Effects of substrate concentration on bioethanol production from oil palm empty fruit bunches with simultaneous saccharification and fermentation (SSF)

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Abstract. Oil palm empty fruit bunches (OPEFB) are industrial wastes generated from the processing of palm oil. OPEFB has the potential to be feedstock for bioethanol production due to its high cellulose content. In this paper, ethanol production is conducted with Simultaneous Saccharification and Fermentation (SSF) method, which combined hydrolysis (saccharification) and fermentation process simultaneously in one reactor to create a faster conversion time and minimize costs. The aim of the study was to determine the effect of substrate concentration on the growth of microorganisms and the yield of bioethanol produced through the SSF method. Pretreatment of OPEFB to decompose lignin is done by soaking it in alkaline solution. Hydrolysis is carried out enzymatically with cellulase enzyme within 48 hours in incubator shaker. The process is then followed by fermentation within 12 hours by adding *Zymomonas mobilis* inoculum. The research method was done experimentally with descriptive analysis, which included the effects of OPEFB substrate concentration (3%, 6%, 9%, 12%, 15%) on ethanol production. The result shows that the high concentrations of substrate in the medium increase the concentration of glucose, in which the highest concentration is 3.2 g/L. The higher the substrate loading resulted in lower hydrolysis yield and ethanol yield. The highest ethanol concentration is 0.25 g/L, obtained from fermenting 9% OPEFB loading in 3 hours.

1. Introduction

Bioethanol has advantages such as flammability and good combustion properties because it contains oxygen in large quantities. Thus, bioethanol as an alternative to fossil fuels could decrease environmental pollution because of its ability to reduce CO gas emissions. Bioethanol is produced from renewable materials, such as various crops such as wheat, corn, straw, and others. Materials that can be used as bioethanol are substances containing glucose, such as sugar, starch, and cellulose [1].

Oil palm empty bunches (OPEFB) are one of the most widely available agricultural wastes in the palm oil industry. The number of OPEFB may reach 20-22% in each processing of crude palm oil [2]. OPEFB is selected as a substrate in this study because of the high content of cellulose, which is about 35-43% [2]. Generally, conversion of lignocellulosic substrate into ethanol is conducted through several stages such as pretreatment, saccharification (hydrolysis) and fermentation.

The substrate is a material that contains nutrients for the growth of microorganisms during the fermentation process. The increase in substrate concentration could speed up the rate of the hydrolysis because more compounds are bound to the active side of the enzyme [3]. With a fixed concentration of enzymes, a low amount of substrate causes only a few substrates bonded to the enzyme's active site. Thus, the increase in substrate concentration may increase the product formation [3].

Substrate concentrations are important in order to make the process more economical, but a very high substrate concentration may lead to inhibition of end-product formation. It has been reported in [3] that with various OPEFB substrate loading (15% to 25%), the fermentation rate is slower at the highest solid substrate concentration (25%). However, a study of *A. donax* fermentation from the different solid amount (from 5% to 10%) resulted in a high ethanol concentration at 10% substrate [4]. As such, this paper examined the influence of substrates ranged from 3% to 15% on ethanol production.
The fermentation methods could be done by Separated Hydrolysis and Fermentation (SHF) and Simultaneous Saccharification and Fermentation (SSF) [1]. While SSF uses only one reactor for saccharification and fermentation processes, the hydrolysate could be directly converted into ethanol, which enables a higher ethanol yield formation compared to the SHF method. A different of SHF and SSF method for bioethanol production from OPEFB shows that ethanol yield produced by SSF method is higher than that of SHF method [5].

This research is conducted the experiment with simultaneous saccharification and fermentation method, using *Zymomonas mobilis* bacteria to help the hydrolysate conversion. OPEFB, which serves as a substrate, is varied into several assays to examine its influence on bioethanol production.

2. Materials and Methods
2.1. Raw materials
OPEFB was collected from PTPN VIII Cikasungka Bogor in West Java, Indonesia. Before used, the OPEFB was cut into pieces and washed off with water. Later on, OPEFB was oven dried at 105 °C overnight and ground. The OPEFB powders are then sieved at 40 mesh size, as recommended in the attempt of higher ethanol concentration [6]. The lignocellulosic composition of OPEFB was examined with Chesson-Datta method [7]. Results showed that OPEFB used in this research contains 33.83 % of cellulose, 17.07 % of hemicellulose and 26.71 % of lignin.

2.2. Pretreatment of OPEFB
Pretreatment of OPEFB proposes to reduce the lignin structure, thus making the enzymatic hydrolysis of cellulose composition easier. Using a beaker glass, OPEFB powder was submerged with 5 M NH4OH solution at 1:5 (weight to liquid) ratio for 24 hours. The ammonia solvent was then separated by filtration and the OPEFB was washed with distilled water until the pH remains neutral. To avoid the remnants of ammonia that may still be contained, re-dry the material in the oven for 24 hours at a temperature of 50 °C [8]. Cellulose content after pretreatment was determined using a method adapted from Chesson-Datta [7].

2.3. Simultaneous saccharification and fermentation
2.3.1 Saccharification
In this study, enzymatic hydrolysis or saccharification was performed with various substrate concentrations, as in Figure 1. Pretreated OPEFBs were divided into several concentrations such as 3%, 6%, 9%, 12% and 15% (w/v) of the volume of the buffer. For example, if the buffer volume is 100 mL, then 3% substrate (w/v) equals 3 g OPEFB. Each substrate was put into a different Erlenmeyer flask. To maintain the pH of the process, citrate buffer with pH 5.0 was added with the same amount for each substrate.

After that, each Erlenmeyer was added with the enzyme as much as 5% (v/v) of the buffer volume. Cellulase enzyme Cellic Htec was purchased from ITB. The saccharification process is conducted using an incubator shaker for 48 hours. The rotation speed is controlled to 130 rpm and the temperature is set at 50°C [9]. Measurement of sugar content was determined using DNS method. The measurement of hydrolysis yield was calculated as Eq. (1) [4].

\[
Hydrolysis \ yield \ (\%) = \frac{Gluc_t}{Glucose} \times 100\%
\]

Where Glu is the glucose concentration (g/L) produced at time t and Glucose is the theoretical glucose from biomass.
2.3.2. Fermentation

In the SSF process, the prior hydrolysis result is not filtrated nor centrifuged before undergoing fermentation. SSF was conducted in one reactor for hydrolysis and fermentation. Hydrolysate was immediately added with inoculum to start the SSF process. In this study, the culture used was *Zymomonas mobilis* obtained from Department of Biology Universitas Padjadjaran. *Zymomonas mobilis* was cultivated in YEPD agar medium (yeast extract peptone dextrose) that contains 4 g of yeast extract, 2 g of KH₂PO₄, 3 g of (NH₄)₂SO₄, 1 g of MgSO₄·H₂O, 3.6 g of peptone and 20 g of agar/L distilled water [10].

The inoculum was made by inoculating the *Zymomonas* culture in slanted agar. The culture was then incubated for 24 hours at 30°C. After that, the culture was diluted with distilled water and cell density was adjusted with McFarland 3 by checking it on spectrophotometer UV-Vis at λ = 600 nm. The experiment was done until enough inoculum provided, that is 10% (v/v) inoculum solutions to each hydrolysate. Hydrolysates were added with fermentation medium to enrich the process and to help the growth of microorganisms. The fermentation medium contains 4 g of yeast extract, 2 g KH₂PO₄, 3 g (NH₄)₂SO₄, 1 g MgSO₄·H₂O, and peptone of 3.6 g/L of distilled water. The flasks were put into a shaker incubator for 12 hours to start fermentation. The agitation was set to 100 rpm and the temperature at 30°C [10]. During the fermentation process, sampling was done at every few hours to calculate the number of microorganisms and ethanol formation.

The number of microorganisms was determined with Total Plate Count. Growth of microorganisms was calculated from biomass concentration data during the exponential phase, following Eq. (2) [11].

\[
\mu X = \frac{dX}{dt}
\]  

(2)

In which \( \mu \) is the specific growth rate (h⁻¹).

The concentration of ethanol was determined using dichromate oxidation method [9]. The yield of product to substrate concentration will be performed as \( Y_{(p/s)} \) in Eq. (3) [10].

\[
Y_{(p/s)} = \frac{\Delta P}{\Delta S} = \frac{P_t - P_o}{S_o - S_t}
\]

(3)

Where:

\( Y(p/s) \) = The yield of ethanol product to substrate

3. Results and Discussion

3.1 Pretreatment of raw materials

According to the result of OPEFB characterization using Chesson-Datta method, the composition of OPEFB consists of 33.83 % of cellulose, 17.07 % of hemicellulose and 26.71 % of lignin. Strongly-bonded cellulose with lignin has to be pretreated to make the hydrolysis process easier [1]. The initial treatment stage in the production of bioethanol aims to destroy the lignin structure, reduce the crystalline properties of cellulose and facilitate the enzyme to work [12]. This process will open the cellulose structure in the substrate. The result of pretreatment in this research is in Table 1.

| Components of oil palm empty fruit bunch. | Content (%) | Untreated OPEFB | Pretreated OPEFB |
|-----------------------------------------|-------------|-----------------|-----------------|
| Cellulose                               | 33.83 ± 1.02| 46.85 ± 0.15    |                 |
| Hemicellulose                           | 17.07 ± 0.98| 12.08 ± 0.14    |                 |
| Lignin                                  | 26.71 ± 0.83| 18.43 ± 0.75    |                 |

Alkaline chemicals are reported to have several advantages. One of them is that alkaline could break hydrogen bonds in cellulose; thereby reducing the nature of crystalline. Other advantages include breaking ester bonds, dissolving lignin, damaging crystalline structures, and increasing the access of enzymes to the cellulose surface [12]. Commonly used compounds are NaOH and ammonia or NH₄OH solutions.

Table 1 shows that the lignin compound in OPEFB is reduced up to 8%, with delignification degree of 31%. This value is significantly lower from the previous study, which got a delignification percentage up to 60%. These findings could be due to the difference of initial lignin from the untreated OPEFB, in which the previous study has 17.79 % lignin compound in the research [8]. The result also showed that ammonia-pretreated OPEFB has a lower hemicellulose compound than the untreated OPEFB. This might
be due to the characterization of ammonia pretreatment, in which the ammonia reduces lignin content and removes some hemicellulose while de-crystallizing cellulose [12].

3.2. Effect of substrate concentration on enzymatic hydrolysis

Enzymatic hydrolysis is a fundamental step for the SSF method. In this process, the initial glucose for ethanol conversion will be produced. Thus, in this study, the influence of various solid loadings on percent hydrolysis and glucose concentration is examined. Figure 2 shows that the increase in OPEFB concentration was negatively correlated with the hydrolysis yield. The maximum hydrolysis yield (14.5%) is shown to be at the lowest biomass loading.

There was a reduction of hydrolysis yield up to 4-5% shown from 3% solid loading to 6%. In fact, over 8% decrease of yield is recorded between the lowest to highest OPEFB concentration. This could happen due to several factors. If the solid loading is too high, a problem with enzyme accessibility, it could inhibit the conversion of cellulose. The increasing of viscosity material could also affect to reduce of enzyme mobility. There is also a chance of end-product inhibition that could prevent the hydrolysis [5].

The result of this research is similar to previous studies, in which an increase of pretreated Arundo donax (from 10% to 20%) resulted in the decrease of hydrolysis yield [13]. It has been reported that an increase in OPEFB (from 5% to 15%) showed a similar outcome in which the highest hydrolysis yield was released from the lowest substrate loading [2].

By contrast, the increasing of OPEFB loadings resulted in a rising of glucose concentrations. Figure 3 shows that the highest concentration of glucose is reached at 15% substrate concentration. The amount of sugar is 2.9 g/L at 24-hour saccharification and improved to 3.2 g/L at 48-hour saccharification.

As the hydrolysis yield in 3% substrate loading also increased from 12.1% to 14.5% at respectively 24 hours and 48 hours of saccharification, this phenomena indicates that addition of reaction time gave an increase in hydrolysis yield and glucose concentration. These results are consistent with previous studies that showed the higher substrate loading and longer reaction time, the higher amount of glucose released [4, 19].

3.3. Effect of substrate concentration on bacteria growth

In this set of experiments, the OPEFB concentration is varied as a substrate while the initial concentration of Zymomonas mobilis was kept constant at 10% (v/v). The growth rate of Zymomonas mobilis in this study varied, as the substrate concentrations are different. Figure 4, 5, 6, 7 and 8 show the growth of Zymomonas mobilis on various OPEFB concentrations and fermentation times.
Figure 4. The profile of cell growth on 3% substrate concentration.

Figure 5. The profile of cell growth on 6% substrate concentration.

Figure 6. The profile of cell growth on 9% substrate concentration.

Figure 7. The profile of cell growth on 12% substrate concentration.

Figure 8. The profile of cell growth on 15% substrate concentration.

The specific growth rate in 3% OPEFB concentration is 0.88 h\(^{-1}\) and increased at 0.94 h\(^{-1}\) with 9% substrate concentration. The highest substrate concentration, 15%, resulted in a decrease in growth rate; which is 0.76 h\(^{-1}\). This shows that the maximum specific growth rate of *Zymomonas mobilis* is reached at 9% OPEFB loading in this study.

When substrate concentration is lower, the specific growth rate would positively correlate with the substrate, whereas if the substrate is too high, the specific growth rate would not be dependent on substrate loading [14]. This suggests that in this study, the specific growth rate in substrate concentration over 9% was not influenced by the amount of solid concentration. In natural environment, the growth rate could also be affected by of pH, light intensity, DO and temperature [14].

3.4. Effect of substrate concentration on ethanol concentration

This study measure ethanol concentration and ethanol yield to examine the level of SSF performance. Ethanol yield is a ratio between the amount of produced ethanol from the sample and the theoretical ethanol possibly produced from various concentrations of substrate. As in Figure 9, the maximum ethanol concentration (0.25 g/L) was found at 3-hour fermentation with substrate loading 9%.
However, the highest final ethanol concentration (0.2 g/L) was reached by fermenting the 15% OPEFB substrate. The highest final ethanol concentration is derived from the 15% substrate concentration. The figure showed that within 6 hours of fermentation, most substrate loadings (3%, 6%, and 12%) had an increase in their ethanol production. Then, as the fermentation keeps on going longer, the ethanol production became stagnant or even decreased. This might be caused by the formation of inhibitory compounds, such as furfural, acetic acid, and phenolic. Phenolic compounds are produced from partial lignin damage, which could occur during a pretreatment process [15]. This corresponds to the low delignification degree, hence the remains of pretreatment process caused inhibition. During fermentation, a decrease in ethanol could also be caused by the composition of the substrate, reduction of the enzyme’s active sides, and the inefficiency of mass transfer [4].

Figure 10 indicates when using a 15% pretreated OPEFB loading, the ethanol yield was lower than with 3% substrate loading. These results might have been caused by other substances formed during hydrolysis that could inhibit the fermentative metabolism of microbial strain [4].

The maximum ethanol yield is 0.096 (g/g) which was achieved from 3% substrate loading. In this study, the ethanol yield tends to decrease with a higher substrate concentration. Similar outcome was recorded in the previous study [3]. This could be due to several reasons, such as the difference in cellulose content, enzyme concentrations, and yeast type.

4. Conclusion
This research showed that the increasing of OPEFB concentration as substrate influenced the production of bioethanol. This research found that 15% substrate loading gave the highest initial glucose for fermentation, which was 3.2 g/L. The high concentration of OPEFB solid loading resulted in low hydrolysis and ethanol yield. The highest ethanol concentration is 0.25 g/L at 3 hours of fermentation with 9% of substrate. The highest ethanol yield is 0.096 (g/g) at an initial concentration of 3% substrate.

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