulting from Alkali pretreatment, which showed that the former was proven to be more optimum.

Keywords: Lignin degradation, *Aspergillus niger*, *Phanerochaete chrysosporium*, alkali (Ca(OH)2), Napier grass

## 1. Introduction

The need for fuel (gasoline) in developing countries such as Indonesia is increasing in parallel with the technological developments, while petroleum energy reserves are decreasing on a daily basis. To resolve the need for fuel oil, the government has drafted an energy policy planned in Presidential Instruction No. 1 of 2006 and Presidential Regulation No. 5 of 2006, which stipulate the importance of alternative energy, especially biofuels. Biofuels in this context are defined as fuels in the form of solids, liquids and gases that are produced from organic materials [1].

One of the new renewable fuels/energy is obtained by converting biomass into bioethanol. Biomass is a term used to classify organic materials from plants or animals that are rich in energy reserves, hence the converted product is called bioenergy. Bioethanol is an alternative fuel that has several advantages compared to fuel oil. Bioethanol emits 19 to 25% lower CO gas when compared to fuel oil [2]. Bioethanol can also be produced from materials that contain lots of cellulose. Cellulose is found in agricultural and plantation waste. This resource has not been used optimally.

Bioethanol produced today is made from starch (corn) or sugar (sugar cane) which is basically a food commodity in


https://doi.org/10.5614/3bio.2020.2.1.5

Indonesia. Producing bioethanol from human food can cause the next problem which is the food crisis. Therefore, the production of second generation bioethanol using non-food biomass should be a major concern to be a future of an alternative energy.

One non-food ingredient that has the potential to supply bioethanol from cellulose is Napier grass (*Pennisetum purpureum* Schumach.). Napier grass can thrive in tropical environments such as the one in Indonesia. With a relatively shorter growing period (40-90 days), Napier grass shows the potential in meeting the availability of raw materials for producing up to 78 tons/ha/year of bioethanol, more than other similar grasses [3]. Aside from being an animal feed and companion plant, Napier grass has not been widely used.

Bioethanol production from cellulose materials requires several stages prior to fermentation. This is because according to Isroi (2008), cellulose materials consist of lignin-covered and hemicellulose-bound twisted fibers that are difficult to process [4]. One important step to break down lignin protection is by pretreatment. Lignocellulosic biomass cannot be hydrolyzed by enzymes without pretreatment, mainly because the lignin in plant cell walls forms four types of barriers to withstand enzyme activity [5].

This pretreatment aims to break down the lignin barrier, change the structure of lignocellulose, and make cellulose and / or hemicellulose more easily hydrolyzed [6]. Ideal pretreatment will reduce the lignin content and crystallinity of cellulose and increase the surface area for enzyme activity [7].

Pretreatment can be done with physical, chemical, biological, or even a combination of these methods. However, amongst the other type of pretreatments, Biological pretreatment is claimed to be the most efficient and eco-friendly. Biological pretreatment is a promising alternative because biological agents commonly have lignolytic enzymes capable of breaking down complex lignin structures. Biological pretreatment is also a sustainable method, has a high energy efficiency (because it does not require large energy), and also cost efficient. Biological pretreatment has the advantage of a physiochemical approach to processes that can occur under normal conditions and do not produce by-products that cannot be tolerated by the environment (hence, eco-friendly) [8]. Despite these major advantages, the use of biological pretreatment on a large scale of production is limited by several barriers, namely the reaction rate is generally very slow and requires a long time [8]. Therefore, research is needed to optimize the concentration and time of biological pretreatment of chemical pretreatment to get the best pretreatment method in increasing the accessibility of enzymes to cellulose.

## 2. Materials and Research

### *2.1. Materials Used*

The materials used include PDA (Potato Dextrose Agar) medium, PDB (Potato Dextrose Broth) medium, distilled water, filter paper, fatty cotton, 18M concentrated sulfuric acid, acetic acid, spiritus, falcon tube, 96% alcohol, Tween80, NaCl, fungi medium, DNS reagent, distilled water, NaOH solution, HCl solution, and Solid $Ca(OH)_2$ [9][14].

### *2.2. Pure culture of Aspergillus niger and Phanerochaete chrysosporium*

Pure culture of *Aspergillus niger* and *Phanerochaete chrysosporium* fungi were obtained from the Microbiology Laboratory, School of Life Science and Technology, Bandung Institute of Technology - Ganesha Campus in a test tube containing tilted agar (PDA). The pure culture was then stored in a refrigerator at around 4 ºC to maintain viability.

### *2.3. Napier grass substrate*

Napier grass (*Pennisetum purpureum* Scumach.) obtained was 2.5 - 3 months old after planting. After harvesting, it was immediately put into plastic and dried using shade drying machine at the Laboratory of Natural Product Isolation and Analysis, School of Life Science and Technology.

### *2.4. Napier grass sample preparation*

Napier grass (*Pennisetum purpureum* Scumach.) that has been aged for 3 months was sun-dried for one day to reduce its water content from 89% to around 20-30% [23]. Napier grass was then oven roasted for 16 hours at 105 ºC, then cut into pieces and mashed with a blender (the size is between 10 and 35 mesh, about 2 mm) [9]. The material was then weighed every 15 minutes until it reached a constant mass.

### *2.5. Lignocellulose component analysis by the Chesson-Datta method*

Lignocellulose fractionation from Napier grass was carried out using the Chesson-Datta method [10]. A total of 1 gram of dry sample (1 gram is the value (a) on the Chesson-Datta fraction calculation) was refluxed in 150 ml of distilled water at 100 ºC for two hours. The reflux solution was filtered with a Buchner funnel and the residue was dried in an oven at 150 ºC to a constant weight (weighed every 30 minutes). Dried residues which mass has stabled were weighed and a value of (b) was obtained.

The dry residue was then refluxed again using 150 ml of 0.5 M $H_2SO_4$ at 100 ºC for 2 hours. This reflux solution was rinsed with hot distilled water to neutralize the pH, and then filtered with Buchner, while the residue was dried in the oven to 105ºC until the residual mass was constant (weighed every 30 minutes). The constant residual mass is the value (c).



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The residue was then immersed in 10 ml of 72% v/v $H_2SO_4$ at room temperature for four hours and diluted to become 0.5 M $H_2SO_4$ solution, and then refluxed again for 2 hours at 100 ºC. The sample solution was rinsed with hot water and filtered with Buchner, and then dried in the oven for 2 hours or until the mass of residue was constant at 105 ºC. The constant residual mass is the value (d). The residue was then purified at 575 ºC. After obtaining the values a, b, c, d, and e, we then calculated the presence of hemicellulose, cellulose and lignin using the formulae as follows:

$$\text{Hot } Water\ Soluble\ Content\ (\%wt) = \frac{a-b}{a} x\ 100\% \quad (2.1)$$

$$Hemicellulose(\%wt) = \frac{b-c}{a} x\ 100\% \quad (2.2)$$

$$Cellulose\ (\%wt) = \frac{c-d}{a} x\ 100\% \quad (2.3)$$

$$Lignin\ (\%wt) = \frac{d-e}{a} x\ 100\% \quad (2.4)$$

$$Ash\ (\%wt) = \frac{e}{a} x\ 100\% \quad (2.5)$$

With :

- $a$ : Initial sample dry weight (grams)
- $b$ : Residual dry weight after refluxed in distilled water (grams)
- $c$ : Residual dry weight after refluxed in 0.5 M sulfuric acid (grams)
- $d$ : Residual dry weight after treatment in 72% sulfuric acid, dilution to a concentration of 0.5 M, and then refluxed in 0.5 M sulfuric acid (grams)
- $e$ : Ash weight after it treated in the furnace (gram)

### 2.6. Developing *Phanerochaete chrysosporium* Viability Curves

This Viability Curve was made using the spread plate method [11]. Petri dishes containing PDAs were prepared under 27 sterile conditions. First, we made pure cultures from *P. chrysosporium* as many as 6 test tubes and cultured at the same conditions and times. The spore suspension was then harvested on a daily basis starting from day 2 of the PDA with the addition of 5 mL of harvest solution (0.85% NaCl w/v + Tween80 0.1% v/v) into the test tube. The spores were then harvested by gently rubbing the Ooze stick onto the PDA. The suspension was then homogenized using a vortex and 0.1-0.3 mL of it was taken to count the spores using a hemocytometer. The calculated suspensions were then diluted to a concentration of $10^3$, $10^2$, and $10^1$ spores / mL (dilution with 0.9 mL suspension and 0.1 mL of harvest solution). After being diluted, 0.1 mL of each suspension was then taken to be put into a petri dish and spread evenly throughout the PDA. The same thing was done for days 3, 4, 5, and 6 with 2 repetitions for each day and concentration.

### 2.7. Biological Pretreatment

The first biological pretreatment used two species of fungi, namely soft-rot fungi (*Aspergillus niger*) and white-rot fungi (*Phanerochaete chrysosporium*), to degrade the lignin content in Napier grass. To carry out this pretreatment, it is first necessary to prepare a sample of Napier grass which is aged approximately 2-3 months and the propagated fungal spore as pretreatment materials.

Soft-rot fungi (*Aspergillus niger*) and white-rot fungi (*Phanerochaete chrysosporium*) cultures which have been inoculated on PDA medium in test tubes were then harvested with harvesting solution. The harvesting solution was made by mixing 0.85 grams of NaCl dissolved in 100 mL of distilled water (physiological solution of 0.85% NaCl). Then 0.1 mL of Tween80 (surfactant) was added and stirred until the solution became homogeneous. The homogenous solution was then put into the Erlenmeyer and covered with aluminum foil. The harvest solution was sterilized in an autoclave at 121 ºC (1.5 atm) for 15 minutes. *Aspergillus niger* would be harvested at its maximum growth time, which is four days when grown on the tilted PDA medium [12]. Likewise, *Phanerochaete chrysosporium* would be harvested in 5 days according to its maximum growth time.

Harvested fungal spores (5 mL of solution) were then counted by the number of fungal spore cells using a hemocytometer. Fungal spore concentrations were calculated and adjusted according to variations in fungal concentrations in **Table 1**.

Pretreatment time was determined on the basis of Ayed et al's (2013) study, which states that the greatest acquisition of LiP (Lignin Peroxydase) enzymes is in the pretreatment for 72 hours or 3 days, and according to Valencia and Meitiniarti (2017), in which the ability of *Aspergillus niger* biodelignification will continue to increase until the 7th day, which shows that research is needed for the 9th day as well [13] [15]. Furthermore, Napier grass which had been cut into pieces with a size of about 2 mm was sterilized by pasteurizing the 70 ºC (Low Heat Temperature) method for 3 times on manual heating, each for 15 minutes. Then, as much as 4 grams of Napier grass was put into a Rox bottle and additional nutrient solution was added until the Napier grass had an MC of 80% or with a nutritional ratio of 3.5 gram : 1 mL [16]. The Rox bottle containing Napier grass was inoculated with a ratio of 2 mL inoculum to 10 grams of Napier grass [17]. Additional nutrients given to Napier grass are listed in **Table 2**.

The Rox bottle was then put into the oven at 35 ºC and left for the treatment time [13]. During the treatment, the substrate was dried before it was analyzed for lignocellulose content using the Chesson data method. The pretreated grass was then rinsed with 1:50 of water between the acetate buffer (0.2 M, pH 4.5) and the grass then stirred at 130 rpm for 30 minutes to dissolve the reducing sugar found in the grass by



https://doi.org/10.5614/3bio.2020.2.1.5

soft-rot fungi (*Aspergillus niger*) [18]. Each pretreatment was done 2 times to get the best results with the smallest margin of error

### *2.8. Chemical Pretreatment*

The second pretreatment given was using alkali ($Ca(OH)_2$). To carry out the chemical pretreatment, it is first necessary to prepare a sample of Napier grass aged approximately 2-3 months and also pretreatment materials that have been dissolved in accordance with certain variations in concentration. The variations used in this experiment are shown in **Table 3**.

**Table 1** Time variation for pretreatment in Napier grass [13][14]

| Type Treatments | Total Cell (cell/ g substrat) | Time (day) |
|---|---|---|
| JA1 | | 1 |
| JA2 | $10^6$ | 3 |
| JA3 | $10^7$ | 5 |
| JA4 | $10^8$ | 7 |
| JA5 | | 9 |
| JP1 | $10^6$ | 28 |

Information : JA = *Aspergillus niger* JP = *Phanerochaete chrysosporium*

**Table 2** Additional nutrients in pretreatment with fungi on Napier grass [17]

| Nutrient | Weight (gram/L) |
|---|---|
| Yeast Extract | 5 |
| $(NH_4)_2SO_4$ | 1 |
| $KH_2PO_4$ | 0.5 |
| $K_2HPO_4$ | 0.5 |
| $MgSO_4$ | 0.2 |

**Table 3** Variation of Alkali ($Ca(OH)_2$) pretreatment in Napier grass [19]

| Variation | Component $Ca(OH)_2$ g/g on the substrat | Time |
|---|---|---|
| B1 | 0.02 | 6,24, and 96 hour |
| B2 | 0.1 | |
| B3 | 0.5 | |

Information : B = Alkali.

After the Napier grass powder pertains a constant mass, the powder was then given a treatment using $Ca(OH)_2$ with a variation ratio of the concentration of chemical solvents and Napier grass powder according to **Table 3**. According to Xu et. al, (2008), the ratio between pretreatment solution and Napier grass substrate is 1: 10. This means that for every 1 gram of Napier grass substrate, a 10 mL pretreatment solution is needed [19]. A mixture of grass and water will form slurry or mud-like form. The substrate used in this study was 4 grams, so the required pretreatment solution was 40 mL. Repetition for each treatment was 3 times in order to obtain optimal results.

After the pretreatment was carried out in accordance with the time listed in **Table 3**, the solution and substrate of Napier grass were separated using a filter paper. The filtrate was then collected and continued with the reducing sugar test. The pretreated Napier grass substrate was then washed using 400 mL of distilled water to remove excess lime, reducing sugars, and by-products that could inhibit the hydrolysis process using enzymes [19]. After that the Napier grass was dried using an oven. After the Napier grass



https://doi.org/10.5614/3bio.2020.2.1.5

reached a constant weight, lignin, hemicellulose, and cellulose levels were analyzed using the Chesson-Datta method.

### *2.9. Sugar Reduction Analysis*

Reducing sugar was analyzed using the DNS (dinitrosalycilic acid) method. Reducing sugar was measured on a spectrophotometer at a wavelength of 575 nm [20]. Reduction sugar analysis was carried out before and after the pretreatment to calculate the reduction of sugar contained in the treatment mixture.

The method for analyzing reducing sugars is as follows. First, 3 mL of the sample solution was put into a test tube and mixed with 3 mL of reagent DNS. Then the solution was closed and heated at a temperature of 90 ºC for approximately 5-15 minutes. The heated solution was added with 1 mL of Rochelle salt with a concentration of 40%. The solution was immediately cooled to room temperature and absorbance measured using a spectrophotometer at a wavelength of 575 nm.

## 3. Results and Discussion

### *3.1. The kinetics of the growth of the Phanerochaete chrysosporium*

In this study, the kinetics of fungal growth was analyzed using *Phanerochaete chrysosporium* as a biological agent in biological pretreatment. There are many methods that can be used in experiments on the growth kinetics of an organism, such as the gravimetric, the counting chamber, the spectrophotometric method, and so on. The method used in this study is the Total Plate Count (TPC) method. TPC method is the most widely used method in this kind of analysis, because the colony can be seen directly with the eye without using a microscope. Another advantage of the spread plate method is that the grown fungi / bacteria can be spread evenly on the surface of the agar and this facilitates the calculation of the number of mold spores [21].

Based on data processed using the spread plate method, a fungi growth curve can be made by creating a viability curve between percent viability (%) of time and growth curve using a hemocytometer between cell counts / mL x $10^4$ against time. Through these two curves, the time at which maximum growth occurs can be calculated. The two curves can be seen in **Figure 1** and **Figure 2**.

In **Figure 1**, it can be seen that the viability curve increases on days 2 and 3, while decreasing on day 4. This can be caused by the condition whereby the *Phanerochaete chrysosporium* culture on day 4 has a different treatment from the condition of the fungi culture on day 2, 3, 5, and 6. Differences in these conditions include initial culture conditions, PDA medium, and temperature of TPC testing which can cause a decreased growth. On the 5th day, the percentage of viability was obtained at the maximum value of 93.3%. This is supported by the growth curve in **Figure 2**, which produces the best growth on day 5.

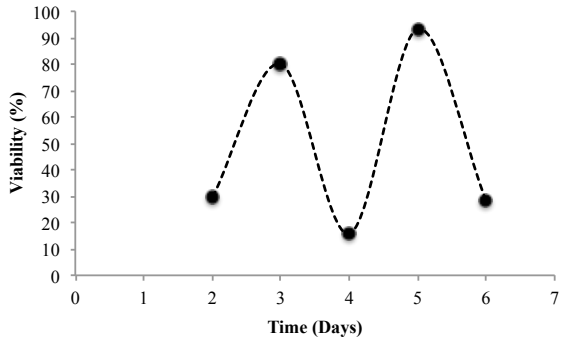


**Figure 1** Viability (%) *P. chrysosporium*

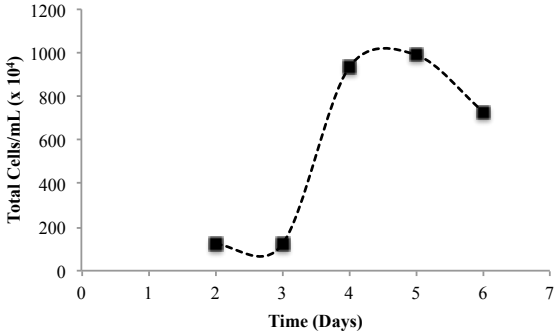


**Figure 2** Growth Curve of *P. chrysosporium*

Furthermore, on the growth curve, it can be seen that on days 2 and 3, the growth of the *Phanerochaete chrysosporium* is still in the lag phase. This is in accordance with the study done by Yao and Nokes (2014) which states that in days 2-3, *Phanerochaete chrysosporium* is still in its lag phase of growth [22]. In the lag phase, the *Phanerochaete chrysosporium* is still adapting to its environment. It was further explained that the fungi enter an exponential phase on days 4-5. Here, a phase of slowing growth occurs.

In the deceleration phase, the fungi begin to run out of nutrients which causes the growth of biomass begins to decrease. In this study, the maximum growth was obtained on the 5th day, but it was different from the literature wherein the fungi continue to experience growth until the 11th day. This could be due to several factors such as the source of nutrition, temperature, and the strain of fungi used [23]. In our study, the medium used is slanted agar containing PDA medium. On the 5th to the 6$^{th}$ day, the fungi enter the death phase, where there is recorded decrease in cell count.



https://doi.org/10.5614/3bio.2020.2.1.5

From the two curves, the same results showed that the maximum growth of *Phanerochaete chrysosporium* was on the 5th day after inoculation. Fungal spore growth on PDAs was carried out at 37 ºC because it is the optimal temperature for the growth phase of *Phanerochaete chrysosporium* mycelium [24].

### *3.2. Effect of Variations in Alkali pretreatment on Lignocellulose Levels*

The pretreatment process aims to break the lignin bond (delignification), in removing the lignin and some of the hemicellulose contents, damaging the crystalline structure of cellulose and increasing the porosity of the material [25]. The principle of lime ($Ca(OH)_2$) pretreatment is to degrade amorphous substances (such as lignin and hemicellulose) which makes the crystallinity increases. Lime ($Ca(OH)_2$) increases lignin degradation which makes enzymes work effectively by eliminating non-productive parts of the adoption and also increasing access to cellulose and hemicellulose [26]. Napier grass consists (wt%) of three main ingredients, namely hemicellulose at 37.5% cellulose, 26.5% hemicellulose and 14.9% lignin depending on the type and component of nutrients obtained [27].

From this study, measurements of lignocellulose component levels were carried out in the control and also in three variations of the concentration of lime pretreatment. All pretreatments in this study were carried out at the laboratory room temperature at Jatinangor, which ranged from 23 to 25 ºC and humidity ranging between 61 and 63%. The lignocellulose fraction in Napier grass can be seen in **Table 4**.

**Table 4** Lignocellulose fraction of Napier grass (*Pennisetum purpureum* Scumach.)

| Component | Content (%) |
|---|---|
| HWS | 27.96 |
| Hemicellulose | 20.01 |
| Cellulose | 33.10 |
| Lignin | 17.82 |
| Ash | 1.12 |

The measurement of lignocellulose component levels was carried out using the Chesson-Datta method. The following is a display of lignocellulosic data resulting from pretreatment with a variation of lime concentration in **Figure 3**.

According to Mosier et al. (2005), pretreatment with lime ($Ca(OH)_2$) can impact the chemical composition of lignocellulose and change the chemical / physical structure of lignocellulose [6]. Lime pretreatment is proven to have a huge impact in increasing the area of cellulose that can be accessed by enzymes. In addition, lime pretreatment can also degrade lignin and destroy the hard lignin structure. However, lime pretreatment only affects slightly on the degradation of hemicellulose [6].

Delignification carried out by the lime pretreatment process in Napier grass samples in this experiment reduced some components of HWS, lignocellulose, cellulose, and lignin, as shown in **Figure 3**. The best delignification conditions can be achieved if the results show low lignin values and high cellulose values, or a low ratio of lignin to cellulose. The factors which also affect the value of lignin and cellulose are lignin degradation and the recovery of solids and cellulose. This needs to be taken into account [28].

The top priority of the lignocellulose component is lignin. Alkali pretreatment can cause swelling of biomass, which can make enzymes or other biocatalyst agents to access biomass more easily [29]. Alkali pretreatment allows breaking of the lignin bonds and changing the crystalline structure of cellulose.

In **Figure 3**, it can be clearly seen that the control sample has a high lignin level of 17.82 wt% and cellulose content of 38%. All lime pretreatments with concentrations of 0.5, 0.1 and 0.02 on average reduce the levels of lignin and HWS. This is due to the nature of lime that can degrade lignin by destroying its structure within a relatively high pH with concentrations of 0.5 g/g, 0.1 g/g, and 0.02 g/g are 12.3 wt%, 12.8 wt%, and 13.2 wt%, respectively [30]. This is also true to the hemicellulose content obtained in this study, where the result only differs slightly from the control. This is in accordance with Mosier, et al. (2005) who asserts that the impact of lime pretreatment on hemicellulose only slightly degrades hemicellulose [6].

In **Figure 3**, it is also seen that the smallest lignin content is found in lime pretreatment with a concentration of 0.5 g / g substrate in 96-hour time with 7.4% lignin level. However, the level of cellulose obtained was only 25% and relatively small when compared with controls. The largest cellulose content was found in lime pretreatment and proves to produce the largest lignin reduction of 58.47%.

It will be difficult to determine pretreatment with maximum concentration and time when viewed from each aspect of lignocellulose. This is because the results are tightly close and hard to compare. As an alternative, the difference can be seen by reviewing the aspect of lignin to cellulose ratio, considering that the aim is to obtain as lowest lignin value and highest cellulose value as possible. **Figure 4** shows the ratio between lignin and cellulose in each treatment.

**Figure 4** shows that the concentration of lime pretreatment with the highest cellulose content and lowest lignin content was 0.1 gram / gram of Napier grass in 24 hours, with a ratio of 24.56%. These results differ only slightly with the concentration of 0.02 gram / gram of Napier grass in 96 hours, as well as with a concentration of 0.5 gram



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/ gram of Napier grass in 6 hours, with ratios of 24.67% and 26.79%, respectively. This is also supported by the results of the reducing sugar contained in the pretreatment hydrolyzate in **Figure 5**.

The results shown in **Figure 5** also demonstrate that the concentration of lime pretreatment with the highest reducing sugar content is 0.1 gram / gram of Napier grass within 24 hours of treatment. These results were obtained in accordance with the results of lignin degradation with lime on switchgrass, with the best concentration of 0.10 g/gram of biomass for 24 hours at 50 ºC [19].

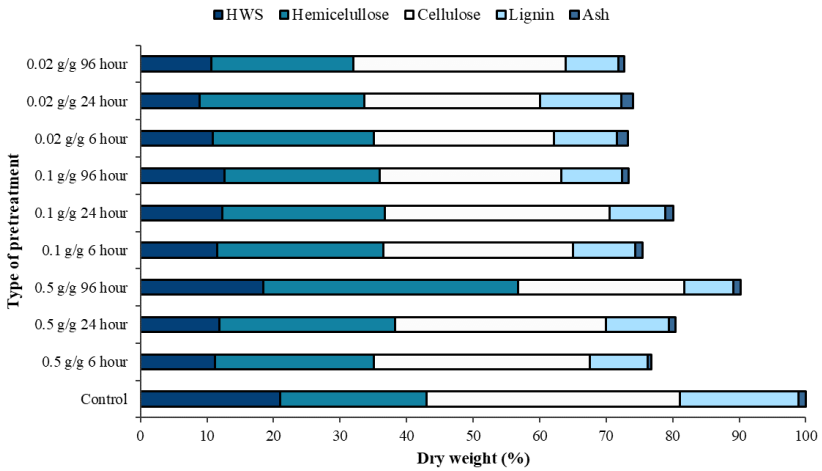


**Figure 3** Lignocellulose fraction in lime ($Ca(OH)_2$) pretreatment

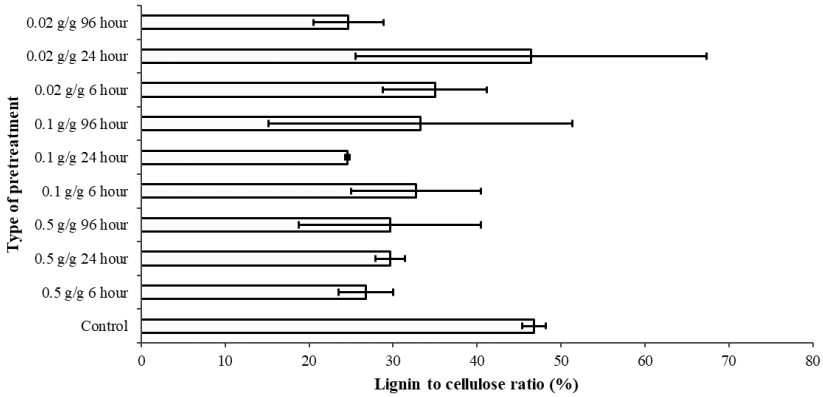


**Figure 4** Lignin to Cellulose Ratio on the lime pretreatment result'

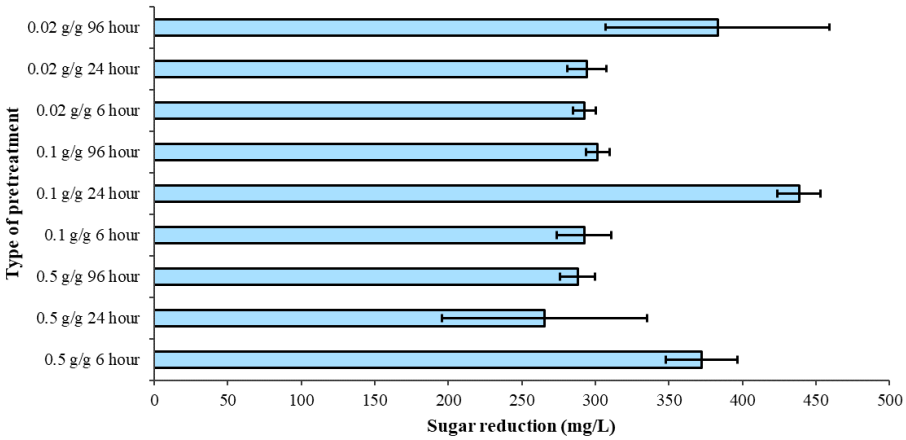


**Figure 5** Reducing Sugar Level (mg/L) on hydrolyzate from the lime pretreatment



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### *3.3. Effect of variations in concentration and time of biological pretreatment with Aspergillus niger on levels of lignocellulose*

Biological pretreatment is a preliminary treatment of lignocellulosic biomass to break the hard and crystal-shaped lignin bonds. The process uses biological agents (fungi) that possess enzymes as weapons to break down lignin [31]. Biological pretreatment is an efficient, environmentally friendly, and inexpensive pretreatment [32]. Microorganisms such as brown- and white-rot fungi are commonly used to degrade lignin and hemicellulose in waste biomass such as agricultural waste [33]. On the other hand, soft-rot fungi, which are fungi of the ascomycetes class, has a very strong property of degrading carbohydrates in wood, and some of these fungi can show a significant ability in breaking down the structure of lignin [34]. However, lignin degradation by soft-rot fungi is still little studied.

In general, the enzymes produced by these fungi are one of the three main enzymes that degrade lignin, namely LiP, MnP, and Laccase [35]. **Figure 6** to **13** illustrates lignocellulosic component data ranging from HWS, hemicellulose, cellulose, lignin and ash resulting from pretreatment with *Aspergillus niger* with varying fungal concentrations and pretreatment times.

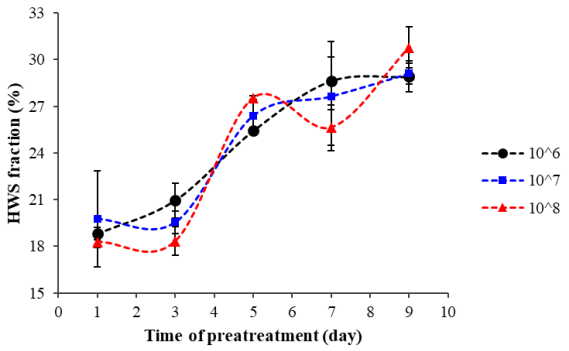


**Figure 6** HWS Fraction at each fungi concentration

Data on the treatment of *Aspergillus niger* in HWS fraction was analysed using the Duncan's Multiple Range Test ($p < 0.05$), and showed a significant increase between day 1 and day 9. This could be due to *Aspergillus niger* proven to be able to produce cellulose- and hemicellulose-breaking enzymes, one of which is xylanase [36] [37]. The enzyme can break down cellulose and hemicellulose into simple sugars that can be dissolved in the HWS fraction, thus causing the value of the HWS fraction to increase. HWS consists of several simple components that dissolve easily in water, such as some simple carbohydrates, proteins, and inorganic compounds [4].

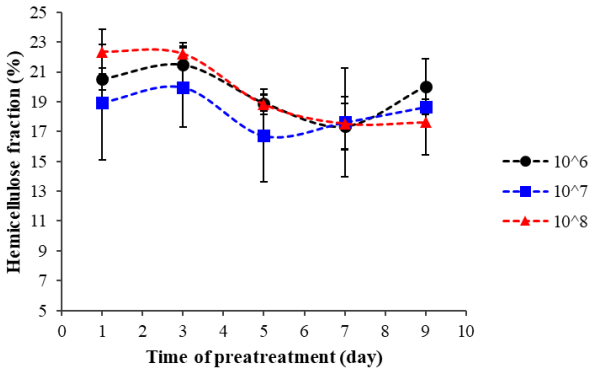


**Figure 7** Hemicellulose fraction at each fungi concentration

When viewed from the hemicellulose fraction, the results show that there was a significant decrease between day 1 and day 9. This can be explained due to the ability of the *Aspergillus niger* to break down hemicellulose into simple sugars (pentose (xylose and arabinose), hexose (manose, glucose, and galactose), and sugar acids [38], which will be used as a source of C in its growth [39].

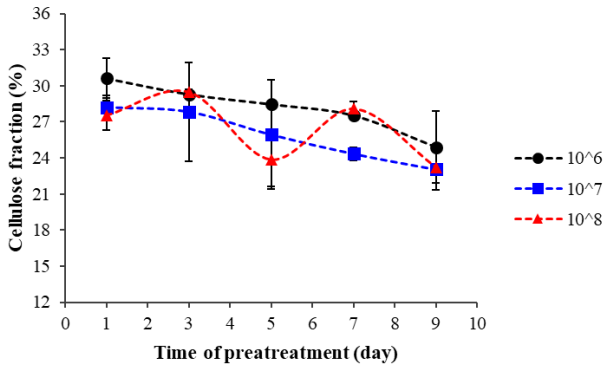


**Figure 8** Cellulose fraction at each fungi concentration

The same holds true with the cellulose fraction, where a significant decrease was recorded on day 1 and day 9. This is also due to the *Aspergillus niger* having the ability to convert the cellulose fraction into simple sugars (D-glucose) using cellulase enzymes such as xylanase [36]. This is what causes cellulose fraction content to decrease from day 1 to day 9. In the pretreatment process, the cellulose fraction is expected not to experience a reduction because if cellulose is reduced in the pretreatment process, the saccharification process in the fermentation will not run optimally [25].

Data on lignin fraction that have been analysed using Duncan's Multiple Range Test ($p < 0.05$) showed that a significant decrease from day 1 to day 9 occurred. Lignin fraction itself is the main parameter in the (chemical and biological) pretreatment process. Some journal articles state that lignin levels have a considerable influence on the acquisition of ethanol from pretreated biomass [40].



DOI: 10.5614.3bio.2020.2.2.2

Although lignin is the main parameter, many other factors such as lignin composition, chemical structure of lignin, as well as complex bonds between lignin and carbohydrates in biomass can have an important impact on biomass digestibility. Although lignin is the hardest component in biomass, it can be concluded that reducing lignin levels can improve the ability to access substrate in bioethanol production [41].

It should be noted that the main purpose of pretreatment is to obtain a low ratio of lignin to cellulose. **Figure 10** illustrates the ratio between lignin and cellulose in each fungal treatment.

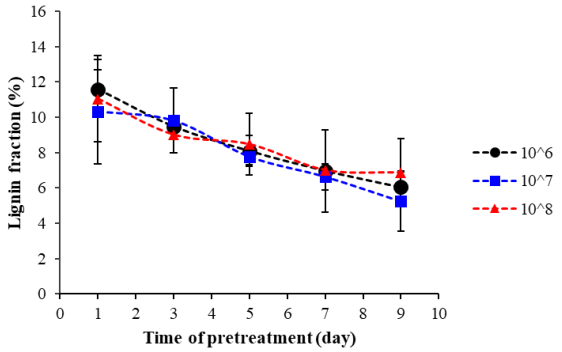


**Figure 9** Lignin fraction at each fungi concentration

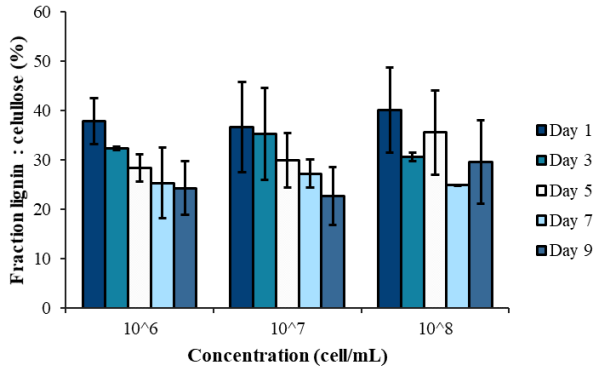


**Figure 10** Lignin : Cellulose ratio in pretreatment with *Aspergillus niger*

By analyzing the data using Duncan's Multiple Range Test ($p < 0.05$) on the ratio of lignin to cellulose, it was shown that the smallest value was found on the 9th day at a concentration of $10^7$. In this test, the smaller the value of the ratio of lignin to cellulose, the smaller the lignin level is compared to the cellulose fraction, and the more effective a pretreatment is. This means that the best pretreatment using *Aspergillus niger* in this study was 9 days pretreatment using $10^7$ concentrations. This is supported by observations of *Aspergillus niger* growth at $10^7$ concentrations that are more evenly distributed throughout the surface of Napier grass. At concentrations of $10^6$ and $10^8$, fungi growth was concentrated only at a few points of Napier grass. **Figure 11** documents the results of the pretreatment with the *Aspergillus niger* on day 9.

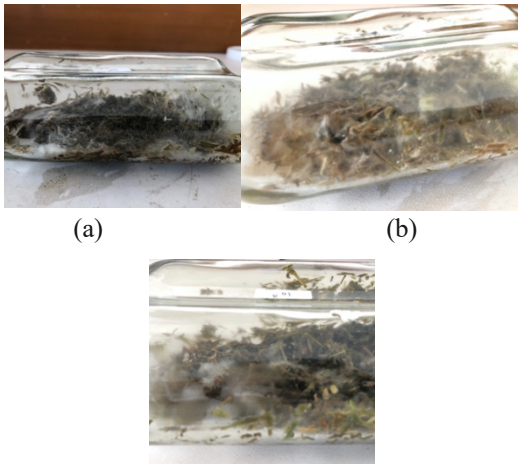


**Figure 11** Pretreatment with *Aspergillus niger* spore at concentrations (a)$10^6$, (b)$10^7$, (c)$10^8$ cells/mL and day 9

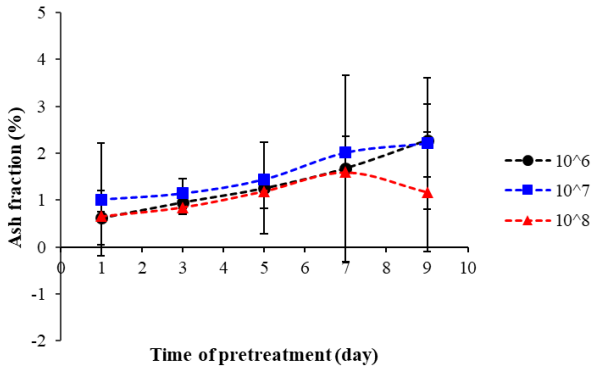


**Figure 12** Ash fraction at each fungi concentration

The results of data analysis using Duncan's Multiple Range Test ($p < 0.05$) in the ash fraction showed a significant increase on day 1 and day 9. The ash fraction showed the presence of inorganic compounds in Napier grass cells. Inorganic compounds such as minerals (calcium, magnesium, phosphorus, chlorine, sodium, and sulfur) are present in several parts of the cell wall [42]. If the cell wall component is broken down / extracted, the inorganic compounds such as minerals will come out. Therefore, in this study the ash fraction content tends to increase due to the *Aspergillus niger* which damages cell walls and makes mineral levels increase from day 1 to day 9.

Reducing sugar is a sugar (carbohydrate) class that has the ability to reduce electron-accepting compounds [43]. Examples of sugars that include reducing sugars are glucose, mannose, fructose, lactose, maltose, and others. Generally, the reducing sugars produced are closely related to enzyme



https://doi.org/10.5614/3bio.2020.2.1.5

activity, where the higher the enzyme activity, the higher the reducing sugars produced [44]. With the results of data analysis using the Duncan's Multiple Range Test ($p < 0.05$) on the reducing sugar fraction, we can see that there is an increase in reducing sugar levels from day 1 and day 9. This is because the *Aspergillus niger* can produce enzymes to break down the cellulose and hemicellulose components, one of which is xylanase into simple sugars [36] [37].

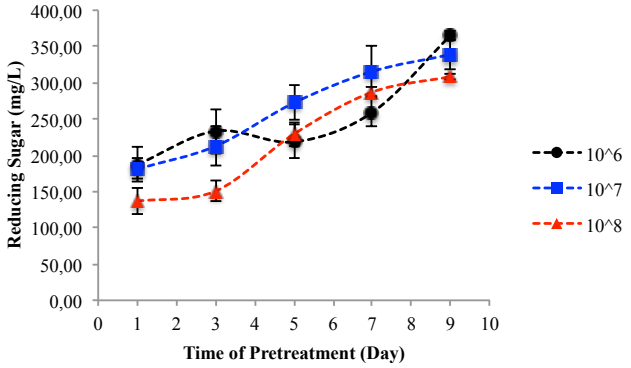


**Figure 13** Reducing Sugar Levels at each fungi concentration

### 3.4. *Biological Pretreatment Comparison between Phanerochaete chrysosporium and Aspergillus niger*

This study uses two species of fungi from different classes, namely white rot fungi and soft rot fungi. The *Phanerochaete chrysosporium* has become a model of organism from which we can learn the extent to which fungi are able to degrade lignin [45]. In this sense, *Phanerochaete chrysosporium* is used in this study as a comparison to the main biological agent used, which is *Aspergillus niger*. This study seeks to prove that *Aspergillus niger* has the potential to be used as a biodelignification agent.

In this study, the treatment of *Phanerochaete chrysosporium* was carried out under the same conditions as that of *Aspergillus niger* and used at optimal concentration, temperature, and moisture content [14]. **Table 5** shows a comparison of lignocellulosic components along with reducing sugars between *Aspergillus niger* and *Phanerochaete chrysosporium* under the same conditions at 35 °C, with concentrations of $10^6$ cells / mL and the same medium.

**Table 5** Comparison of lignocellulosic component and reducing sugar between *Aspergillus niger* and *Phanerochaete chrysosporium*

| Component | *Aspergillus niger* | *Phanerochaete chrysosporium* |
|---|---|---|
| Time of pretreatment (day) | 9 | 28 [14] |
| HWS (%) | 28.904 ± 0.997 | 28.081 ±0.899 |
| Hemicellulose (%) | 20.022 ± 1.867 | 12.150 ± 0.631 |
| Cellulose(%) | 24.880 ± 2.991 | 10.380 ± 0.976 |
| Lignin (%) | 6.044 ± 0.905 | 3.181 ± 0.222 |
| Ash (%) | 2.275 ± 0.778 | 0.407 ± 0.331 |
| Reducing sugar (mg/L) | 365.43 ± 5.534 | 325.45 ± 7.566 |

When viewed from hemicellulose and cellulose content, pretreatment using *Aspergillus niger* is superior to *Phanerochaete chrysosporium* because the former relies on a wide spectrum of sugar sources. Likewise, the reducing sugar produced by the *Aspergillus niger* is superior to that of *Phanerochaete chrysosporium*. This is because the optimal pretreatment time of the *Phanerochaete chrysosporium* is longer than the *Aspergillus niger,* which causes greater cellulose consumption compared to the latter. The rate of sugar consumption possessed by both fungi is relatively the same [46].

As for the lignin parameter, when the level of lignin is compared with the amount of cellulose, *Aspergillus niger* is superior to *Phanerochaete chrysosporium*, with the percentage of lignin to cellulose to be 24.3% and 30.645%, respectively. This value indicates that *Aspergillus niger* is able to process more cellulose as raw material for producing ethanol in the saccharification and fermentation process.

### 3.5. *Comparison between Biological and Alkali Pretreatment*

In this study, two types of pretreatment are compared on the basis of optimal time and concentration. The parameters used to compare the effectiveness of pretreatment are lignocellulosic components which consist of HWS,



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Hemicellulose, Cellulose, Lignin, Ash, and Reducing Sugar fractions. Comparison between Biological pretreatment using the *Aspergillus niger* and Alkali pretreatment using lime ($Ca(OH)_2$) is shown in Table 6.

When viewed from the components of hemicellulose, cellulose, and reducing sugars, Alkali pretreatment is superior to Biological pretreatment. This is because Alkali pretreatment can strongly affect lignin degradation but only slightly degrades hemicellulose, cellulose, and other sugars [6]. As explained by Liong et al. (2012), biological pretreatment is definitely lower than alkali pretreatment when it comes to glucose acquisition [14]. In short, the reason is that fungi need a longer time to consume lignin.

However, because the main parameter of this study was the ratio of lignin to cellulose, the biological pretreatment using *Aspergillus niger* was superior compared to the Alkali pretreatment, with a ratio of 22.7% and 24.5%, respectively. This can be caused by three things. First, the enzyme produced by *Aspergillus niger* will achieve maximum activity on day 7, therefore on days 7 to 9, *Aspergillus niger* is in maximum condition to produce cellulase, hemicellulase, or delignification enzymes [47]. The second reason is Alkali's ability to degrade lignin. In the reaction between alkali and lignocellulose, the main goal is degradation of lignin, but bases can also degrade cellulose and hemicellulose. This can reduce the ability of alkalis to degrade lignin [48]. Furthermore, because the lignin fraction has a reactive group, the condensation reaction between lignin components can inhibit the ability of alkali in delignification. Lastly, in the alkali pretreatment of lignin, conjugated acids can form. When acid is formed, it can cause the consumption of alkaline components and lower the pH value [48].

pH testing was also carried out on lime ($Ca(OH)_2$) pretreatment to determine the formation of conjugated acid. The pH at the optimal alkali pretreatment conditions with lime ($Ca(OH)_2$) in a concentration of 0.1 gram / gram substrate for 24 hours was 7.397 ± 1.060. Meanwhile, in the initial pretreatment conditions, the pH given was around 12.8. It can therefore be concluded that the formation of conjugated acid causes pH to decrease by 5.403. The lime ($Ca(OH)_2$) pretreatment does not run optimally under these conditions.

## 4. Conclusions

From our study, we can see that the most optimal biological pretreatment time using *Aspergillus niger* is 9 days with the most optimal fungal spore concentration to degrade lignin is 107 cells/mL. Comparison between pretreatment with *Aspergillus niger* and *Phanerochaete chrysosporium* towards better delignification ability in optimal conditions shows that the former could give better results, using hemicellulose, cellulose and reducing sugar concentration parameters. In alkali pretreatment using lime (Ca(OH)2), the results show that the most optimal time and concentration of pretreatment in degraded lignin is 0.1 gram / gram substrate with a pretreatment time of 24 hours (1 day). Comparison between Alkali pretreatment using (Ca(OH)2) and Biological pretreatment using *Aspergillus niger* show that the latter was better, seen using the main parameter of ratio between lignin and cellulose content.

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