Valorization of oil palm empty fruit bunch for bioethanol production through separate hydrolysis and fermentation (SHF) using immobilized cellulolytic enzymes

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Abstract: Oil palm empty fruit bunch (EFB) is a lignocellulosic waste from oil palm industry. This biomass is composed of cellulose, hemicellulose, and lignin which could be a good feedstock for second-generation bioethanol production. The cellulolytic enzyme is one of important biocatalyst for conversion cellulose into glucose that subsequently could be fermented to ethanol. However, instability of enzyme is considered as a barrier for large-scale production. Enzyme immobilization is believed can obtain the high stability of enzyme. Therefore, this study explores the immobilization of cellulolytic enzyme on mixed Ca-alginate-activated carbon beads for separate hydrolysis and fermentation (SHF) process of EFB. The mixed of 3% w/v of sodium alginate and 3% w/v of activated carbon was used in immobilized enzymes. Variation of substrate concentration (50 g/L, 100 g/L, and 150 g/L) was conducted in this study. Hydrolysis process was carried out at 50°C, pH 4.8 and 150 rpm of agitation for 96 h. Furthermore, the hydrolyzate was fermented using yeast Saccharomyces cerevisiae to produce ethanol. As results, immobilized of cellulolytic enzyme could convert cellulose into glucose in hydrolysis. The highest glucose yield of 75.48% was provided from 150 g/L of substrate loading concentration. Furthermore, ethanol yield of 78.95% could be provided in fermentation process of 150 g/L of EFB. These results indicate the use of immobilized enzymes could be applied in hydrolysis for bioethanol production.

1. Introduction
Increasing energy demand is an important issue in the world. Energy consumption worldwide grew by 2.3% in 2018, nearly twice the average rate of growth since 2010 [1], wherein fossil fuel still led in the energy consumption scheme. In Indonesia, energy consumption (excluding firewood) in 2016 was still dominated by oil fuels by 47%. Moreover, the transportation sector has the largest share of 42% [2].

However, fossil fuels have some drawbacks as main sources, i.e., categorized as non-renewable energy, limited stock, and also serious implications for the environment. Therefore, achieving energy security and environmental safety became important goals for countries in the world. They take an effort to reduce fossil fuel dependency by developing renewable energies.

Among the renewable energies, bioethanol is considered as an important renewable fuel to substitute fossil-derived fuels, especially gasoline. Bioethanol can be generated from lignocellulosic biomass, is known as the second-generation bioethanol. This biomass is a promising bioethanol feedstock due to their abundant availability in the world. Lignocellulose materials have been projected as a carbon-neutral renewable source, which can decrease CO₂ emissions and atmospheric pollution [3]. Indonesia as one of the biggest producer of palm oil produces huge lignocellulose biomass from oil palm waste such as oil palm empty fruit bunch (EFB), oil palm frond, and oil palm trunk. These biomasses are potential as raw material for second-generation bioethanol production.

Bioethanol production from lignocellulose biomass has complex processes. Typically, the conversion of lignocellulose to ethanol consists of the following steps: pretreatment, hydrolysis, fermentation, and purification [4]. However, significant challenges must be overcome for realizing lignocellulose as a sustainable and profitable resource. In the particular case of hydrolysis process, the cost of cellulose-degrading enzyme, cellulase, is major defiance. The high cost of these enzymes has
limited the economic viability of their use in industrial bioprocesses [5]. One study examined biofuel production at second-generation ethanol plants determined that enzyme cost accounted for 32% of the total cost [6]. Moreover, these enzymes are also short-lived, low catalytic efficiency and low stability, which makes their long-term storage difficult, increasing cost and reducing production efficiency in industrial scale [7,8]. One of the methods that considered can tackle the enzymes problem is immobilization of enzymes.

There are some advantages of immobilized enzymes, that are the ability to stop the reaction rapidly by removing the enzyme from the reaction solution, stabilization of enzymes by bounding, prevention of contamination of product with the enzyme, elimination of reagent preparation in order to improve the functionality and performance of enzymes for bioprocessing applications, and multiple or repetitive use of a single batch of enzymes [9]. Based on the physical mechanism applied, the immobilization methods can be divided into four major categories: (a) entrapment/encapsulation, (b) adsorption or attachment on solid carrier surfaces, (c) cross-linking, and (d) covalent bonding [10]. Several studies were conducted on hydrolysis of EFB using cellulase immobilized on multi-wall carbon nanotubes [8] or polyethylene glycol (PEG) [11]. However, these immobilizations still need high cost. Therefore, this work explores the immobilization of cellulolytic enzymes on alginate-activated carbon beads for bioethanol production from EFB with separate hydrolysis and fermentation (SHF) method. Alginate beads are one of the most commonly used in immobilization which are easy availability, ease of preparation and low cost. Furthermore, adding activated carbon could provide valuable support for enzyme immobilization.

2. Materials and Methods

2.1. Materials

Oil palm empty fruit bunch (EFB) in this study was collected from a palm oil plantation in Palembang, South Sumatra. The cellulase enzymes were obtained from Novozymes that consist of Cellic Ctec2 and Cellic Htec2. The enzyme activity was 126.05 FPU/mL for Cellic Ctec2. One unit of activity in FPU is equal to 1 mol of glucose produced per minute per milliliter of the enzyme from filter paper. Commercial instant dry yeast Saccharomyces cerevisiae were applied in fermentation process. Sodium alginate and activated carbon were provided by Merck. Other chemicals and all reagents used in this study were of analytical grade.

2.2. Pretreatment Process

EFB was chopped and milled into particle size about 3 mm, then dried up to about 10% of moisture content. Furthermore, EFB was pretreated using 10% NaOH solution. The ratio of EFB and NaOH solution was 1:5. The condition of pretreatment was carried out at temperature 150°C and 4-7 kg/cm² of pressure for 30 minutes. EFB-treated was washed by water until neutral pH and dried until moisture content about 10%.

2.3. Enzyme immobilization

Figure 1 shows the procedure for immobilizing cellulolytic enzymes. 30 FPU/g substrate of Ctec2 (cellulase complex) and 20% v/v of Htec2 (this value was based on the volume of Ctec2 loaded) was mix with citrate buffer until the total volume of 20 mL. Furthermore, 20 mL of enzymes solution was mixed with 3% w/v each of sodium alginate and activated carbon which has been autoclaved at 121°C for 15 mins. The sodium alginate and activated carbon mixture was thoroughly stirred with the enzymes solution until dissolution. The obtained solution was, then, withdrawn with 10 mL sterile syringe and extruded drop-wise into 200 mL of sterilized 0.2 M CaCl₂ solution. The beads containing the immobilized enzymes were formed instantaneously. They were allowed to harden in the CaCl₂ solution for at least 45 mins. Then, they were washed twice with sterilized distilled water to rinse away the CaCl₂ solution.
2.4. Separate hydrolysis and fermentation process (SHF)
SHF consists of two sequential stages, enzymatic saccharification and fermentation. The total volume was 100 mL with the variation of substrate loading were 50, 100, 150 g/L. The substrate and 0.05 M citrate buffer was loaded in Erlenmeyer flask until reach the total volume of 100 mL and, then, it was sterilized at 121°C for 15 mins in autoclave. Furthermore, immobilized enzymes was added in the substrate solution. The saccharification was conducted at temperature 50°C, pH 4.8 with agitation of 150 rpm for 96 h. Replication was applied for each SHF.

After 96 h of saccharification, the hydrolyzate was separated with immobilized enzymes. The hydrolyzate was added 1% w/v of dry yeast for fermentation. The fermentation was carried out at temperature 32°C, 150 rpm for 72 h in a shaking incubator. Sample 1 mL was withdrawn from medium of hydrolysis and fermentation every 24 h for analyzing product.

2.5. Analytical methods
Chemical compound (cellulose, hemicellulose, and lignin) of EFB-untreated and EFB-treated were determined using standard biomass analytical procedures from National Renewable Energy Laboratory (NREL)[12].

The product composition (glucose, xylose, and ethanol concentration) was determined by high-performance liquid chromatography (HPLC). HPLC (waters, USA) system was equipped with a Hi Plex Ca (Duo) column and analyzed with refractive index (RI) detector. The eluent used as mobile phase was water at a flow rate of 0.4 mL/min.

The product yields were adjusted to an initial volume of broth according to equation [13]:

\[
\% \text{ glucose yield} = \frac{[\text{glucose}]_e - [\text{glucose}]_i}{(f[\text{biomass}] 1.11)} x 100\% \quad (2-1)
\]

\[
\% \text{ ethanol yield} = \frac{[\text{ethanol}]_f - [\text{ethanol}]_i}{0.51(f[\text{biomass}] 1.11)} x 100\% \quad (2-2)
\]

Where:

\([\text{glucose}]_e = \text{glucose concentration at the end of saccharification (g/L)}\]
\([\text{glucose}]_i = \text{glucose concentration at the beginning of saccharification (g/L)}\]
\([\text{biomass}] = \text{dry biomass concentration at the beginning of saccharification (g/L)}\]
\(f = \text{cellulose fraction of dry biomass (g/g)}\]
\(1.11 = \text{equivalent conversion factor of cellulose to glucose}\]
\([\text{ethanol}]_f = \text{ethanol concentration at the end of fermentation (g/L)}\]
\([\text{ethanol}]_i = \text{ethanol concentration at the beginning of fermentation (g/L)}\]
\(0.51 = \text{conversion factor for glucose to ethanol based on stoichiometric reaction}\)
3. Results and discussion

3.1. EFB characterization

Table 1 shows the comparison of chemical component of EFB before and after pretreatment using alkali explosion. Based on the results, cellulose was the main component in EFB followed by lignin and hemicellulose. The percentage of chemical composition of EFB was changed after pretreatment. Cellulose content increased to 61.64%, while hemicellulose and lignin declined to 20.86% and 11.01%, respectively. The reduction of lignin is the main purpose of this pretreatment. Alkali explosion has been recognized as one of the best chemical pretreatment methods for delignification of lignocellulose.

| No. | Component      | EFB-untreated (%) | EFB-treated (%) |
|-----|----------------|-------------------|-----------------|
| 1   | Lignin         | 26.53             | 11.01           |
| 2   | Cellulose      | 36.59             | 61.64           |
| 3   | Hemicellulose  | 24.97             | 20.86           |
| 4   | Ash            | 1.79              | 0.38            |

The mechanism of alkali pretreatment is saponification of intermolecular ester bonds crosslinking xylan and other components, such as lignin and other hemicelluloses. The removal of these crosslinks has enhanced the porosity of lignocellulosic biomass [14]. The use of NaOH provokes swelling, rising the internal surface of cellulose and reducing the degree of polymerization and crystallinity, that causes lignin disruption [15]. Most of lignin and slight hemicellulose can be dissolved in alkali solution, thus increasing the percentage of cellulose content in the solid phase. The reduction of lignin and hemicellulose content makes the access of cellulolytic enzymes to cellulose easier in hydrolysis.

3.2. Separate hydrolysis and fermentation using immobilized cellulolytic enzymes

The shape of immobilized beads with 3% each of alginate and activated carbon concentration was spherical (figure 1). According to Chen et al., 2-3% sodium alginate concentration can produce spherical and strong beads [16], thus, 3% was selected as the alginate and activated carbon concentration to immobilize the cellulolytic enzymes.

![Figure 2](image-url)  

**Figure 2.** The condition of (a) substrate, citrate buffer, and beads before hydrolysis, and (b) hydrolyzate and beads after 24 h of hydrolysis.
Figure 2(a) and 2(b) demonstrates the mix of substrate, citrate buffer, and immobilized cellulolytic enzyme before and after hydrolysis. Before hydrolysis, the hydrolyzate was still in solid form, and the hydrolyzate became slurry after 24 h of hydrolysis. It indicated that cellulolytic enzymes in beads could work to convert cellulose into glucose, thus, the product was soluble in the citrate buffer and the beads still remains in solid spherical phase. After hydrolysis, the immobilized enzymes was separated from hydrolyzate and showed that the shape of beads was still spherical but beads strength was reduced. Viet et al. [17] reported, the activity of the cellulose-immobilized enzyme was not significant different with the free enzyme. Immobilized enzyme was more stable at high pH and temperatures [17,18].

Figure 3 shows the change of glucose concentration during hydrolysis on the variation substrate concentration of 50 g/L, 100 g/L, and 150 g/L by immobilized cellulolytic enzymes. The hydrolysis results indicated that EFB could be hydrolyzed by immobilized enzymes to produce glucose. These hydrolyze produced the highest glucose concentration for each variation substrate concentration after 72 h process. The highest glucose concentration obtained from 150 g/L substrate reached 83.5 g/L. The glucose yield for hydrolysis of 50 g/L, 100 g/L, and 150 g/L substrate were 79.69%, 82.58%, and 82.10%, respectively, in 72 h of hydrolysis. The glucose yield of 82.10% in the 150 g/L substrate was not much different if compared to the EFB hydrolysis with free cellulolytic enzymes in the similar substrate loading, the use of 40 FPU/mL Ctec2 and Htec2 produced about 87% of glucose yield [19].

![Figure 3](image-url)

**Figure 3.** The change of glucose concentration during hydrolysis with variation of substrate concentration of 50%, 100%, and 150%

Table 2 shows the product yield (glucose) after 96 h of hydrolysis and product yield (ethanol) after 72 h fermentation. These results indicated that hydrolysis of 100 g/L of substrate provided the highest glucose yield as compared with others substrate loading concentration. Although the fermentation of 150 g/L of EFB gave the highest ethanol concentration reached 31.50 g/L, but the highest ethanol yield was obtained in fermentation of 100 g/L of EFB (78.95 %). The process using 100 g/L substrate gives optimum results in both glucose yield and ethanol yield as compared with others substrate concentration. Ethanol production with immobilized-enzymatic hydrolysis was slightly lower than process with free enzyme [20].
Table 2. Product (glucose and ethanol) yield in hydrolysis and fermentation of EFB with the variation of substrate loading concentration

| No. | Substrate concentration (g/L) | Condition process | Theoretical product concentration (g/L) | Final product concentration (g/L) | Yield (%) |
|-----|-------------------------------|-------------------|----------------------------------------|----------------------------------|-----------|
|     |                               | Hydrolysis        |                                        |                                  |           |
| 1   | 50 g/L                        | T= 50°C,          | 33.90                                  | 23.61±0.3                        | 69.65     |
| 2   | 100 g/L                       | 150 rpm,         | 67.80                                  | 51.18±0.2                        | 75.48     |
| 3   | 150 g/L                       | pH= 4.8, t= 96 h | 101.71                                 | 66.52±0.8                        | 65.40     |
|     |                               | Fermentation      |                                        |                                  |           |
| 1   | 50 g/L                        | T= 32°C,          | 17.29                                  | 10.20±0.2                        | 58.99     |
| 2   | 100 g/L                       | 150 rpm,         | 34.58                                  | 27.30±0.7                        | 78.95     |
| 3   | 150 g/L                       | t= 72 h          | 51.87                                  | 31.50±0.2                        | 60.73     |

4. Conclusion

Immobilized cellulolytic enzymes using sodium alginate and activated carbon could hydrolyze EFB to glucose and subsequently produce ethanol with adding *Saccharomyces cerevisiae* in fermentation process. The highest glucose yield of 75.48% and ethanol yield of 78.95% was obtained from 100 g/L of substrate loading concentration. These results indicated that immobilized enzymes could be a promising method for hydrolysis and can assist to reduce the cost of bioethanol production due to the easy separation between enzyme and hydrolyzate. Future investigations will endeavor at exploring the stability of beads and recycling immobilized enzymes.

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6. References

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