Isolation of Microfibrilated Cellulose from Oil Palm Empty Fruit Bunches (EFB) Through Peracetic Acid Delignification and Enzyme Hydrolysis

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Abstract. Oil palm empty fruit bunches (OPEFB) have high cellulose content, that is equal to 35.66%-57.75% wt. This research aims to convert cellulose fibre from OPEFB into advance biomaterial such as micro and nanofibrilated cellulose through peracetic acid delignification and enzyme hydrolysis. Peracetic acid (PAA) is a strong oxidizer which has been used by previous researchers for pretreatment of lignocellulosic biomass, can act as a bleaching agent and safe for the environment. The delignification process was done with PAA concentration varied from 2.5-7.5% v/v, within 2-6 hours at 80-85°C. Enzyme hydrolysis with cellulase complex at a concentration 10-25% w/w for 12-48 hours was done to reduce the particle size of cellulose. The cellulose fibre treated with 2.5% v/v PAA for 2 hours delignification time has the highest cellulose content of 81.01% wt. The best enzyme hydrolysis treatment obtained from a 15% enzyme concentration for 24 h hydrolysis time, with the dispersion stability of 60% and average particle size of 822.7 nm. The highest product crystallinity was 25.3%, obtained after 36 h hydrolysis time. Based on the particle size and SEM images result, it was known that enzyme hydrolysis can transform cellulose into microfibrilated cellulose but it is not strong enough to produce nanocellulose.

1. Introduction
Crude palm oil (CPO) production generates solid waste consisting of oil palm empty fruit bunch (OPEFB), mesocarp fibre, shells and wet decanter solid. The amount are 23%, 13%, 6.5% and 4% of fresh fruit bunch (FFB) respectively. OPEFB is an organic material with macro and micro nutrients content such as C, N, P2O5 and K2O which are very important for plant growth. Therefore, most palm oil mill use OPEFB as fertilizer that can be returned to the soil as a nutrition sources. On the other hand, the use of OPEFB as fertilizer through improper methods actually has a negative impact to the environment. It requires a longer degradation time, causing the accumulation of OPEFB on the plantation were triggers to an anaerobic decomposition process. It can causes formation of gas pollutants, as well as leachate which can contaminate groundwater and surface water [1]. Therefore, it
is necessary to develop alternative products derived from OPEFB to reduce the accumulation of biomass significantly.

Microfibrilated Cellulose (MFC) is a purified cellulose fibers, typically consist of partially disintegrated microfibril aggregates with diameter in the range of 20-40 nm and length of several micrometer [2]. MFC have been used as one of a potential bio-reinforcing material due to it’s high surface area, aspect ratio and specific strenght. MFC is composed of both amorphous and crystalline parts. It can be produced from all natural cellulose sources such as plants, bacteria, tunicates and algae [2,3]. Acid treatment and mechanical processes are the method commonly used by previous scientist to convert cellulose into microfibrilated cellulose as well as nanocellulose [4,5,6].

MFC isolation through mechanical processes generally uses high shear homogenization, ultrafine grinding [7], cryocrushing [8] and ball mill [9]. The disadvantage of this technique is the high energy requirements. Therefore, mechanical processes generally combined with chemical pre-treatments such as alkaline delignification and acid hydrolysis using sulphuric acid [4] or hydrochloric acid [3]. The liquid acid hydrolysis method is easy and inexpensive, effectively produce micro/nanofibrilated cellulose with a negative charge on its surfaces so that the dispersion of product is more stable. However, this method causes other problems such as heavy corrosion to the metal equipment, because it used high acid concentration (54-65%/v/v). It is also requires large amounts of water for the neutralization step, which causes the formation of large amount of liquid waste [10] and difficult to process. Therefore in this study we used a combination of acid and enzyme to maximize the hydrolysis of amorphous part of cellulose without damaging the crystalline area. Besides, it is also expected to reduce the concentration of sulphuric acid needed for cellulose hydrolysis.

This research aims to convert cellulose fibre from OPEFB into micro/ nanofibrilated cellulose through peracetic acid delignification and enzyme hydrolysis. Biomass delignification using Peracetic Acid (C₂H₃O₄) was conducted in the pre-treatment stage. Delignification is an important step to reduce lignin, thereby facilitating the penetration of catalysts used in the hydrolysis process. The results of previous studies on the effect of biomass pre-treatment with a combination of peracetic acid (PAA) and [Emim] [OAc] on pine biomass showed that both are synergized to produce regenerated biomass which can be easily enzymatically hydrolyzed [11].

2. Materials and Method

2.1. Materials

Cellulose pulp used as raw material in this study were alkali delignified OPEFB pulp, obtained from the Indonesian Biotechnology and Bioindustry Research Center. Peracetic acid (C₂H₃O₄) for the advanced delignification process was obtained from Solvay Peroxythai, Co. LTD. Acid hydrolysis of nanocellulose using sulphuric acid (H₂SO₄) from Merck Millipore, whereas in the enzyme hydrolysis process used cellulase complex from Novozymes and citrate buffer pH 5.

2.2. Method

2.2.1. Peracetic Acid Delignification

Five g of OPEFB pulp were added with 100 ml of PAA solution with a concentration of 2.5-7.5% v/v. The mixture was then heated at a temperature of 75-85°C for 2-6 hours, with a mechanical stirring of 1 000 rpm. After that, it was cooled immediately to reach room temperature [11]. Washing step with demineralized water was carried out until the pH was neutral. Parameters analyzed included yield, cellulose, hemicellulose, lignin and ash content with the Chesson method [12].

2.2.2. Acid Hydrolysis

First step of hydrolysis processes was acid hydrolysis by using sulphuric acid with a concentration of 55% (v/v), pulp and acid ratio of 1:10. It was done at temperature of 35°C for 6 hours. The suspension centrifuged at 6 000 rpm for 40 minutes to separated acid and product. The precipitates then washed
repeatedly with distilled water until the pH was neutral, and stored in cold temperatures before being used in the next hydrolysis process.

2.2.3. Enzyme Hydrolysis
Ten g of precipitate from acid hydrolysis were added by cellulase complex enzymes in citrate buffer (pH 5). The enzyme dose was 10, 15, 20 and 25% w/w. Enzyme hydrolysis carried out in a water bath shaker at temperature of 50°C for 12, 24, 36 and 48 hours. The suspension was centrifuged at 6000 rpm for 40 minutes to obtained the product. Product characterization included yield, crystallinity by X-ray Diffraction, particle size by Particle Size Analyzer and surface morphology with Scanning Electron Microscopy.

3. Result and Discussion
3.1. Fibre Composition of Cellulose Fiber
Cellulose is the most abundant biological raw material in the world, naturally binds with hemicellulose and lignin to forms main structure of the plants. The presence of lignin is not desirable in the hydrolysis process of lignocellulosic biomass because it will inhibit the catalyst penetration. Delignification aims to remove lignin component in biomass. There are several techniques that can be used in the delignification process, including biological delignification using enzymes, bacteria and fungi, or chemical delignification with acids or alkali. Chemical delignification widely used in many industries, especially pulp and paper [13]. Disadvantage of alkali delignification technique is the process conditions that take place at high temperatures and pressures. In addition, it also requires a bleaching process to remove unwanted dark brown colour of delignified pulp.

The fibre composition of cellulose pulp treated with PAA delignification can be seen in Table 1. Cellulose pulp from alkaline delignification and OPEFB was also analyzed as a comparison. It is known that alkaline delignification can significantly reduce lignin content from 28.84% in OPEFB to 4% in alkali delignified cellulose fibre, so that cellulose content was increased from 36.56% wt to 73.01% wt. Lignin are more soluble in alkaline condition, but cellulose not soluble in alkaline. OPEFB treatment by NaOH in high pressure and temperature will decrease the lignin content [14]. Delignification also causes a decrease in hemicellulose, from 22.10% wt in OPEFB to 17.31% wt in alkali delignified cellulose fibre.

Further delignification with peracetic acid aims to maximize lignin elimination and purify cellulose, thus can increasing cellulose conversion into micro/nanofibrilated cellulose. From the fibre composition analysis, it was found that the highest cellulose content was 81.01% wt, obtained from the treatment of 2.5% v/v PAA for 2 hours delignification time. PAA delignification was also decreased hemicellulose levels. Hemicellulose is a polysaccharide that composes the plant cell walls along with cellulose and lignin. The composition of hemicellulose in lignocellulosic biomass is around 30% wt. It is more easily hydrolyzed by acid in mild process conditions. Therefore this process is usually used as a pretreatment of biomass to produce sugar in the ethanol production process [15].

The use of higher acid concentrations in longer delignification times causes a significant decrease in lignin content. The lowest one obtained from the treatment of 7.5% PAA in 6 hours delignification time, which was 1.72% wt. PAA is a strong oxidizing compound that is reported to be able to degrade 95% lignin at 5% acid concentration and facilitate enzyme penetration in the wood biomass hydrolysis [11]. However, it should be noted that the used of higher acid concentrations and longer hydrolysis times actually decreasing cellulose content, which can be caused by the partial hydrolysis of cellulose component. Ash content and hot water soluble component also increases with increasing PAA concentration and delignification time. The increase in hot water soluble component influenced by the presence of simple sugars resulting from cellulose hydrolysis.
### Table 1. Fibre composition of cellulose pulp

| Treatment                  | HWS (%) | Hemicellulose (%) | Cellulose (%) | Lignin (%) | Ash (%) |
|----------------------------|---------|-------------------|---------------|------------|---------|
| OPEFB                      | 15.21±0.06 | 22.10±0.58     | 36.56±0.55   | 24.84±0.96 | 1.29±0.23 |
| Alkali Delignified Pulp    | 5.49±3.05  | 17.31±4.84       | 73.01±5.43   | 4.00±2.41  | 0.20±0.06 |
| PAA 2.5%, 2 h              | 6.60±1.31  | 7.54±1.50        | 81.01±1.18   | 4.70±0.98  | 0.16±0.01 |
| PAA 5%, 2 h                | 5.79±0.33  | 9.16±1.44        | 78.59±0.62   | 6.34±1.72  | 0.12±0.02 |
| PAA 7.5%, 2 h              | 5.93±0.07  | 13.98±2.66       | 73.44±2.58   | 6.44±0.06  | 0.22±0.09 |
| PAA 2.5%, 4 h              | 7.74±0.79  | 9.95±1.61        | 77.23±2.32   | 4.92±0.12  | 0.15±0.04 |
| PAA 5%, 4 h                | 9.65±1.61  | 8.74±0.34        | 74.63±0.62   | 6.81±2.49  | 0.17±0.07 |
| PAA 7.5%, 4 h              | 10.51±1.30 | 8.50±0.37       | 77.71±1.10   | 3.14±0.13  | 0.14±0.04 |
| PAA 2.5%, 6 h              | 10.68±0.72 | 11.70±1.31      | 73.45±2.71   | 3.98±2.13  | 0.18±0.01 |
| PAA 5%, 6 h                | 10.54±0.55 | 10.78±0.21      | 75.32±0.29   | 3.12±0.11  | 0.25±0.06 |
| PAA 7.5%, 6 h              | 11.02±0.74 | 9.97±0.11       | 76.28±0.93   | 1.72±0.09  | 0.36±0.01 |

3.2. Surface Morphology of Product

Surface morphology of product resulted from this study can be seen in Figure 1. From this visual it is known that alkali delignified pulp as starting material has a diameter of 6721 nm while the diameter of product from acid hydrolysis was 2708 nm. Further hydrolysis using cellulase complex with 20% enzyme concentration for 12 hours hydrolysis time showed a reduction in diameter size. It was 869 nm, 960 nm, 1121 nm and 1365 nm.

![SEM images of alkali delignified pulp (A), nanocellulose from acid hydrolysis (B) and nanocellulose from acid-enzyme hydrolysis with diameter of 869, 960, 1121 and 1365 nm respectively (C-F)](image)

The plants cell walls arranged by elementary fibril with diameter 2-20 nm and length of a few micrometer. These cellulose microfibrils can be considered as CNFs, considering the definition of nanofiber size <100 nm in one dimension [6]. Based on these definition and the SEM imaging result, it was known that the products from the acid-enzymatic hydrolysis process in this study was still in the form of microfibrillated cellulose. The hydrolysis technique was not strong enough to produce nanostructured cellulose. Therefore, further treatment e.g with mechanical disintegration is needed to reduce the cellulose particle.
Previous researcher was successfully isolated cellulose nanofibre from different lignocellulosic sources by enzymatic hydrolysis with endoglucanase, xylanase or pectinase followed by mechanical treatment such as high pressure homogenization or sonication. The product has a diameter in the range of 10-20 nm with a length of 400 nm to several micrometers [16,17,18,19].

3.3. Weight of Product
Weight of product referred to the measurement of the weight of product resulted from acid-enzyme hydrolysis process, after being separated from the supernatant by centrifugation. These parameter was analyzed to identify how much cellulose fiber converted into micro/nanofibrilated cellulose through the hydrolysis process. Cellulase complex enzyme was reported can be used to hydrolyze the amorphous parts of cellulose fibers and capable of producing nanocellulose with a diameter of 5-10 nm and a length of 200 nm [20]. The treatment of 10% enzyme concentration after 12 hours hydrolysis time has the largest product weight, amounting to 9.41 grams. The use of a longer hydrolysis time affects the product weight loss (Figure 1).

The product weight referred to in this discussion is the overall weight of cellulose microfibrils obtained after acid-enzyme hydrolysis. Products separation based on its particle size was not carried out in this study. Acid hydrolysis followed by enzymes hydrolysis will degrade the amorphous part of cellulose, which causes a decrease in overall product weight. Higher enzyme concentration and longer hydrolysis time causes the higher product weight loses.

3.4. Particle Size Distribution
In this study, the size of nanocellulose was observed using Particle Size Analyzer. Treatment of 20% enzyme concentration and 12 hours hydrolysis time showed the smallest average particle size, which was 521.3 nm (Figure 2). Nanocellulose terminologically refers to cellulose which has a size of less than 100 nm at least in one dimension [6]. So, the particle size of product obtained in this study was still large. Based on the morphological evaluation by SEM as mentioned earlier, it was known that the product still in the form of microfibrilated cellulose. Particle size analysis with PSA are irrelevant when used for MFC which has a structure like spider-web.

![Figure 2. Weight of product](image-url)
3.5. Product Crystalinity

The crystallinity evaluation was done using X-ray diffraction. This test identifies the crystallinity index of product in crystalline and amorphous parts [21]. Alkali delignified pulp used as raw materials in this study have a crystallinity of 25%. After acid hydrolysis, the crystallinity index was 25.5%. After continued hydrolysis with enzymes, the highest crystallinity of nanocellulose was only 25.3% (Table 2). These results can be influenced by several factors. First, MFC naturally have both amorphous and crystalline parts which cause low crystallinity of product. It is different from nanocrystal cellulose, where the amorphous part has been removed so that the crystallinity index is very high. Previous study which was isolated cellulose nanocrystal from banana racis, sisal, kapok and pineapple leaf by acid hydrolysis with oxalic acid produced CNC with crystallinity index ranging from 80.9-92.3% [22].

| Treatment | Crystalline | Amorff |
|-----------|------------|--------|
| 12 h      | 24.3%      | 75.7%  |
| 24 h      | 24.7%      | 75.3%  |
| 36 h      | 25.3%      | 74.7%  |
| 48 h      | 23.3%      | 76.7%  |

Second, the presence of water and water-soluble solids such as salt and monosugar resulted from acid-enzymatic hydrolysis of cellulose allegedly also affects the low crystallinity of the product. Purification of nanocellulose through dialysis can be done to reduce the influence of dissolved solids in measuring the crystallinity of product.

3.6. Dispersion Stability

The easy way to identify nanocellulose is through its dispersion stability. Nanocellulose has the ability to form a stable suspension, depends on its functional properties, aspect ratio, and solvent ability to form hydrogen bonds between hydroxyl groups [10,23]. Table 3 shows the dispersion stability of MFC product, while the visual appearance of dispersion stability test can be seen in Figure 3. From the observation, it was known that the samples with code numbers 6 and 10 have the highest dispersion stability. Samples 6 were MFC obtained from the treatment of 15% enzyme concentration for 24 hours hydrolysis time, with average particle size was 822.7 nm. While sample 10 was MFC obtained from the treatment of 15% enzyme concentration for 36 hours hydrolysis time, with average particle size was 607.6 nm. The dispersion of all treatments was stable after 24 hours observation.
4. Conclusion
Peracetic acid (PAA) at low concentration can be used to maximize elimination of lignin from biomass. The use of acid concentration more than 5% was able to eliminate lignin significantly, but it is necessary to consider the decrease in cellulose content due to the hydrolysis process. The combination of acid and enzyme hydrolysis can be done to reduce the concentration of sulphuric acid but there is a tendency of a decrease in product crystallinity. The treatment of enzyme concentration and the duration of enzyme hydrolysis give different responses to the parameters of particle size, product weight, crystallinity and dispersion stability. It can be concluded that the acid- enzymatic hydrolysis method used in this study can produced microfibrilated cellulose but not for nanocellulose. The process needs to be improve in terms of the type of enzyme used, the condition of the hydrolysis process, and further treatment by mechanical treatment to maximize defibrillation and disintegration of cellulose particles

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