Optimization of Cellulose Nanofiber Production from Oil Palm Empty Fruit Bunch Using *Trichoderma* sp. with the Solid State Fermentation Method

Pingkan Aditiawati, a Rudi Dungani,a,* Redian Mayo Fikri,a and Sri Hartati b

Cellulose nanofibers were isolated from oil palm empty fruit bunch by delignification using *Marasmius* sp. and subsequently treated by solid state fermentation using *Trichoderma* sp. This method used pH values of 4.8, 5.5, and 7 and temperatures of 28 °C, 32 °C, and 37 °C for 7 d. Scanning electron microscopy, Fourier transform infrared (FT-IR) spectroscopy, X-ray diffraction (XRD), and particle size analysis were performed to determine the properties of the isolated cellulose nanofibers. Results showed that chemical analysis by FTIR, lignin was completely removed from the EFB. It was found that the combination of pH 5.5 and incubation temperature 37 °C were favorable for the SSF process to isolate cellulose nanofibers from oil palm EFB fiber. SSF processing resulted in smooth morphological structures, with a fiber diameter range of 32.6 nm to 36.4 nm.

Keywords: Nanocellulose; Enzymatic; Delignification; Marasmius sp.; Cellulase; Trichoderma

Contact information: a: School of Life Sciences and Technology, Institut Teknologi Bandung, Bandung 40132, West Java, Indonesia; b: Faculty of Agriculture, Padjadjaran University, Bandung 45363, Indonesia; *Corresponding author: rudi@sith.itb.ac.id

INTRODUCTION

Recently, lignocellulosic materials from oil palm empty fruit bunch (EFB) have emerged as an abundant resource because of the increase in oil palm tree plantations (Dungani et al. 2013). It has been reported that Indonesia produces approximately 21% of the total annual production of palm EFB (Yuliansyah et al. 2009). Indonesia produced 28.6 million MT of EFB in 2014 and 30.6 million MT in 2015 (Hambali and Rivai 2017). Some portion of EFB is used as fuel and other oleochemical substances, and the remaining part is combusted for fertilizer or discarded. Therefore, an alternative ecofriendly way of treating EFB is required. Empty fruit bunch waste has potential and can be converted into different products. This is mainly because of its lignocellulosic content, which is up to 50% cellulose. One of the potential products is nanocellulose, which has a promising prospect in nano-based industries (medical, automotive components, pharmacy, etc.) because of its unique characteristics, such as renewability, biodegradability, and non-toxic properties. Therefore, it can be used to manufacture products that range from food packaging to biomaterials for surgeries (Dufresne 2013). Numerous studies have been conducted on the isolation of EFB nanofibers via various methods, such as chemical (Abdul Khalil et al. 2012), mechanical (Abdul Khalil et al. 2014), and chemo-mechanical treatments (Fatah et al. 2014).

Microbiological delignification is a relatively new approach that depends on the use of ligninolytic enzymes produced by a wide range of microorganisms. Therefore, the study of the delignification ability of each microorganism is important to the development
and further use of this approach. A large number of microorganisms can degrade cellulose; however, only a few of them produce significant quantities of free enzymes capable of completely hydrolysing cellulose (Koomnok 2005). Therefore, the application of fungi in producing cellulase is widely used. The cellulolytic properties of some fungi genera such as Trichoderma sp. and Marasmius sp. are well known (Aditiawati et al. 2018). Trichoderma viride, Aspergillus niger, and Fusarium oxysporum (Kadarmoidheen et al. 2012), also have been reported. The production of cellulase enzyme depends upon a complex relationship involving a variety of factors including inoculum size, temperature, pH value, medium additives, presence of inducers, aeration, growth time, and so forth (Immanuel et al. 2006). As the demand for nanocellulose increases, an environmentally friendly large-scale method for nanocellulose production is needed.

Meanwhile, the process of delignification is a biological pretreatment that must be carried out to reduce the content of lignin, which tends to hinder access of enzymes to the cellulose. There were many kinds of white rot fungi that have been used for bio-delignification, such as Marasmius sp. (Darsih et al. 2015; Aditiawati et al. 2018). This fungus has the ability to produce enzymes that can break down organic matter with high levels of lignin and cellulose (Harvey et al. 1993)

One solution is the use of solid state fermentation (SSF) with Trichoderma sp. to produce nanocellulose enzymatically. This method has the advantage of reduced energy consumption compared with mechanical processes; it is an environmentally friendly approach (no corrosive waste is produced); and the nanocellulose product obtained is superior (Henriksson et al. 2007). This SSF method has been shown by many researchers to be better for filamentous fungi (Mojsov 2010; Sun et al. 2010; Briiwani and Vadlani 2011; Nadagouda et al. 2016). Studies on SSF and some of their results have provided a substantial contribution to the improvement of the existing and widely used technology. Therefore, it is necessary to optimize the production of nanocellulose in terms of various parameters that greatly affect culture growth of Trichoderma sp. such as pH and temperature. Previously studies on the use of pH values have been done, namely at pH 4.8 by Vigneshwaran and Satyamurthy (2016); pH 5.5 by Ahmed et al. (2009); and pH 7 by Ibrahim et al. (2013). This is also in accordance with Singh et al. (2014), where Trichoderma can grow in a pH range between 4 to 8. Determining the optimum incubation temperature also follows from the growth temperature range of Trichoderma sp., which is at 7 to 42 °C (Domingues et al. 2016).

The present work was carried out to optimize the pH and temperature of growth of Trichoderma sp. for cellulose nanofibers (CNF) production from EFB through SSF method. The optimization was measured based on the characteristics of the CNF production by particle size analyzer (PSA), scanning electron microscopy (SEM), X-ray diffractometer (XRD), and Fourier transform infrared (FT-IR) spectroscopy.

**EXPERIMENTAL**

**Materials**

The oil palm EFB sample was collected from Riau, Indonesia. The EFB was cut into small pieces and soaked in water. The EFB was dried until the overall moisture content reached 5% ± 0.2% and was then cut into 10-mm to 20-mm pieces. In this study, the brown-rot fungi Trichoderma sp. and Marasmius sp. (Mycology Laboratory, Biological Department, Institut Teknologi Bandung, Indonesia) were grown in Roux bottles
containing a potato dextrose agar (PDA) medium. The harvested spores were collected and counted with a haemocytometer until the spore count was approximately $10^6$ spores/mL. One hundred microliters of spore suspension were inoculated on a PDA slant in a reaction tube. The spores were harvested every 12 h for counting and were spread in a petri dish containing a PDA medium with serial spore dilutions of $10^1$ spores/mL, $10^2$ spores/mL, and $10^3$ spores/mL. The spore viability was calculated using Eq. 1:

$$\text{Spore Viability (\%)} = \frac{\text{Colony Count (CFU/mL)} \times \text{Dilution Factor}}{\text{Spore Count}}$$

Methods

Dry weight curve analysis

*Trichoderma* sp. fungus was grown in Roux bottles containing a PDA medium. The spores were harvested and counted until a count of $10^7$ spores/mL was obtained. The spores were inoculated in 20 Erlenmeyer flasks, where each contained 100 mL of potato dextrose broth medium, and were then agitated at 150 rpm. Each Erlenmeyer flask was sampled every 12 h using filtration with Whatman filter paper no. 1 in a Buchner funnel to separate the medium and mycelia.

The filter paper was dried in a 50 °C oven for 24 h, and this was followed by desiccation for 24 h. It was then weighed to determine the dry weight, which was calculated using Eq. 2:

$$\text{Dry Weight (g)} = \frac{\text{Weight of Filter Paper after Desiccation (g)}}{\text{Initial Weight of Filter Paper (g)}}$$

Delignification

*Marasmius* sp. fungus was grown in a PDA medium for 24 h, transferred to a Cerealia medium, and then incubated for 86 h at 25 °C with triplicate measurement. Exactly 4 g of mycelium was then inoculated in a mixture containing 20 g of EFB, 20 mL of basal medium, and 0.1 g of sugar in a baglog, which was followed by 28 d of incubation. After 28 d, the lignin, cellulose, and hemicellulose contents were measured using Chesson method with fractionation sequentially to various components (Datta 1981). Triplicate experiments for each component fractionation experiment were performed and the reported data are the average values.

Solid state fermentation

Solid state fermentation was used to hydrolyze cellulose from the EFB using cellulolytic enzymes from *Trichoderma* sp., which were grown for 24 h in a Roux bottle containing a PDA medium. The spores were then harvested and counted until a count of $10^6$ spores/mL was attained.

Approximately 100 μL of spores were inoculated in 100-mL Erlenmeyer flasks containing 1 g of EFB and 1 mL of basal medium with three different pH values (4.8, 5.5, and 7). The EFB was then incubated for 7 d at three different temperatures ($\pm 28$ °C, 32 °C, and 37 °C). Thus, there were nine variations with three replications (Table 1). The enzyme activity was determined by the dinitrosalicylic acid method (Wang *et al.* 2015).
Table 1. Conditions of pH and Temperature of *Trichoderma* sp. Incubation

<table>
<thead>
<tr>
<th>Label</th>
<th>Variations Condition</th>
<th>pH</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td></td>
<td>4.8</td>
<td>± 28</td>
</tr>
<tr>
<td>1.2</td>
<td></td>
<td>4.8</td>
<td>32</td>
</tr>
<tr>
<td>1.3</td>
<td></td>
<td>4.8</td>
<td>37</td>
</tr>
<tr>
<td>2.1</td>
<td></td>
<td>5.5</td>
<td>± 28</td>
</tr>
<tr>
<td>2.2</td>
<td></td>
<td>5.5</td>
<td>32</td>
</tr>
<tr>
<td>2.3</td>
<td></td>
<td>5.5</td>
<td>37</td>
</tr>
<tr>
<td>3.1</td>
<td></td>
<td>7.0</td>
<td>± 28</td>
</tr>
<tr>
<td>3.2</td>
<td></td>
<td>7.0</td>
<td>32</td>
</tr>
<tr>
<td>3.3</td>
<td></td>
<td>7.0</td>
<td>37</td>
</tr>
</tbody>
</table>

*Post-treatment*

The EFB was cryocrushed with liquid nitrogen in a mortar and pestle (Dhandapani and Sharma 2014). It was then mixed with 30 mL of Aqua Des™ (Indo Daisun Sakti, Jakarta, Indonesia) in an Erlenmeyer flask and agitated for 24 h so that it was swollen. The EFB sample was then sonicated for 15 min. The process was performed at a frequency of 25 kHz with an output power of 200 W. The ultrasonic fibrillation was conducted in an ice bath (0 °C) to overcome the possibility of overheating throughout the entire process. Thereafter, the suspension was separated by centrifugation at 4500 rpm and 4 °C for 15 min (Zhou et al. 2012). A nanocellulose suspension (1.5 mL) was then collected for the PSA with the dynamic light scattering (DLS) method. The suspension was dried in a 50 °C oven for SEM imaging (Hitachi SEM SU3500, Tokyo, Japan), diffractograms the particle size using XRD (PHILIPS PW 1050 X-pert Diffactometer, Germany), and FT-IR characterization (Nicolet Avatar 360, New York, USA).

**RESULTS AND DISCUSSION**

**Spore Viability**

Figure 1 shows that the spore viability fluctuated from 0 h to 36 h and was relatively stable from 36 h to 120 h. Figure 1 also shows that the spore viability was reduced in the duration from 0 until 12 h. This is because at time 0 h there were spores coming from the inoculum, which was transferred to the Roux bottle. The spore viability decreased because of the germination of the spores into mycelium. The highest spore viability was achieved at 24 h and was 23.2%. Commonly, the spore count increases with a longer incubation time. In this study, a count of 10⁹ spores/mL was obtained, which was the highest spore viability. From these criteria, the 24-h incubation time was preferred because it had the highest viability and a spore count of approximately 10⁹ spores/mL.

This result was supported by a previous study (Daryaei et al. 2016), where the germination proportion became high after 22 h. Hence, the viability also increased and the spore count reached 10⁸ spores/mL in 3 d. Because nutrient depletion is directly connected with the spore count, the spore count achieved in 24 h could prevent immediate nutrient depletion (Gopalakrishnan et al. 2016).

The dry weight was plotted after 228 h of incubation with sampling times every 12 h. The activity of cellulase enzyme, can be seen 4 growth phases of *Trichoderma* sp. namely the log phase, the logarithmic phase, the stationary phase, and the death phase. A lag phase was observed from 12 h to 36 h. In this phase, cell duplication occurred regularly...
and constantly (Wang et al. 2015). From dry weight, optimum inoculum age was determined using the half log phase at 24 h.

![Spore viability curve](image)

**Fig. 1.** Spore viability curve of the *Trichoderma* sp. after 120 h

**Delignification**

The results of the delignification process were examined morphologically with SEM analysis, and chemically examined using the lignin, cellulose, and hemicellulose content changes. The SEM results are shown in Fig. 2.

![SEM micrographs](image)

**Fig. 2.** SEM micrographs of the EFB: (a) before delignification; (b) after 28 d of delignification; and (c) EFB surrounded by mycelia after 28 d

Figure 2a shows that the surface of the EFB was still intact and silica particles were present. After delignification, the surface structure was degraded and the silica particles were removed from the surface (Fig. 2b). The purposes of delignification is the removal or breakdown of lignin, which in the process results in the removal of silica particles, which are usually found in fibers such as EFB. The removal of silica particles is an important process that enables fibers to be efficiently treated biochemically with enzyme (Omar et al. 2014). Silica removal is also done in some pulping processes. Meanwhile, Figure 2c shows that mycelium from *Marasmius* sp. stick to the EFB fibers that have not been cleaned. This indicates the occurrence of the activity of the fungus.

The EFB was also examined chemically after delignification via measurement of the lignin, cellulose, and hemicellulose content changes. Table 2 shows that the lignin, cellulose, hemicellulose, and silica were reduced by 15.5%, 7.4%, 10.0%, and 11.6%, respectively. Analysis of EFB fibers for chemical composition showed significant differences between the control (before delignification), and after delignification. Although the main goal of the delignification process was to reduce the lignin content, the cellulose,
hemicellulose, and silica contents were also reduced (New et al. 2019). This was because of the nature of Marasmius sp., which also produces cellulase enzymes (Kamcharoen et al. 2014).

**Table 2.** Lignin, Cellulose, and Hemicellulose Content Changes in the EFB Sample

<table>
<thead>
<tr>
<th></th>
<th>Lignin (%)</th>
<th>Cellulose (%)</th>
<th>Hemicellulose (%)</th>
<th>Silica (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>16.88 (0.37)*</td>
<td>44.42 (1.01)*</td>
<td>21.39 (0.68)*</td>
<td>0.43 (0.24)*</td>
</tr>
<tr>
<td>After Delignification</td>
<td>14.27 (0.34)</td>
<td>41.13 (0.74)</td>
<td>19.24 (0.67)</td>
<td>0.38 (0.22)</td>
</tr>
<tr>
<td>% Reduction</td>
<td>15.46 (0.66)</td>
<td>7.41 (0.29)</td>
<td>10.05 (1.01)</td>
<td>11.63 (0.87)</td>
</tr>
</tbody>
</table>

*Values in parentheses are standard deviations

**Nanocellulose Analysis**

After SSF by Trichoderma sp. for 7 d, the cellulase enzyme activity was measured and is shown in Fig. 3. The particle size was measured after the EFB underwent delignification, SSF, cryocrushing, and sonication. Table 3 shows that there were three variations with invalid results, which were 1.2, 2.2, and 3.3.

**Fig. 3.** Cellulase enzyme activity of the *Trichoderma* sp. after 7 d of incubation

**Table 3.** Particle Size Distributions and P.I for All of the Variations

<table>
<thead>
<tr>
<th>Sample</th>
<th>Label</th>
<th>D (10%) (nm)</th>
<th>D (50%) (nm)</th>
<th>D (90%) (nm)</th>
<th>P.I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Control</td>
<td>K_A</td>
<td>278.7</td>
<td>332.2</td>
<td>482.9</td>
<td>0.648</td>
</tr>
<tr>
<td>Delignification</td>
<td>K_D</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH = 4.8</td>
<td>± 28 °C</td>
<td>1.1</td>
<td>88.2</td>
<td>99.9</td>
<td>138.8</td>
</tr>
<tr>
<td></td>
<td>32 °C</td>
<td>1.2</td>
<td>88.2</td>
<td>99.9</td>
<td>138.8</td>
</tr>
<tr>
<td></td>
<td>37 °C</td>
<td>1.3</td>
<td>69.4</td>
<td>78.4</td>
<td>108.7</td>
</tr>
<tr>
<td>pH = 5.5</td>
<td>± 28 °C</td>
<td>2.1</td>
<td>262.3</td>
<td>310.3</td>
<td>452.8</td>
</tr>
<tr>
<td></td>
<td>32 °C</td>
<td>2.2</td>
<td>100.8</td>
<td>113.9</td>
<td>157.9</td>
</tr>
<tr>
<td></td>
<td>37 °C</td>
<td>2.3</td>
<td>32.6</td>
<td>32.6</td>
<td>36.4</td>
</tr>
<tr>
<td>pH = 7.0</td>
<td>± 28 °C</td>
<td>3.1</td>
<td>222.8</td>
<td>265.4</td>
<td>389.5</td>
</tr>
<tr>
<td></td>
<td>32 °C</td>
<td>3.2</td>
<td>531.5</td>
<td>637.7</td>
<td>929.6</td>
</tr>
<tr>
<td></td>
<td>37 °C</td>
<td>3.3</td>
<td>14.1</td>
<td>16.4</td>
<td>23.3</td>
</tr>
</tbody>
</table>

D – particle size distribution

These invalid results were observed because of the absence in the particle size distribution graph. For variations 2.2 and 3.3, the invalid results may have been because the *Polydispersity Index* (P.I) number was outside of the measurement limit for the DLS.
method (0 to 1). Therefore, the optimum variation was 2.3, which had the lowest particle size distribution (90%). This was supported by the mean data that is shown in Fig. 4.

The mean particle size (Fig. 5) supported variation 2.3 (pH = 5.5; 37 °C) as the optimum variation because it had a mean value of 33.4 nm, which was one of the lowest sizes among all of the variations. These findings were different from those of Domingues et al. (2016), who found that the optimum temperature for Trichoderma sp. growth is 27 °C. This may have been because of the difference in optimum temperatures for Trichoderma sp. growth and cellulase enzymes. The cellulase enzyme has an optimum activity over a temperature range of 40 °C to 50 °C (Pardo and Forchiasiin 1999). This temperature range effectively optimized the hydrolysis of cellulose, which was higher than the optimum growth temperature and was close to the cellulase activity.

**Fig. 4.** Mean value of the particle size for each experimental variation

The nanocellulose yield obtained was calculated with the DLS data. Nanocellulose is defined as a particle with a maximum particle size of 100 nm in at least one dimension (Jonoobi et al. 2011). The data obtained are presented in Fig. 5.

**Fig. 5.** Amount of nanocellulose (< 100 nm) obtained for each experimental variation
Figure 5 shows that variation 2.3 produced 100% nanocellulose, which meant that the suspension contained 100% nanocellulose regardless of its amount. The similarly results are also in variation 3.3 which produce 100% nanocellulose. This is in accordance with the study by Singh et al. (2014), which showed the optimum pH value of *Trichoderma* sp. in the range of 5.5 to 7.5. However, the temperature that produces the smallest particle size is not in accordance with the optimum temperature of growth of *Trichoderma* sp. that is at 27 °C (Domingues et al. 2016).

Figure 6 shows that nanocellulose with sizes of 41.0 nm and 45.0 nm were present. This confirmed the PSA result that a nanocellulose size of less than 100 nm was found. There was also a bigger particle size detected because of the agglomeration of cellulose and the mandatory drying process prior to the SEM examination, which formed hydrogen bonds (Peng et al. 2012). From the SEM the show that image does not look clear, this is due to the re-aggregation of nanocellulose particles. Aggregation can occur starting shortly after the enzymatic process from cellulase is terminated until the process of drying nanocellulose in the process of preparing SEM samples (Karim et al. 2016). Figure 6 also revealed that the nanocellulose was dispersed uniformly, showing rod-shaped particles.

![SEM micrograph of the sample from the optimum experimental variation (pH = 5.5; temperature = 37 °C)](image)

The X-ray diffraction (XRD) spectra of nanocellulose are shown in Fig. 7. The fiber crystallinities gradually increase at each stage of the process. Delignification *Marasmius* sp removes lignin and hemicelluloses, so that the percentage of the crystalline regions in cellulose increased. Nanocellulose produced using solid state fermentation method and through cryocrushing, and sonication process accelerated the cleavage of the cellulose molecular chains within the amorphous regions resulting in the further increase of the crystallinity (Joshi et al. 2018). The diffractograms are shown in Fig. 7. The nanocellulose specimens exhibited two diffraction peaks; they were $2\theta = 16.2^\circ$ and $23.5^\circ$. Cellulose has amorphous and crystalline parts; the amorphous will be more vulnerable to isolation process (Brinchi et al. 2013). The degradation by using cellulolytic enzymes from *Trichoderma* sp. will break the amorphous region of cellulose to produce nanocellulose with higher crystallinity index. The increase in crystallinity from 26.65% to 76.15% was also due to the refining, cryocrushing, and sonication.
Fig. 7. X-ray diffraction spectra (XRD) of nanocellulose

Fig. 8. FT-IR spectra of various samples. (a) Initial control; (b) delignification control; (c) nanocellulose

The sample was also characterized by FT-IR spectroscopy to determine the functional groups. Figure 8 shows various peaks in the initial control, delignification
control, and nanocellulose samples. From these peaks, the initial control samples had peaks with a lower transmittance, except at 1400 cm$^{-1}$, where the nanocellulose sample had the lowest transmittance (Fig. 8a). The nanocellulose sample had the highest cellulose content, which was expected. The peak at 1050 cm$^{-1}$ was due to the presence of C-O stretching vibration all three polymers, namely lignin, cellulose, and hemicellulose (Loow et al. 2018).

The result of FT-IR for delignification control sample shows five main functional groups that are attributable to cellulose (Fig. 8b). The O-H group (3345 cm$^{-1}$) is a hydrogen bond that functions to bind the cellulose microfibril structure to one another, allowing it to remain structured and compact. The CH$_2$ group (2898 cm$^{-1}$ / 1314 cm$^{-1}$) is a carboxyl group that can be used to estimate the level of cellulose crystallization. The H-O-H group (1644-1650 cm$^{-1}$) was used to determine the level of water adsorption in the sample. The C-O group (1107 cm$^{-1}$) is a polyhydroxyl group and shows that the delignification control sample is formed from glucose or its derivatives. C-O-C group (1050-1055 cm$^{-1}$) is a glycosidic bond that plays a role in the bonding of glucose polymers to form cellulose (Mohammadkazemi 2015).

It can be seen that all the main functional groups associated with the delignification control sample were also associated with the nanocellulose samples (Fig. 8c). This result indicates that the nanocellulose sample is indeed cellulose. Changes in cellulose size also affect H-O-H groups in cellulose, where the adsorption of water in cellulose becomes smaller (Aditiawati et al. 2018).

**CONCLUSIONS**

1. Cellulose nanofibers were successfully isolated from the EFB, which is an abundant biomass in Indonesia, through delignification using *Marasmius* sp. and the SSF method using *Trichoderma* sp.

2. The combination of pH 5.5 and incubation temperature 37 °C were found to be favourable for solid-state fermentation (SSF) to isolate cellulose nanofibers from oil palm empty fruit bunch fiber. With this SSF combination, a 100% nanocellulose content was achieved having a mean particle size of 33.4 nm.

3. The results showed that the SSF method with various pH values and incubation temperatures was an important factor in determining the properties of the CNFs from the EFB.

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