Comparison of Pretreatment Methods on Vetiver Leaves for Efficient Processes of Simultaneous Saccharification and Fermentation by Neurospora sp.

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Abstract. Lignocellulosic biomass is a potential raw material for bioethanol production. Neurospora sp. can be used to convert lignocellulosic biomass into bioethanol because of its ability to perform simultaneous saccharification and fermentation. However, lignin content, degree of polymerization, and crystallinity of cellulose contained in lignocellulosic biomass can inhibit cellulosic-biomass digestion by Neurospora sp, so that a suitable pretreatment method of lignocellulosic biomass is needed. The focus of this research was to investigate the suitable pretreatment method for vetiver leaves (Vetiveria zizanioides L. Nash) used as a raw material producing bioethanol in the process of simultaneous saccharification and fermentation (SSF) by Neurospora sp.. Vetiver plants obtained from Garut are deliberately cultivated to produce essential oils extracted from the roots of this plant. Since the vetiver leaves do not contain oil, some of harvested leaves are usually used for crafts and cattle feed, and the rest are burned. This study intended to look at other potential of vetiver leaves as a source of renewable energy. Pretreatments of the vetiver leaves were conducted using hot water, dilute acid, alkaline & dilute acid, and alkaline peroxide, in which each method was accompanied by thermal treatment. The results showed that the alkaline peroxide treatment is a suitable for vetiver leaves as indicated by the increase of cellulose content up to 65.1%, while the contents of hot water soluble, hemicellulose, lignin, and ash are 8.7%, 18.3%, 6.8%, and 1.1%, respectively. Using this pretreatment method, the vetiver leaves can be converted into bioethanol by SSF process using Neurospora sp. with a concentration of bioethanol of 6.7 g/L operated at room temperature.

Keywords: vetiver leaves, chemical pretreatment, SSF, Neurospora sp.

1. Introduction

Based on the feedstocks of bioethanol production, there are three generations of bioethanol [1, 2], i.e. (a) the first generation, which is produced from sugar plants and starch; (b) the second generation, which is produced from lignocellulosic biomass, such as household waste, wood, and grass; and (c) the third generation, which is produced from (micro)algae. This research focuses on second-generation bioethanol production process because Indonesia has abundant sources of lignocellulosic biomass
derived from agroindustrial waste. Unlike the first generation bioethanol, the feedstock of second-generation bioethanol production process is not used for food, so that this process does not compete with the food industry [3].

One of agroindustrial wastes that is potentially used as a source of lignocellulosic biomass in Indonesia is a waste of vetiver essential oil industry. Indonesia is one of vetiver oil-producing country in the world [4]. Vetiver essential oil obtained from the roots of vetiver grass (Vetiveria zizanioides L. Nash). This essential oil is used in various industries for perfumes, cosmetics, aromatherapy, food and flavouring productions. Vetiver grass is usually planted at the beginning of the rainy season. Harvesting can be done after the plant was 12-16 months. Before the root of vetiver grass can be harvested, the leaves of vetiver grass is usually cut every few months to encourage the growth of seedlings and to reduce the fire hazard in the dry season. Amount of cut vetiver leaves are more than 10 tons/Ha. Some vetiver leaves are used as animal feed (especially young leaves), crafts, and as compost. A large amount of left residues, however, are not used and usually just burned at the edge of the farm field. Therefore, the utilization of lignocellulosic content of vetiver leaves residues into a high value-added chemical such as bioethanol is very important to be studied. The process of conversion of lignocellulosic biomass into ethanol generally consists of four processes, namely, biomass pretreatment, saccharification, fermentation and product separation. Kuhiran and Punnapayak (2000) reported that 13% of bioethanol yield from dry vetiver leaves was obtained after separation process using one-cycle column distillation. They run a series of conversion process from vetiver leaves into bioethanol started from alkali treatment for the leaves. The next processes were the saccharification and fermentation, which were carried out simultaneously. This process is known as a simultaneous saccharification and fermentation (SSF). For the purpose of saccharification to obtain fermentable sugars, they used cellulose enzyme derived from Trichoderma reesei. Simultaneously, they used yeast for the glucose fermentation into bioetanol in the same reactor. The SSF process was carried out for seven days at 40°C and pH 5.0 [5]. The cost and energy used in SSF process is much lower than the conventional process that separates saccharification and fermentation using two different reactors. In addition, the fermentable sugars formed in the SSF process will soon be fermented into ethanol so as to avoid inhibition of the hydrolysis process [6].

Biomass pretreatment plays an important role in the conversion of lignocellulosic biomass into bioethanol. This pretreatment aims to increase the pore size, reducing the content of lignin, and reduce the crystallinity of cellulose. The pretreatment can be performed by using chemical and heat treatment. Typically, acid pretreatment is used to hydrolyze the hemicellulose coating, whereas alkali pretreatment is mainly used to remove lignin. Therefore, pretreatment is highly important to allow cellulase access the cellulose fibers in saccharification process [1]. The pretreatment products can certainly affect the product yield of bioethanol.

This study aimed to investigate the effect of pretreatment method chosen to increase bioethanol yield from vetiver leaves through the SSF process by Neurospora sp. Neurospora sp. is a fungus that can be obtained from traditional Indonesian fermented food, known as oncom. These fungi have the ability to produce the enzymes needed for saccharification and fermentation processes simultaneously, so that the process is no longer required the addition of (hemi-)cellulose enzymes. However, lignin content, degree of depolymerization (DP), and the crystallinity of cellulose can inhibit cellulosic-biomass digestion by Neurospora sp., so that the suitable pretreatment method of lignocellulosic biomass is strongly needed. This study started by investigating the contents of lignocellulose in vetiver leaves, then applied some method of acid or alkaline pretreatment, or any combination thereof, as well as thermal treatment. The pretreated vetiver leaves from different pretreatment methods were applied into SSF process and the bioethanol yields were then determined.
2. Materials and Methods

2.1. Microorganism

*Neurospora* sp. used in this study was isolated from the fungus on oncom, a traditional Indonesian fermented food. Stock cultures were maintained on potato dextrose agar slants at 4°C. *Neurospora* sp. is kept and cultivated in Bioconversion Laboratory, Biosciences and Biotechnology Research Centre, Institut Teknologi Bandung, Indonesia.

2.2. Lignocellulosic biomass

Vetiver grass (*Vetiveria zizanioides* L. Nash) used as lignocellulosic biomass feedstock was obtained from Leles Garut, West Java, Indonesia with the growing age of 6 months. The grasses were washed and dried in sunlight. Dried vetiver leaves were milled using a blender and then sheaved to obtained the particle size of +120/-230 mesh.

2.3. Pretreatment methods

2.3.1. Hot water pretreatment. 100 mL of water was added into 500 mL Erlenmeyer flask containing 10 g of milled vetiver leaves. The mixture was then autoclaved at 130°C and 24.5 psi (relative to atmosphere) for 1 h. Before the autoclaved leaves can be used as feedstock for SSF process, they were filtered, washed with distilled water, sterilized using autoclave (121°C for 20 min), and dried at 60°C for 16 h.

2.3.2. Dilute acid pretreatment. About 7.83 g dry solid (DS) of milled vetiver leaves were slurried with 1L of 1.5% v/v dilute sulfuric acid. The mixture was autoclaved at 130°C and 24.5 psi (relative to atmosphere) for 30 min. After acid pretreatment, pH of vetiver slurry was adjusted to pH 5.0 with 10 M NaOH [7]. After pH adjustment, the vetiver leaves were filtered, washed, sterilized, and dried at 60°C for 16 h.

2.3.3. Alkaline & dilute acid pretreatment. This method has two steps of treatments. In the first step, 10 g of milled vetiver leaves were mixed with 1.0 M NaOH and then incubated at room temperature for 24 h. Before going to the second step, the leaves were washed, filtered, and dried overnight at 60°C for 16 h. In the second step, three gram of pretreated vetiver leaves were hydrolyzed with 2.0% v/v of sulfuric acid at 111°C for 30 min using autoclave. Before applying to SSF process, the treated leaves were filtered, washed, sterilized, and dried again at 60°C for 16 h [8].

2.3.4. Alkaline peroxide pretreatment

This method was adopted and modified from the one described by Saha and Cotta (2007) [9]. 15 g milled vetiver leaves were suspended in 1 L of 7.5% v/v H₂O₂. Alkaline level was adjusted to 11.5 using NaOH. The samples were shaken (160 rpm) and incubated at 35°C for 48 h. The samples were then filtered and solid residues were washed up to neutral pH. Similar with other methods, before the samples applied to SSF process, they were sterilized and dried at 60°C for 16 h.

2.4. Simultaneous saccharification and fermentation (SFF) using *Neurospora* sp.

*Neurospora* sp. was initially cultivated in a 50 mL vial containing 10 mL potato dextrose broth (PDB) medium. The culture was shaken (150 rpm) and incubated at room temperature for 48 h in aerobic condition. The aerobic condition was intended for the growth of *Neurospora* sp. After gaining the proper weight of biomass, 0.1 g of pretreated vetiver leaves was fed into fermenter. In order to increase the yield of bioethanol, the fermentation was continued in anaerobic condition for 24 h.

2.5. Analytical method

Lignocellulosic contents of vetiver leaf were analyzed according to the Chesson-Datta method [10]. SSF 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 (5 mM H$_2$SO$_4$) was adjusted at 0.6 ml/min and the temperature at 60°C.

3. Results and Discussion

3.1. The influence of pretreatment methods on the composition of lignocellulosic vetiver leaves

Vetiver leaves are an attractive feedstock for bioethanol production, because they contain high hemicellulose and cellulose. The mass fraction of hemicellulose and cellulose on fresh vetiver leaves (no pretreatment) were 0.35 and 0.31, respectively as shown in Table 1. Besides polysaccharides, lignin, hot water soluble (HWS), and ash are also detected in the lignocellulose content analysis of vetiver leaves. Lignin, which surrounds the cellulose, can inhibit enzymatic hydrolysis of cellulose. Besides lignin contents, enzymatic hydrolysis of lignocellulosic biomass is inhibited by crystallinity of cellulose, DP, surface area, and moisture contents [11 - 14]. In this research, several methods of pretreatments have been conducted in order to enhance the digestible contents of vetiver leaves as biomass feedstock in simultaneous saccharification and fermentation. Lignocellulose composition measured after pretreatment are shown in Table 1.

| Table 1. Lignocellulosic contents of vetiver leaves with different pretreatment methods. |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Lignocellulose Contents         | No Pretreatment | Hot Water       | Dilute Acid     | Alkaline & Dilute Acid | Alkaline Peroxide |
|                                |                  |                 |                 |                   |                 |
| HWS (%-w/w)                    | 14.01 ± 0.65    | 7.33 ± 0.65     | 10.33 ± 0.58    | 38.33 ± 0.58      | 8.67 ± 0.58     |
| Hemicellulose (%-w/w)           | 34.55 ± 0.58    | 35.67 ± 0.58    | 9.00 ± 1.00     | 16.33 ± 0.58      | 18.33 ± 0.58    |
| Cellulose (%-w/w)               | 31.39 ± 0.26    | 34.33 ± 0.58    | 54.67 ± 0.58    | 40.67 ± 0.58      | 65.07 ± 0.12    |
| Lignin (%-w/w)                  | 17.58 ± 0.44    | 20.50 ± 0.58    | 23.33 ± 0.44    | 2.33 ± 0.58       | 5.46 ± 0.25     |
| Ash (%-w/w)                     | 2.47 ± 0.23     | 2.17 ± 0.29     | 2.67 ± 0.58     | 2.34 ± 0.58       | 2.47 ± 0.23     |

The examined pretreatment methods in this study are hot water, dilute acid, alkaline & dilute acid, and alkaline peroxide methods. All those pretreatment methods begin with mechanical treatment by milling the dried vetiver leaves for reducing the particle size. Mechanical treatment may cause an increase of surface area, reduction of DP, and shearing of the biomass. All of these things can lead an increase of hydrolysis product yield of lignocellulosic biomass [15]. The lignocellulosic composition of pretreated vetiver leaves with hot water does not show a significant difference to the composition of untreated vetiver leaves (see Table 1). Temperature used in hot water pretreatment is 130°C, which is not high enough temperature to solubilize hemicellulose and lignin. Some research reported that hemicellulose starts to dissolve at temperature more than 150°C [16], while lignin can be solubilized if the lignocellulose biomass is heated up around 180°C [17]. Other research reported that liquid hot water pretreatment conducted at the range of temperature 220-280°C could increase the enzymatic hydrolysis of 2-5-fold for their lignocellulosic biomass [18].

Dilute acid pretreatment was intended to solubilize the hemicellulose, so that the (hemi-)cellulase of Neurospora sp. could freely access the (hemi-)cellulose. Solubilized hemicelluloses can be hydrolyzed in dilute acid treatment. Presented in Table 1 that the composition of hemicellulose after dilute acid pretreatment is lower than the one without pretreatment. Products of hydrolysis of hemicellulose can be monomer, hydroxymethylfurfural (HMF), furfural, and the products of other volatiles [15]. HMF, furfural, soluble lignin compounds, and other inhibitors produced from pretreatment are not desired in the fermentation step [19]. In this study, the pretreated vetiver leaves were washed before being fed to the bioreactor so that the remained pretreatment chemicals and the inhibitors produced during pretreatment do not interfere the SSF process. Some of hydrolysis products were brought along with washing water. Only the insoluble compounds in cold washing water are remained on the pretreated sample. As a result, the mass fraction of HWS after dilute acid treatment in Table 1 is lower than the one without pretreatment. The loss of some compounds of this hydrolysis product causes the proportion of cellulose and lignin increased.
Alkaline & dilute acid pretreatment is a combination between alkaline and acid pretreatments. This method aims to improve the anaerobic digestibility. In this method, an alkaline pretreatment was previously applied to the vetiver leaves before a dilute acid pretreatment was applied. Compared with the previous method, in this method, there is an additional alkaline pretreatment. Alkaline pretreatment can cause swelling of the biomass, which can later be accessed by enzymes or other biocatalyst agent [15]. Alkaline pretreatment allows the dissolution of lignin and change the state of crystalline cellulose [20]. As explained earlier that the dilute acid would hydrolyze the soluble hemicellulose so that the combination of these two pretreatment methods can eliminate lignin and hemicellulose gradually. As a result, the cellulose content increased after alkaline & dilute acid pretreatment compared with the content without pretreatment as can be shown in Table 1.

In the last method, an oxidizing compound, hydrogen peroxide, is used. It is intended to remove the hemicellulose and lignin so that biomass can be digested by a biocatalyst. At a pH of 11.5 - 11.6, hydroxyl ions could be expected to do delignification. Gould (1984) reported that approximately 50% lignin can be dissolved by applying this method for about 20 hours [21]. In this study, the use of alkaline peroxide method can eliminate approximately 70% lignin in biomass (see Table 1).

3.2. Simultaneous saccharification and fermentation of pretreated vetiver leaves using Neurospora sp.

The pretreated vetiver leaves were fed into the bioreactor as a carbon source and substrate for Neurospora sp. to produce bioethanol. Neurospora sp. is a biological agent that is capable of producing enzymes for (hemi-)cellulose hydrolysis and converting fermentable sugars into bioethanol [22]. The ability of Neurospora sp. to convert the pretreated vetiver leaves into bioethanol was assessed for each pretreatment methods that previously done.

![Figure 1](attachment:bioethanol_yield.png)

**Figure 1.** Yields of bioethanol from different pretreated vetiver leaves by Neurospora sp. Bioethanol yield is in g bioethanol/g dry solid (DS) of vetiver leaves/L fermentation mixture.

The bioethanol yields (per liter) from fermentations of each lignocellulosic vertiver coming from the different treatments are given in Figure 1. Results indicate that the type of pretreatment method can affect the performance of Neurospora sp. in producing bioethanol. Alkaline peroxide method provides the highest yield of bioethanol, i.e. 67% (g/g DS), followed by a dilute acid, alkaline & dilute acid, and hot water pretreatment methods, subsequently. Production of inhibitor compound and the loss of fermentable sugars frequently occur during pretreatment, hence the yield of bioethanol is still low. As reported by other researcher that the use of an acid during the pretreatment can lead to the formation of volatile compounds that can inhibit the fermentation process, while the use of an alkaline can produce
other inhibitor compounds which can interfere the fermentation process [15]. The use of oxidative compounds can cause non-selective oxidation so that lot of sugar is lost [15].

4. Conclusions

Several methods for pretreatment of lignocellulosic biomass, such as pretreatments with hot water, dilute acid, alkaline and dilute acid, and alkaline peroxide have been done for vetiver leaves. The success of pretreatment stage in reducing the lignin content and also increasing the polysaccharides and oligosaccharides contents significantly affects the performance of Neurospora sp. in digesting the pretreated vetiver leaves and carrying out saccharification and fermentation. In this study, pretreatment methods using alkaline peroxide could reduce lignin content of vetiver leaves by 69% and increase the total cellulose and hemicellulose content of 26% compared to the lignocellulose contents of the leaves without any pretreatment. The vetiver leaves after alkaline peroxide pretreatment can be converted by Neurospora sp. into bioethanol with a yield of 67% (g/DS).

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