Production of Bioethanol from Napier grass: Comparison in Pre-treatment and Fermentation Methods

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Abstract. Bioethanol as alternative renewable energy can be derived from lignocellulosic biomass. One of the potential biomass that can be used for bioethanol is Napier grass (Pennisetum purpureum Schumach). Napier grass has a high content of cellulose which can be utilized as a substrate for fermentation process. A better understanding of bioconversion of Napier grass into bioethanol is essential for enhancing its performance. In this study, three biological agents were involved, Aspergillus niger and Phanerochaete chrysosporium for delignification and Neurospora sitophila for cellulase and bioethanol production in solid-state and submerged fermentation. Alkaline pretreatment using Ca(OH)₂ and NaOH were conducted in this study. The result showed biological pretreatment using Aspergillus niger gave a higher delignification yield compared to alkaline pretreatment. For cellulase production, solid-state fermentation resulted in a higher enzyme activity compared to submerged fermentation and for bioethanol production, submerged fermentation yielded higher bioethanol compared to solid-state fermentation system but the difference was not too significant.

1. Introduction
Napier grass (Pennisetum purpureum) is known to contain high sugar level, high cellulose content and low lignin content (8-21%) [1]. Napier grass also known to have efficient photosynthetic activity and water consumption which makes this grass easy to be cultivated[2]. Bioconversion of lignocellulosic Napier grass into bioethanol requires several steps including pretreatment to remove lignin and increase cellulose content, cellulose hydrolysis into simple sugars[3,4] and bio-utilization of these sugars by appropriate microorganisms.

A pretreatment to reduce lignocellulosic material is needed in order to obtain maximum product. This pretreatment method includes physical, chemical, biological, or the combination of some of these methods. The most common pretreatment method is the physical comminution; which includes chipping, grinding, and/or milling can be applied to reduce lignin and cellulose crystallinity[4]. Among the available pretreatment methods, biological pretreatment is considered as the most economically favourable, environmentally friendly, without high corrosive chemicals requirement, lesser waste stream generated and almost no fermentation process inhibitor produced [5]. Biological pretreatments to degrade lignin, cellulose and hemicellulose in planty materials usually using microorganisms or its enzymes such as brown rots fungi which mainly attack cellulose compound of the cell wall, whereas white and soft rots degrade both the cellulose and lignin compound [6]. Phanerochaete chrysosporium is widely used as a model organism for studying lignin degradation process by white-rot fungi [7]. However, pretreatment using microorganism could cost carbohydrate loss due to the consumption of simple sugars by the fungi. The hydrolysis rate is also relatively low...
compared to physical and chemical pretreatments, which makes the biological method is industrially unattractive. Nevertheless, biological pretreatment has some advantages because of it requires a low amount of energy and also less expensive compared to the other pretreatments.

The integration of saccharification and fermentation process into one single process is called simultaneous saccharification and fermentation. This technique is used to improve ethanol yield. This process is based on the use of an enzymatic complex to hydrolyze cellulose and convert the simple sugar into various products[8]. This process technology has been developed in recent years because of its potential to reduce investment cost, energy-saving capability, and higher ethanol production productivity [9]. N.sitophila is classified as a mesophilic mould which has the ability to produce hydrolytic enzymes and conducting fermentation process[10].

Simultaneous saccharification and fermentation usually conducted in submerged fermentation (SmF). This process is conducted by specific microorganism in a closed container. The advantage of this technique is that the purification of the products is much easier compared to other techniques [11]. The utilization of solid-state fermentation (SSF) has another advantage since its produce higher enzyme activity [12].

Both SSF and SmF have been widely used at laboratory scale; some techniques yielded better results than others. Further research needs to be done to identify the best technique to optimize the cellulase and bioethanol production from Napier grass using N. sitophila. The present research aims to determine the best pre-treatment options for napier grass and to compare the solid-state fermentation and submerged fermentation for cellulase and bioethanol production using N. sitophila.

2. Materials and Methods

2.1. Microorganism
There are three microorganisms used in this study: Neurospora sitophila, Aspergillus niger, and Phanerochaete chrysosporium. Stock cultures for all microorganisms were obtained from the Microbiology Laboratory Culture Collection, School of Life Science and Technology, Bandung Institute of Technology. For SmF system, N.sitophila spore in the Potato Dextrose Agar (PDA) slant was harvested using sterile 0.1% tween 80 solutions and transferred into Potato Dextrose Broth (PDB) for pellet formation. This inoculum was cultivated for three days on a benchtop orbital shaker at 120 rpm at room temperature. The fungal pellet formed was used as the inoculum for cellulase and bioethanol production with SmF system. For the SSF system, the spore was kept on the PDA slant. A. niger was harvested at the maximum viable growth which is on the 4th days after the inoculation [13]. For P. chrysosporium, the maximum viable growth is on day 5 after inoculation.

2.2. Napier grass
Harvested napier grass (three months old) were washed and dried using shade drying method. Dried Napier grass leaves were milled using a blender and then meshed through 10, 35, and 60 mesh number. For the alkaline and biological pretreatment, Napier grass used was then milled and retained on the mesh number 10 and 35 and for the fermentation process, Napier grass used was the one that retained on the mesh number 35 and 60.

2.3. Neurospora minimal medium
The growth medium for Neurospora under SmF and SSF process was the Neurospora minimal medium. This medium composition is as listed in the National Collection of Industrial Microorganisms (NCIM) as optimum for initial revival or subculture of each strain [14]. For SSF, the use of biotin was changed with SCR and the medium was supplemented with Yeast Extract (6.4% v/v).
2.4. Biological and alkaline pretreatment of Napier grass

The pretreatment condition conducted in this study can be seen in Table 1. The biological pretreatments (using A. niger and P. chrysosporium) were conducted in the SSF system and the pretreatment concentration was chosen based on preliminary research.

| Pretreatments | Concentration | Time Period |
|---------------|---------------|-------------|
| Ca(OH)₂       | 0.01 g/g substrate (12.5 g/L) | 24 h |
| NaOH          | 15 g/L        | 120 h       |
| A. niger      | 10⁷ cells/g substrate | 9 days |
| P. chrysosporium | 10⁶ cells/g substrate | 28 days |

Sterilization process was conducted using a low temperature pasteurization process. Four grams of sterilized Napier grass was inserted into a roux bottle and an additional nutrient solution was added until the moisture content reached 80%. The nutrient solution used was the medium for fungal cultivation on SSF system [20]. After the inoculation, substrate was incubated in an oven at 35°C for 9 days [15]. The pre-treated Napier grass was washed with acetate buffer (0.2 M, pH 4.5) and agitated at 130 rpm for 30 minutes [16]. This experiment was repeated twice.

The chemical pretreatment was conducted using Ca(OH)₂ and NaOH solution. The variation of Ca(OH)₂ and NaOH concentration used in this research are listed on Table 1. This experiment was repeated 3 times. After the chemical pretreatment was done, the sample was being washed using demineralized water. The filtrate was tested for its reducing sugar content and the residue was tested for its lignocellulosic content.

2.5. Enzyme and ethanol production: SmF system

Simultaneous saccharification and fermentation on SmF system was carried out in a 250 ml Erlenmeyer flask. One gram of pretreated napier grass was put into the flask. This substrate was mixed with 90 ml of Neurospora minimal medium (NMM). The mixture was sterilized with the low-temperature pasteurization process. Ten ml of PDB broth containing Neurospora pellet suspension was put into the flask and pH was adjusted to 6.8-7.0. The culture was shaken at 130 rpm and incubated at room temperature for 72 h in anaerobic condition. After 72 hours of cultivation, the oxygen supply was stopped. The culture was harvested periodically every 24 hours for 120 hours for fungal growth analysis, substrate residual concentration, enzyme activity, and end-product analysis. Each flask represents one experimental point.

2.6. Enzyme and bioethanol production: solid-state fermentation system

Fermentation process on a solid substrate was done in two steps as described by Rao et al.[17] in 1983 and Dogaris et al. in 2009 [10]. The moisture content of the substrate was adjusted using glucose (5 g/L), yeast extract (6.4 g/L), and NMM. The fermentation medium was added until the moisture content of the substrate reached 70-80%. One ml of heavy spore suspension of N. sitophila was inoculated into the substrate. The cellulase enzyme activity was analyzed every day for six days. After two days, the cultivation condition was switched into a microaerobic condition with the addition of oxygen absorber and the cultivation was continued for another 6 days. Enzyme extraction was proceeded according to Dogaris et al. [10]

| Variation | Time Periods |
|-----------|--------------|
| SmF       | 30 g/L       | 48 hours aerobic and switched to an anaerobic condition for another 72 hours |
| SSF       | -            | Six days of aerobic condition and switched to an anaerobic condition for another 6 days |
2.7 Analytical Method

2.7.1 Fungal spore viability analysis. Fungal spore viability analysis was conducted using Total Plate Count method as described by Marturin and Peeler [18] and viability calculation as described by Scott [19].

2.7.2 Lignocellulosic and fermentation product analysis. Napier grass lignocellulosic compound before and after pretreatment and fermentation process were analyzed using Chesson-Datta method [20]. SmF products were analyzed using high-performance liquid chromatography (HPLC) equipped with an ion moderated partition chromatography column, Aminex HPX-87H (Bio-Rad, CA). The HPLC was used in combination with a Waters (2414) refractive index detector. The flow rate of the mobile phase (5mM H2SO4) was adjusted at 0.6 ml/min and the temperature at 60°C. Reducing sugar was determined by Dinitrosalicylic Acid (DNS) method [21]. Protein concentration was measured according to the Bradford method [22]. Cellulase activity was determined according to Ghose [23]. For the SSF system, the analysis of bioethanol produced was conducted using Crowell and Ough Method [24]. Ethanol was extracted from the fermentation substrate using the distillation method as stated by Devrajan [25].

3. Result and Discussion

3.1. Lignocellulosic fraction of Napier grass
Energy content from the 3 months old Napier grass was the highest compared to the older or younger age Napier grass [26]. The comparison of lignocellulosic compounds compared to other studies can be seen in Table 6.

| Studies            | HWS  | Hemicellulose | Cellulose | Lignin | Ash  | Others |
|--------------------|------|---------------|-----------|--------|------|--------|
| This research      | 23.21| 27.50±        | 36.18     | 12.19  | 0.92 ±| -      |
|                    | ±    | ±             | ±         | ±4.93  | 0.35 |        |
|                    | 8.47 | 5.92          | 5.40      |        |      |        |
| Takara., 2015      | 14.3±| 19.2±1.2      | 38.5±     | 17.9±  | 8.8±3.3| -      |
| Montipo et al., 2018 | 16.43| 20.62         | 33.60     | 18.42  | 12.25 | 0.99   |
| Sladen et al., 1991| -    | 20-31         | 30-37     | 8-21   | -    | -      |

Lignocellulosic content in napier grass showed some variations in percentage compared to other studies (Table 6). This variation is probably caused by the difference in harvesting age, nutrition given, and the cultivation location of Napier grass [29,30]. Harvesting age is an essential variable that can affect the variation of lignin content. As the grass grows older, the lignin content supposed to be increased. Napier grass in this study was three months old, while in Takara study was four months old, therefore its lignin content was less. Lignin content was increased as the grass grows older, although lignin content might also be influenced by the environmental [31,32]. Hemicellulose and cellulose content showed relatively similar to other studies. Apparently, hemicellulose and cellulose content could be varied over time but the total carbohydrate content remains relatively constant [27]. The benefit of having a high content of hemicellulose and cellulose with low percentage of lignin ash make Napier grass an ideal source for bioethanol production.
3.2 Comparison of pretreatment methods of Napier grass
Alkaline delignification conducted on this research exhibited a reducing effect on some lignocellulosic components. The optimum condition would be achieved if the lignin content was reduced while cellulose content was enhanced or stabilized. The most efficient pretreatment agents was sodium hydroxide (NaOH) solution (Fig 1) which showed the lowest lignin: cellulose ratio (11%), while the biological pretreatment using *P. chrysosporium* showed the highest lignin : cellulose ratio (31%). A low lignin: cellulose ratio indicates that the pretreatment was efficient [33]. Beside lignin: cellulose ratio, there are number of considerations when selecting the most suitable pretreatment methods including duration, price of pretreatment agent and its recovery process.

From the duration of the pretreatment, the Ca(OH)$_2$ pretreatment gave a higher result compared to the biological one. Biological pretreatment needs a longer time to complete because as a living organism, pretreatment agents need some time to grow and adapt to the new substrate. For example in this research, it was shown that for a similar lignin to cellulose ratio, Ca(OH)$_2$ pretreatment only took 24 hours to complete while the biological pretreatment using *A.niger* took 9 days to complete.

![Lignin to cellulose ratio of pretreated Napier grass](image)

**Figure 1.** Lignin to cellulose ratio of pretreated Napier grass

Comparing all the pretreatments used in this research, it can be seen that chemical pretreatment gave better results compared to the biological pretreatments. But using biological pretreatments to treat lignocellulosic biomass may have its own perks. Biological pretreatment involves minimal energy input and can minimize the production of inhibitory compounds [34]. Biological pretreatments are also more environmentally safe compared to chemical pretreatment although more detailed research and economic analysis should be conducted to prove its potential to be scaled up.

3.3 Simultaneous saccharification and fermentation: enzyme production by *N. sitophila*
In contrast with SmF system, the SSF system gave a higher cellulose. The highest enzyme activity obtained from SSF system on Napier grass was 0.45 FPU/ml. This result was obtained on the 48 hours after the inoculation (Figure 2). One possible reason why the enzyme activity produced by Neurospora was better when this mold was cultivated with the SSF method is perhaps due to the nature of the fungi itself. The SSF system can maximize its physiological activities relative to the submerged fermentation since it was quite similar with the natural habit of fungi [35]. A study of protein characterization on submerged and solid state fermentation shows that SmF system is most likely to produce a large portion that were related to the fight and stress response while the protein profile for the SSF system was dominated by proteins for growth [36].
3.4. Simultaneous saccharification and fermentation: bioethanol production

The concentration of ethanol produced using SmF method was higher than produced using SSF. The highest ethanol concentration achieved from SmF was about 0.85 g/L while the highest ethanol concentration achieved by solid-state fermentation was only 0.75 g/L (Fig 5). However these two results were lower compared to study by Colvin, which is reported the accumulation of ethanol by Neurospora can reach 4.5 g/L. This was probably due to the culture condition which was still containing oxygen in a high concentration and led to the inhibition of fermentation metabolic pathway [37]. Low ethanol yield indicates that the substrate was not fully converted into ethanol but probably was being used for other cell’s activities [38]. Fermentation products and substrate from SmF system were analysed using HPLC. From the chromatograph, it can be seen that Neurospora also produce a variety of byproducts in fermentation process. Acetic acid is one the byproducts and this also probably were produced in SSF system and lead to lower ethanol yield.
4. Conclusion
Chemical pretreatment gave a higher delignification yield compared to the biological pretreatment. For cellulase production, solid-state fermentation resulted in higher enzyme activity compared to the submerged fermentation system and for ethanol production, the submerged fermentation system yielded a higher ethanol yield compared to solid-state fermentation system but the difference was not too significant.

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