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Effect of Fungal Pretreatment by *Aspergillus Niger* Assisted by Ultrasonication on Microfibrillated Cellulose Obtained from Cocoa Pod Husk

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Abstract – *Microfibrillated cellulose (MFC) can be used in many industrial areas such as composites for construction, automotive, furniture and new materials for electronics and pharmaceutical applications as well as thickener in cosmetic products. It can be isolated from different lignocellulosic materials using numerous technologies. In this study, MFC was produced from cocoa pod husk (CPH) substrate in two distinct processes. Substrate was subjected to fungal-pretreatment at the first stage through a solid state fermentation by Aspergillus niger to promote enzymatic splitting of cellulose. The fungal pretreated substrate was then mechanically processed by the application of high-intensity ultrasonication. The optimum process of the fungal pretreatment stage through a response surface methodology (RSM) by employing central composite design (CCD) using Design expert version 8.0 with substrate raw particle size (RPS) inoculum size (IS) as independent variables were investigated. Statistical result of RSM showed that the model follows a quadratic response with IS as significant term. The 3D model graph exhibits an inverted peak response surface with maximum MFC particle size of 2761 nm at RPH of 850 µm and 5.0% IS. SEM images showed that the structure and morphology of the MFC differed over the various levels of IS with MFC diameter between 30 – 50 µm. Fungal-pretreatment was able to remove amorphous structure of hemicelluloses and lignin giving a loosen structure that less than 100µm. FTIR analyses indicated a prominent spectra change between 3600cm^{-1} and 2600 cm^{-1}. The peak at approximately 1730 cm^{-1} of the raw substrate attributes to the presence of hemicellulose, which this spectrum poses lower intensity and almost disappeared after the fungal treated samples were ultrasonicated. The appearance of 2θ = 150 for both treated MFC was also observed from XRD diffractograms which indicates the high content of crystalline cellulose.*

Keywords: microfibrillated cellulose, fungal pretreatment, ultrasonication, *Aspergillus niger*, cocoa pod husk

1.0 INTRODUCTION

Cocoa pod husk (*Theobroma cacao L.*), *Sterculiaceae* family, is a major waste generated from the cocoa industry. These husks represent between 70 to 75 % of the whole weight of the cocoa fruit, means if per each ton of cocoa fruit there will be between 700 to 750 kg of waste. Improper management of this waste will give huge impacts to the environment such as generating foul odors and propagate diseases. Few studies have been carried out, that utilizes

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these biomass wastes to produce food antioxidants, dietary fibers and animal feed. This study attempts to utilize cocoa pod husk biomass and isolate microfibril cellulose (MFC) from it, which is has the potential as mechanical reinforcement materials in bionanocomposite industry.

Microfibrillated cellulose (MFC) is a nano-cellulosic material that is obtained through fibrillation of lignocellulosic materials via high shear force of mechanical refining. The size of the cellulose depends on the composition of lignocellulosic materials, degree of processing and pretreatment applied prior to mechanical refining. Typically, fibrous cellulose materials obtained from higher plants have a thickness in the range of 2 to 10 nm, with a few tens of microns long. Its thickness could also be in the range of 20–40 nm, as MFC is often composed of cellulose microfibrils aggregates [1]. Other studies reported that this type of nano-cellulosic material has high aspect ratio, with width of 10–100 nm and length 0.5–10'sμm, and contain both amorphous and crystalline regions [2]. Crystalline cellulose has I, II, III and IV polymorphs[4]. Cellulose I is known as "natural" cellulose, since its crystalline cellulose can be naturally found in trees, algae, and bacteria. Its structure is thermodynamically metastable and can be converted to either cellulose II or III [4].

The MFC has attracted a great interest due to its low density, high mechanical properties, biodegradability, renewability and appealing economic value [5]. Its production commonly comes from the abundant source of woody plants, such as kraft pulp, which needs high energy to fibrillate. However, in the case of MFC produced from agroindustry wastes such as corn, wheat, rice, sugar cane, pineapple and coconut crops, their lignin contents are generally less than wood and thus demand less energy. This is because their cellulose fiber is present in the plant's secondary wall, which is easier to separate from the primary wall. Many studies have been done previously where the nanocellulose fibers were isolated using a combination of mechanical and chemical procedures. Pretreatment prior to mechanical treatment (refining/homogenizing process) could significantly reduce energy consumption in the refining/homogenizing process of the feed material. The energy consumption in the latter process is very high due to the predominating hydrogen bonding between the cellulose microfibrils. Strong acidic or alkaline solutions are often used for pretreatment of lignocellulosic biomass. Strongly acidic solutions, combined with sonication, lead to aggressive hydrolysis of the noncrystalline fractions[5]. This result yielded mainly low aspect ratio cellulose I fibril aggregates which are not particularly suitable for mechanical reinforcement [6]. Previous study reported that strong acids are oxidizing agents and would dehydrate and redistribute the biopolymers in lignocellulosic materials [7]. The increment of acid concentrations would also cause the crystallinity index of the cellulose to decrease rapidly. Utilization of alkaline/ acid treatment needs to be carefully controlled to avoid undesirable cellulose degradation. Furthermore, the use of chemical pretreatment would generate toxic byproducts and cause corrosion of equipment, which could translate into high operational and maintenance costs. Thus, less aggressive hydrolysis is required extensively to obtain nano-scale cellulose fibers with entangled networks and improved strength, while maintaining its high aspect ratio. As a result, the isolated cellulose I fibrils will be suitable for use as mechanical reinforcement materials. Previous study showed that the reinforcing effect of nanocellulose materials on the composites depends on the micro/nanofibre entanglements, which are a consequence of their morphology (diameter and length) [8]. On the other hand, the microfibrillated cellulose produced from enzymatically pretreated cellulosic wood fibers yielded a more favourable structure than nanofibers produced by pretreatment of strong acid hydrolysis [9]. Lignocellulose biomass is a cellular complex which consists of cellulose, hemicellulose and lignin, thus a specific process is needed to produce microfibrillated cellulose/ nanocellulose effectively. The application of enzymatic hydrolysis as an alternative

of chemical pretreatment has growing interest due to its highly specific process and can be performed under mild conditions with lower energy consumption as well as less corrosive. This treatment involves a set of cellulases which can be categorized as A- and B- type cellulases (termed cellobiohydrolases) which are able to attack highly crystalline cellulose while C- and D-type cellulases (termed endoglucanases) attacks region of low crystallinity in the cellulose fiber creating. This means the enzymatic pretreatment is mild hydrolysis and the degradation of cellulose can be avoided. This enzymatic pretreatment will also facilitate the subsequent mechanical steps by minimizing the shearing energy through less required passes and preventing the blockage of the homogenizer.

A previous study showed that combining enzymatic hydrolysis with mechanical shearing and high pressure homogenization produces longer and highly entangled nanoscale fibrils, which are more feasible to be used as reinforcement composites materials [4]. They also observed that the resulting cellulose I elements have a higher aspect ratio and high elastic modulus, roughly 2 orders of magnitude larger than the corresponding gels made out of acid hydrolysis[5]. In other studies, the enzyme-treatment was found to facilitate disintegration and the MFC fibers produced also showed higher average molar mass and better aspect ratio than nanofibers resulting from acidic pretreatment [8]. However, the major hindrance for a broader application of these enzymes in the process of cellulose separation is the very high cost of the enzymes themselves [9]. The use of microorganisms, particularly fungi, to degrade lignin and hemicellulose while leaving the cellulose intact is a potential alternative method [10]. Fungal pretreatment, combined with other pretreatments, is expected to result in rapid hemicellulose and lignin hydrolysis. Solid state fermentation (SSF) of suitable agricultural residues by *Aspergillus niger* can produce the required biomass degrading enzymes for the cellulose isolation process.

Regardless of the pretreatment technique, the mechanical step for the MFC production is always required. Recently, ultrasonication is an emerging method to isolate cellulose nanofibers [11]. Cavitation energy from ultrasonication process, which is generated due to the powerful mechanical oscillations and high intensive waves may cause the formation, expansion, and implosion of microscopic gas bubble 34]. This induce the disintegration of micron-sized cellulose fibers into nano-scale fibers. The applied output power of the ultrasonic treatment may influence the extent of the cellulose fiber fibrillation [11]. It was found that the disintegration of micron sized cellulose fibers into nanofibers is efficient when the ultrasonication output power is greater than 1000W [11]. Therefore, this study aims to investigate the effect of fungal pretreatment assisted with ultrasonication on the production of MCF from cocoa pod husk substrate. The raw cocoa pod husk was pretreated with *Aspergillus niger* in SSF at various inoculum and substrate particle size.

2.0 MATERIALS AND METHODS

An optimization process was conducted on the utilization of solid-state fermentation at the pretreatment stage of MFC production followed by mechanical isolation of MFC by ultrasonic treatment.

2.1 Preparation of Raw Materials

Cocoa pod husk (CPH) was obtained from the Cocoa Research & Development Centre of the Malaysian Cocoa Board, Jengka, Pahang, Malaysia immediately after harvesting the fruits. The

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CPH substrate was rinsed water and oven dried at 55° C for 72 hours prior to the size reduction step through a grinding process using a blender into granular form. Substrates were stored in an air-tight container at room temperature.

Aspergillus niger from the Bioenvironmental Engineering Laboratory, Department of Biotechnology Engineering, International Islamic University Malaysia (IIUM) used in the pretreatment stage was uniformly cultured in a potato-dextrose (PDA) agar media for 7 days prior to the fermentation stage. The inoculum was prepared by flooding the agar slants with water and dislodging the spores by gentle scrapping. The inoculum suspension was kept at 4 °C prior to use.

2.2 Fungal Pretreatment

Fungal pretreatment on CPH substrate was performed through solid-state fermentation (SSF) in 250 ml conical flasks by inoculating 8.0 g of CPH with *A. niger* inoculum. Each inoculated flask was incubated for 5 days at 32 ˚C, with initial pH of 5.5, and moisture content of 66.0% (w/w). A modified-basalt media at 5.0% of total fermentation volume was prepared as described by Oyeleke et al. [12] to sustain the growth of the fungus during the fermentation process. The modified-basalt media contained (in g/l) yeast extract (2.0), $NaNO_3$ (5.0), KH_2PO_4 (1.0), $MgSO_4.7H_2O$ (0.5), $FeCl_3$ (0.001).

2.3 Ultrasonic Treatment

Mechanical treatment to obtain the individualized fiber was performed through an ultrasonic treatment (FB-705 Fischer-Scientific™ Ultrasonic Dismembrator) by 70% amplitude and 3 cycles exposure time of 120 seconds per cycle. Fungal pretreated CPH was thoroughly and repeatedly rinsed by sterilized distilled water using filtration and centrifugation processes to remove the fungal spores retained on the surface of CPH. A suspension of 2.0% (w/v) was made by submerging the CPH into sterilized distilled water based on a method described by Dufresne et al., [13]. Ultrasonication was performed by a microtip probe towards the CPH-water suspension insulated in an ice-bath vessel. The resulting MFC suspension was freeze dried to remove water content and to avoid any agglomeration of cellulosic suspension[3]Freeze-dried samples were all kept in a sealed airtight container at -80 ˚C to prevent changes and rearrangement of the structures.

2.4 Optimization of MFC Particle Size

Response surface method (RSM) was used as the experimental design by employing central composite design (CCD) using Design expert version 8.0 towards the SSF pretreatment stage to obtain the optimum MFC particle size. Two independent variables of granular raw particle size (RPS) at 200, 525, and 850 µm of CPH and initial inoculum size (IS) during SSF at 2.0, 3.5, and 5.0% (w/v) were performed. All samples in SSF were made in triplicate and five center-points were used. Statistical analyses were performed by Stat-Ease Design Expert™ Version 7.0.

Individualized-fiber size observation was approached through an observation by Malvern™ Nano ZS. Water was used as the dispersant medium in disposable cuvettes at 25°C and measured viscosity of 0.8872 cP. Measurement angle was set to 173° Backscatter with 3 measurements performed with no delay for each samples.

2.5 Scanning Electron Microscopy (SEM)

The freeze-dried MFC after the ultrasonic treatment underwent an observation through scanning electron microscopy (Hitachi SU-1510). Observations were performed at 15.0 kV towards small gently scattered amount of MFC on top of a carbon tape, and in the absence of gold (Au) coating for all samples.

2.6 Fourier transform infrared (FTIR) spectroscopy

The FTIR spectra were recorded by IRAffinity-1S FTIR Shimadzu™ for mid-IR frequency range (600 – 4,000 cm^{-1}) with a resolution of 4 cm^{-1}. All samples were read in triplicates.

2.7 X-ray diffraction (XRD) spectrometry

The X-ray diffraction (XRD) patterns were measured for raw CPH, ultrasonicated without ultrasonicated MFC and fungal-pretreated-ultrasonicated MFC with D8 Advance x-ray diffractometer (Bruker AXS, Germany). Power was set to 40 kV and 40 mA. Angular reading was set to $\alpha = \beta = \gamma = 90$ for all samples. Crystallinity index (CrI) was calculated from the X-ray diffraction patterns according to the equation performed by Segal et al. [14]:

$$CrI = \frac{I_{002} - I_{am}}{I_{002}}$$

Where I_{002} and I_{am} are the intensities of the peaks at $2 - \theta$ of about 22 and 18, respectively.

3.0 RESULTS AND DISCUSSION

3.1 Microfibrillated Cellulose (MFC) Particle Size and Optimization

In this study, determination of MFC particle size is based on the movement speed of particles which is randomly move in the liquid (Brownian motion). The measurement is conducted using Dynamic Light scattering (DLS). Thus, rather than specifying the particle size as the diameter of the MFC, it might be best to use the data as the approximated length of the MFC measured.

The ANOVA analysis for the optimization of RPS and IS in the isolation of MFC followed a quadratic response with an inverted peak response surface. The model F-Value observed was 9.11 with inoculums size (IS) detected as significant model terms. Lack of fit was not significant and the R-squared was at 0.8668. The 3D model graph is presented in Figure 1.

The maximum MFC particle size obtained was 2761 nm when used 850 µm RPS and 5.0 % IS. At the opposite side, the minimum MFC particle size observed was at 747 nm when used 525.0 µm RPS and 3.5 % IS. Figure 2 presents the contour-plot graph of the resulted model.

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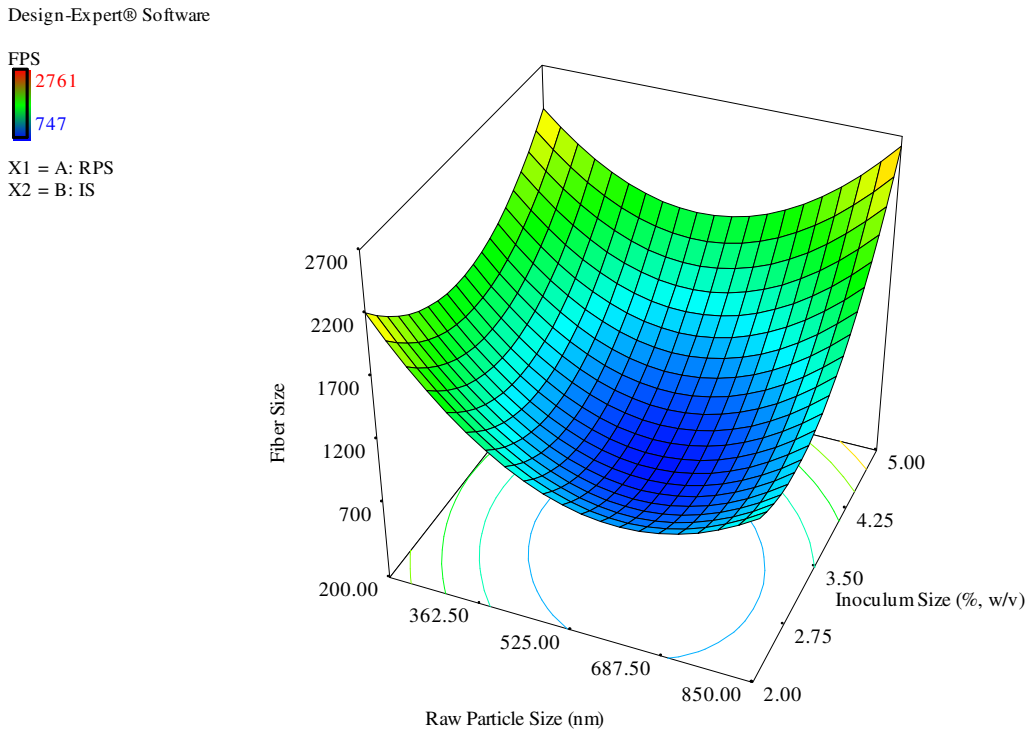

Figure 1: 3D response surface graph on the particle size of MFC with the two factors: raw particle size and inoculums size.

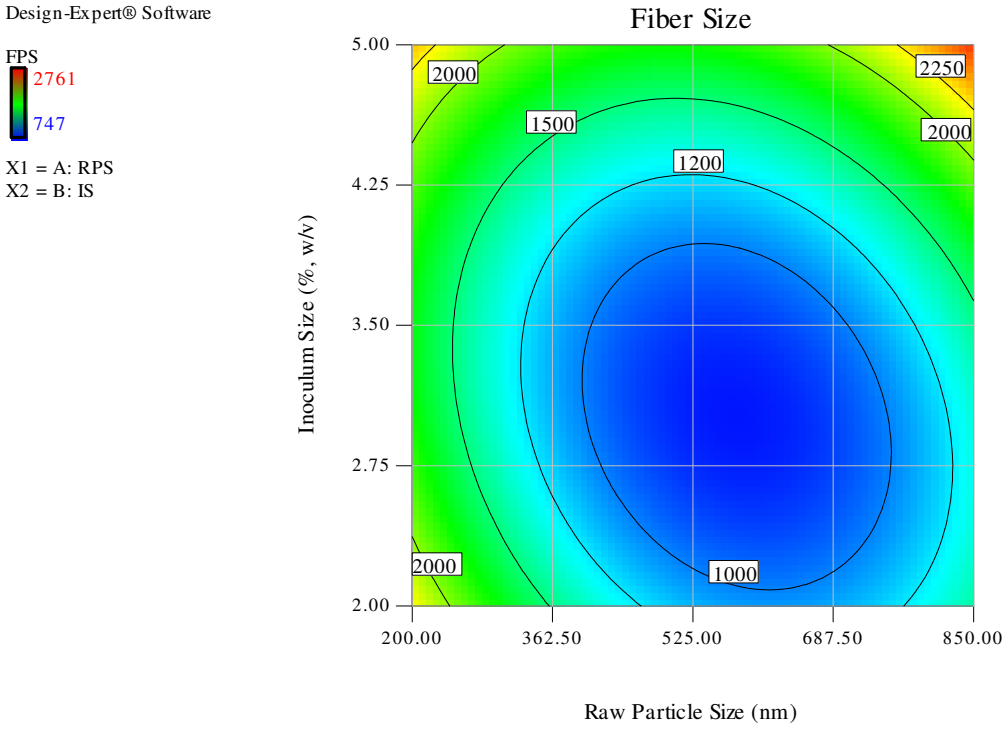

Figure 2: Contour-plot graph on the particle size of MFC with two factors: raw particle size and inoculums size

It was observed that the MFC particle size went lower than 1200 nm when used 320.0 – 390.0 µm RPS and 2.0 – 4.3% IS (w/w). The MFC with particle size bigger than 1500 nm was observed when the RPS less than 250.0 µm or more than 850.0 µm, paired with IS less than 2.0% or more than 4.7% (w/w).

The ANOVA analysis provides four typical solutions where the constraint settings of RPS set to maximize, IS set to minimize, and MFC particle size set to maximize. The predicted result from the suggested ANOVA analysis is showed in Table 1.

Table 1: Optimization results in the utilization of RPS and IS for optimum MFC production as suggested by ANOVA analysis

No	RPS (µm)	IS (% w/w)	Predicted MFC (nm)
1	850.0	2.0	1,412.7
2	847.4	2.0	1,404.4
3	850.0	3.42	1,411.5
4	407.3	2.0	1,343.3

Fungal-pretreatment provides a significant effect towards the MFC particle size produced by providing the required enzyme of cellulase to assist the ultrasonic process to split and reduce the size of individual fiber into the microfibrils size. Previous study showed that combination mild enzymatic hydrolysis with mechanical grinding and a high-pressure homogenization, yielded a controlled fibrillation with nanosized, long and highly entangled cellulose I elements network[5].

From the two independent variables of RSM, only the inoculums size provides the significant effect towards the size of the MFC produced. The preferred size of MFC was beyond 1,000 nm as to differentiate from nanofibrillated cellulose, and to retain the flexibility of the resulted product when used for composite materials.

Based on the observation, the predicted MFC size at optimized condition was beyond 1000 nm with average size of 1392.9 nm. Many researches have shown that enzyme-pretreatment can provide an aggressive conversion of cellulose that might impact the size of MFC. Hassan et al. [15] who studied MFC production from date palm fruit stalks demonstrated that higher dosages of enzyme induce a slight decreased of the MFC length. Their study demonstrated that presence of traces of cellulases in the commercial preparation of xylanase explains that the decreased of the MFC length corresponding to the highest dosages in enzyme that contain the cellulase. This is also supported by the results of Qing et al. [16] and Tibolla et al. [17] who stated that enzymatic pretreatments caused the shorter fibril lengths of several hundred to only 2889.7 nanometers, similarly to cellulose nanocrystals obtained by strong acid hydrolysis. In other words, a mean of control over the activity of each specific enzymes are required to gain the optimum results of particle size produced.

The diameter of MFC less than a hundred nm is in the accordance with the result of Chen et al. [11] at 5 – 20 nm from ultrasonic treatment of wood, and the similar diameter of below 100 nm through fungal-pretreatment by *Ophiostoma ulmi* and high shear refining of bleached kraft pulp by Janardhnan and Sain [18]. In comparison, a specific type of enzyme pretreatment by xylanase towards date palm fruits stalks by Hassan et al. [15] showed that the width of MFC isolated from untreated pulp was 21 ± 9 nm whereas that of MFC isolated from xylanase-treated

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pulps ranged from 81 ± 25 to 60 ± 20 nm.

3.2 Scanning Electron Microscopy (SEM)

Scanning electron micrographs of treated and not treated samples are presented in Figure 3. Observations were focused to the differences between the fungal-pretreated-ultrasonicated and without fungal pretreatment sample. It was observed that sample treated with both methods showed significant effect on the morphology of CPH. It was found that the top surface area of ultrasonicated sample without fungal pretreatment was still rigid and sturdy like crude CPH, while fungal-pretreated-ultrasonicated CPH exposed more significant deformed structure.

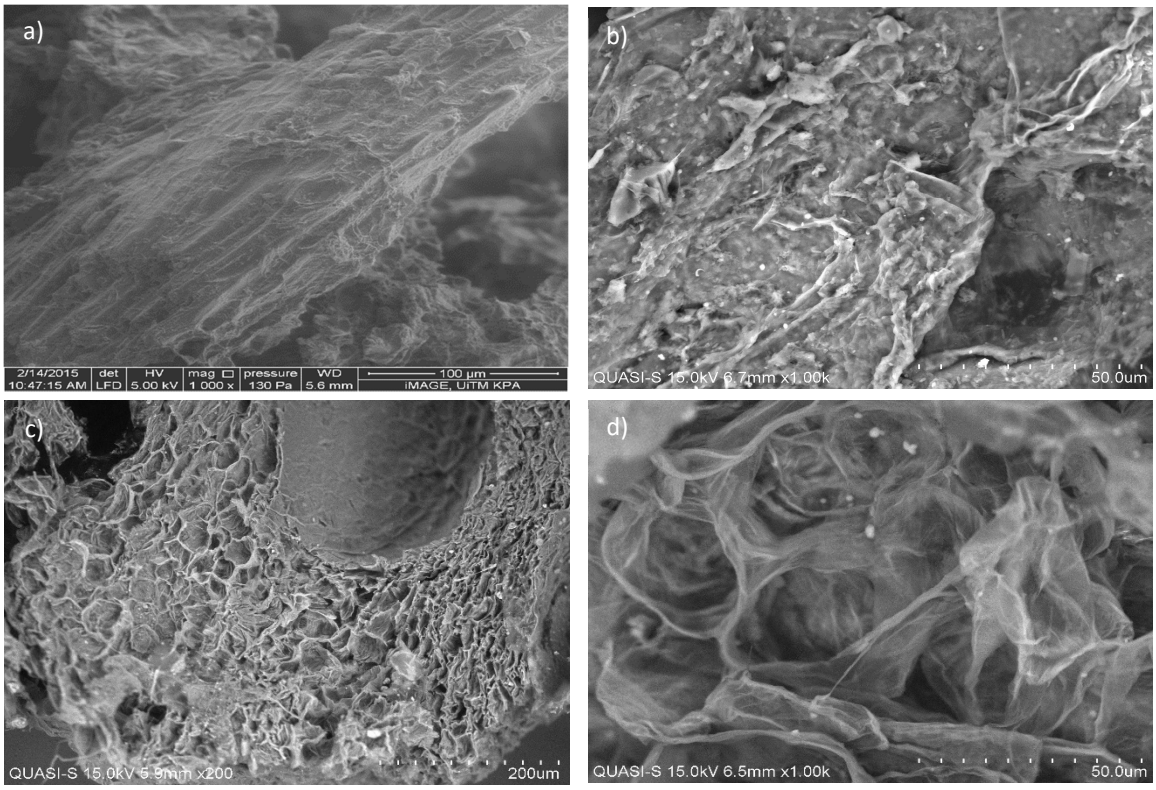

Figure 3: Scanning electron micrographs of (a) CPH without any treatment (control) and (b) ultrasonicated sample without fungal pretreatment, and fungal pretreated-ultrasonicated sample (c) x 200 magnification (d) 1,000 magnification.

SEM images show the differences between control (without fungal-pretreatment) and fungal-pretreated-ultrasonicated MFC. The resulted micrographs provide the information on the morphology, diameter, and length of MFC. However it is more difficult to determine the length because of entanglement and difficulties in identifying both ends of individual fibers [13]. The morphology differences can be clearly seen from the area of liberated cellulose microfibrils structure, where the appearance of fungal-pretreated-ultrasonicated MFC was more disintegrated structure of MFC compare to the one without fungal-pretreatment process. It might be due to the elimination of lignin and hemicelluloses by the enzymes activity released by *A. niger* during SSF. No long and rigid fibers was observed but thin wall of fibres were appeared. This showed that the fungal hydrolysis was able to facilitate ultrasonication process in fibrillation of microsized of fibre.

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3.3 Fourier Transform Infra Red (FTIR) Spectroscopy

Figure 4 showed the results on FTIR spectra. The dominant peak between 3500 and 3200 cm^{-1} was recognized as OH-stretching vibration of the hydroxyl groups in cellulose molecules. A stretching vibration of cellulose C-O in cellulose/hemicellulose and aryl group in lignin can be seen at peak of 1030 cm^{-1}. The peak of 3448cm^{-1} represents a stretching vibration of intramolecular hydrogen bonds of –COH. Additionally, the peak at 2900 cm^{-1} corresponds to the stretching vibration of alkyl groups of C-H in aliphatic bonds of cellulose, lignin and hemicellulose [19]. Other peaks, in the range of 1200 – 1300cm^{-1} corresponding to the aromatic skeletal vibration of lignin [20].

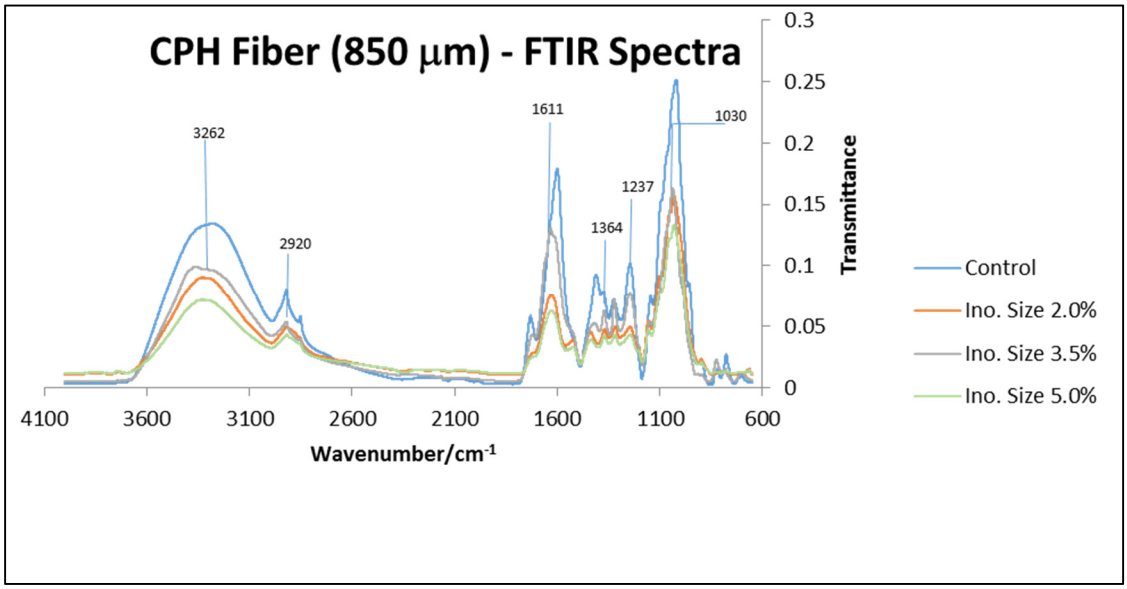

Figure 4: FTIR spectra of MFC at RPS of 825 µm with various level of IS

The absorptions in the range of 1200-1300 cm^{-1} which are corresponding to the aromatic skeletal vibration of lignin were decreased after fungal pretreatment and ultrasonication. A peak located at 1030cm^{-1} which associated with cellulose/hemicellulose and aryl group in lignin was also decreased. These are good evidence that showed the removal of a major portion of lignin from the CPH. These results suggested that spectra pattern of all samples seem similar with slightly change of position or shape of bands.

3.4 X-Ray Diffraction

Figure 5 shows the pattern of XRD from the raw CPH, fungal-pretreated-ultrasonicated MFC, and only ultrasonicated MFC (control). None of the diffractograms exhibit the complete pattern of Cellulose I, particularly at the crystalline cellulose peak in range 2θ from 22^0 to 24^0. However, all samples exhibit a peak around $2\theta = 15^0$ which is assigned to the (1 0 1) and (1 0 ī) lattice planes of cellulose I [21].

Raw CPH diffractogram has a low crystallinity index of 0.13 and its other peak at $2\theta = 21^0$, which peak indicates more to the hemicelluloses structure with some short-range order in the amorphous polymeric structure of the hemicellulose [22]. In the fungal-pretreated-ultrasonicated and only ultrasonicated (control) MFC diffractograms, the peak of raw CPH at $2\theta = 21$ disappeared. However, there was a peak at around $2\theta = 29^0$ appeared for both

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diffractrograms of fungal-pretreated-ultrasonicated and ultrasonicated without fungal-pretreated MFC.

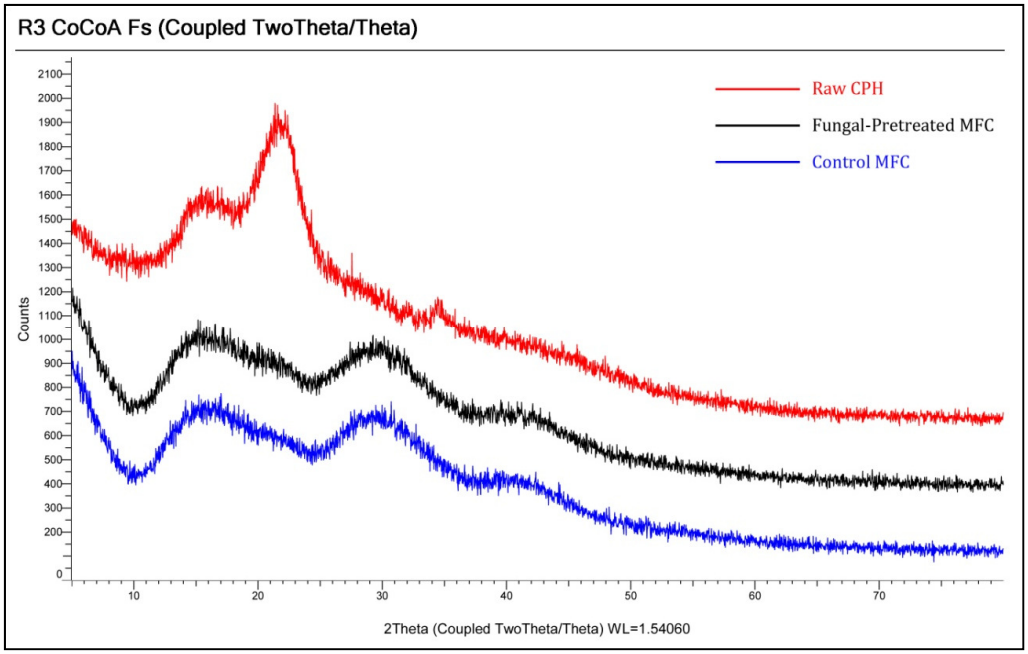

Figure 5: X-ray diffraction spectra of raw CHP, fungal-pretreated-ultrasonicated MFC and ultrasonicated MFC without fungal-pretreated

X-ray diffractograms of raw CPH, fungal pretreated combined with ultrasonicated MFC, and ultrasonicated MFC without fungal pretreated (control) indicate the crystalline behavior of the CPH fibers. A low crystallinity index (CrI) of raw CPH indicates the wider and less clearly refined features in the pattern of diffraction where non-crystalline part of the cellulose structure was accounted for [18]. The CrI of the original cellulose is determined to be 45.3–47.5%. A lower crystallinity index indicated that the regenerated cellulose contains a large amount of amorphous cellulose. The higher value of the crystalline cellulose, the stronger the inter-chain hydrogen-bonding network which leads to a high resistance of enzymatic hydrolysis [23].

The reason of the similar pattern of fungal-pretreated-ultrasonicated and only ultrasonicated (control) of MFC, and their difference towards the raw CPH was accounted to the effect of ultrasonic treatment, rather than fungal-pretreatment process. A study of enzyme-assisted MFC production by Hassan et al. (2014) [15] also showed the similar result with date palm fruit stalks where enzymatic treatment does not significantly affect the crystallinity of the obtained MFC. The disappearance of the sharp peaks at $2\theta = 21^0$ and the emergence of $2\theta = 29^0$ indicates the removal of hemicelluloses amorphous region which suggested that large amount of amorphous regions were removed through the applied methods. It can be seen also the appearance of $2\theta = 15^0$ for both treated MFC which indicated the high content of crystalline cellulose. This is in accordance to several studies [24 – 25] where the removal of amorphous regions and the increase of cellulose crystalline domains are a function of the use of mechanical isolation process and enzymatic pretreatment.

4.0 CONCLUSIONS

Cocoa pod husk can be a good resource for the production of cellulose fibers if handled in an

appropriate way to preserve the cellulose content from degrading into a simpler form. In the production of MFC from this renewable source, fungal-pretreatment provides a significant effect towards the MFC particle size produced by facilitating the splitting and reducing size of individual fiber into the microfibrils size. Based on the study, the optimization process calculates that it is possible to produce MFC with particle size bigger than 1000.0 nm (average size of 1392.9 nm). The results of SEM, FTIR, and XRD also indicated the positive changes on the properties of CPH due to the effect of pretreatment and ultrasonic treatment process. However, the knowledge on how the set of enzymes released by the fungal strain is required to avoid either an incomplete removal of lignin and hemicelluloses, or further splitting of cellulose towards nanoscale size.

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