Cellulase Production from Treated Oil Palm Empty Fruit Bunch Degradation by Locally Isolated *Thermobifida fusca*

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The aim of this research was to evaluate the production of cellulases from locally isolated bacteria, *Thermobifida fusca,* using thermal and chemical treated oil palm empty fruit bunch (OPEFB) as substrate in liquid-state fermentation (LSF). *T. fusca* was successfully isolated and was a dominant cellulase producer in OPEFB composting at the thermophilic stage. Analysis of the surface morphology of OPEFB samples using Scanning Electron Microscopy (SEM) showed that the most significant changes after the combination of thermal and chemical pretreatment was the removal of silica bodies, and this observation was supported by X-ray Diffraction analysis (XRD), Fourier Transform Infrared (FTIR), and Thermogravimetric analysis (TG) showing changes on the hemicelluloses, cellulose, and lignin structures throughout the pretreatment process. As a result of the pretreatment, higher cellulase production by *T. fusca* was obtained. The highest activity for CMCase, FPase, and β-glucosidase using optimally treated OPEFB were 0.24 U/mL, 0.34 U/mL, and 0.04 U/mL, respectively. Therefore, it can be suggested that the combination of chemical and thermal pretreatments enhances the degradation of OPEFB for subsequent use as fermentation substrate, contributing to a higher cellulases yield by *T. fusca*.

Keywords: Cellulases; Thermobifida fusca; Physicochemical characteristics; Oil palm empty fruit bunch

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INTRODUCTION

Oil palm plantations have become the most important economic contributor in Malaysia. However, palm oil mills produce a large amount of solid waste. Currently, the largest solid biomass generated in Malaysia comes from oil palm plantations (Baharuddin *et al.* 2010). The solid biomass from oil palm consists of a multitude of lignocellulosic materials such as fronds, trunks, and empty fruit bunches (EFB). Najafpour *et al.* (2005) reported that the biomass waste contains approximately 23% EFB, 12% mesocarp fibre, 5% shell, and 60% palm oil mill effluent (POME) for every tonne of fresh fruit bunches (FFB) processed in the mills. If this biomass is properly used, it will not only be able to solve the wastes problem but also can create value added products. OPEFB fiber is one class of biomass that is presently burnt for power generation, used to obtain bunch ash, or applied for mulching in the oil palm plantation. However, these approaches can lead to other environmental pollution problems as a result of the burning process.

Plant biomass consists of on average 40% cellulose, 33% hemicelluloses, and 23% lignin (Pereira *et al.* 2003). Cellulose is the most abundant carbohydrate in nature and a major structural material of plant cell walls (Saha *et al.* 2006). Cellulose is a polymer of glucose units connected by β-1,4 linkages. In the fermentation process, cellulose from OPEFB is converted into glucose by cellulase-producing microorganisms. Cellulase is the enzyme commonly utilized for cellulose degradation, and it is able to hydrolyze β-1,4-glycosidic bonds of cellulose to release glucose units (Nishida *et al.* 2007). In practice, cellulases are widely used in various industries such as food, animal feed, brewery, wine, textile, laundry, paper, pulp, and agriculture. They are also used in the bioprocessing of natural fibers, such as for the hydrolysis of cellulose to fermentable sugars and ethanol production. Thus, the demand for cellulases is the main driving force for research on microbial cellulases.

A majority of microorganisms have the ability to produce cellulases. However, only a minority are able to produce a significant quantity of free enzyme that is capable of completely hydrolyzing crystalline cellulose (Koomnok 2005). Immanuel *et al.* (2006) stated that bacteria and fungi are the most common microorganisms that produce cellulases. Although some of the bacteria and actinomycetes were reported to yield cellulase activity, fungi are the major microorganisms in cellulase production. However, bacterial cellulases are thought to be constitutively produced, while fungal cellulase is produced only in the presence of cellulose (Suto and Tomito 2001). *Thermobifida fusca* is a well-known efficient producer of cellulases. It has been described as an aerobic, moderately thermophilic, and filamentous soil bacterium that degrades the plant cell walls in heated organic substances (Lykidis *et al.* 2007). It was also reported that this strain belongs to the phylum of actinobacteria that can grow on most simple sugars and carboxylic acids and is capable of degrading all major plant cell wall polymers except for lignin and pectin (Lykidis *et al.* 2007).

Current research on lignocellulosic biomass has been focused on the development of pretreatment processes. Pretreatment is an important step to alter the structure of cellulosic biomass to enhance the conversion of carbohydrate polymers into sugar monomers by enzymes (Mosier *et al.* 2005). The biomass can be treated using different methods such as physical, thermal, chemical, biological, or a combination of these methods to disrupt the lignocellulosic complex structure. Furthermore, in order to maximize cellulose accessibility and bioconversion yields, increasing the amorphous region of cellulose is required (Kim and Mazza 2008; Ohgren *et al.* 2007). However, in the case of OPEFB, the optimization of the different pretreatments methods and analyzing their effects on increasing the surface area and porosity, modifying the lignin structure, removing lignin and hemicelluloses, and reducing the crystallinity of cellulose to enhance cellulases production has yet to be performed.

Locally isolated cellulase-producing bacteria were introduced in order to degrade the cellulose component found in OPEFB. The effectiveness of thermal treatment and a combination of chemical and thermal treatments were investigated, specifically with respect to the structural alteration of lignocellulosic content and surface morphology of OPEFB fibres. The treated OPEFB fibers were used in the subsequent experiment as the fermentation substrate in order to evaluate the effect of pretreatment on the production of cellulases by *T. fusca*.

EXPERIMENTAL

Microorganism

Thermobifida fusca was used in this study for cellulase production. This bacteria was isolated from the compost of OPEFB. The compost samples were obtained from a previous study done by Baharuddin *et al.* (2010) and stored at -20 °C. This bacteria was grown on a Trypticase Soy (TS) agar at 50 °C for 24 to 48 hours, for subsequent use in inoculum preparation.

Methods of OPEFB Pretreatment

The OPEFB fibres were obtained from the mill in Dengkil, Selangor, Malaysia. The OPEFB fibres were ground using a hammer mill and further treated using chemical and thermal methods. In a combination of chemical and thermal method, 50 g of OPEFB fibres were soaked in 500 mL sodium hydroxide (NaOH) solution (0.5% v/v) at 30 °C for 4 h and then autoclaved at 121 °C, 15 psi for 5 minutes (Umikalsom *et al.* 1997). All the treated OPEFB fibres were filtered and washed with distilled water, until no traces of base could be detected. The solids then were dried in an oven at 105 °C overnight. In the thermal method, 50 g OPEFB fibres were placed in a perforated container, and autoclaved at 121 °C, 15 psi for 5 minutes.

Medium and Fermentation

Trypticase Soy (TS) broth was used as *T. fusca* growth medium. TS broth was prepared in 500 mL shake flasks, then autoclaved at 121 °C, 15 psi for 15 minutes (Wongwilaiwalin *et al.* 2010). The treated OPEFB and microcrystalline cellulose were used as substrates. One percent of substrates were added as a carbon source for cellulases production. In all fermentations, 100 mL of medium was dispensed into a 500 mL shake flask and inoculated with 10 mL of bacteria suspension. The flasks were incubated at 50 °C in a rotary orbital shaker at 250 rpm. Samples of 10 mL were drawn at 0, 6, 12, 24, 36, and 48 h for analysis.

Enzyme Assays

The sample withdrawn during fermentation was centrifuged at 14000 rpm speed for 2 minutes at 4 °C. The clear supernatant was analyzed for enzyme activities. The CMCase, FPase, and β-glucosidase activities were determined using the method described by Wood and Bhat (1988) with some modifications. CMCase activity was determined by measuring the reducing sugar from 1% carboxymethyl cellulose (CMC). FPase activity was determined by estimating the reducing sugars from Whatman filter paper no. 1, while p-nitrophenyl-β-D-glucopyranoside was used as the substrate to determine β-glucosidase activity. The reducing sugars released from the substrates were analyzed by the 3,5-dinitrosalicylic acid (DNS) method. One unit of enzyme activity was expressed as 1 µmol of product liberated per mL of enzyme per minute.

Scanning Electron Microscopy (SEM)

The morphological structures of untreated and treated OPEFB samples were analyzed using a scanning electron microscope (SEM). The samples were mounted on an aluminum stub using double-sided adhesive tape and were sputter-coated (E-1010, Hitachi, Japan) with platinum prior to the morphological examination. The SEM micrographs were obtained at an accelerating voltage of 15 to 25 kV.

X-Ray Diffraction (XRD) Analysis

Measurements of X-Ray diffraction of OPEFB samples were conducted using a Rigaku XRD-DSC-X II diffraction system. Cu-Kα radiation (0.15418 nm) was used as radiation source and 2θ angles were recorded using a scintillation counter in the interval between 10 and 50 °C. The crystallinity of SiO_2 pattern was estimated using the Scherrer equation ($L = K \lambda / \beta \cos \theta$), where λ is a wavelength of X-Ray, β is the full width at half maximum, and θ is the Bragg angle. The value of β was estimated by fitting the XRD pattern obtained from the result.

Fourier Transform Infrared (FTIR) Spectroscopy

FTIR spectra were recorded using a Spectrum TM (Perkin Elmer GX2000, USA) with wave numbers in the range of 800 to 3000 cm^{-1}, and at a resolution of 4 cm^{-1}. This equipment was used in an effort to obtain information about the chemical bonds between functional groups and the effects of surface modification of the OPEFB samples.

Thermogravimetric and Derivative Thermogravimetric Analysis (TG/DTG)

TG/DTG was performed using a thermal analyzer EXTAR 6200 TG/DTG system (Seiko Instrument Inc. Japan) to analyze the thermal behavior of OPEFB samples. The samples were heated from 30 to 500 °C at a heating rate of 9 °C/min under a constant nitrogen flow (100 mL/min^{-1}) to investigate the mass loss of the samples. During the analysis, weight loss and heating rate were continuously recorded.

RESULTS AND DISCUSSION

Lignocellulosic Structural Changes of OPEFB

Scanning Electron Microscopy (SEM)

The surface morphologies of fibres in the untreated and treated OPEFB samples under thermal and a combination of chemical and thermal treatments viewed under the Scanning Electron Microscope (SEM) are shown in Fig. 1. The micrographs in Fig. 1(a) show that the untreated OPEFB surfaces were covered with silica bodies, which attached to circular pores along the OPEFB strands. The presence of silica bodies might hinder the accessibility of enzyme attack, especially to the cellulose layers of the OPEFB. Therefore, the removal of silica bodies would enhance the enzyme penetration into the cellulose of OPEFB. Neethirajan *et al.* (2009) reported that silica bodies act as a shield against fungal attack and give the plant structural support in nature.

Figure 1(b) shows that the thermal treatment had substantially altered the structure of the OPEFB. Although the silica bodies are hard, thermal treatment was able to partially remove the silica bodies. Furthermore, thermal treatment also caused the hemicelluloses and lignin to be easily degraded and transformed into simpler structures, hence increasing the potential for cellulose hydrolysis (Sun and Cheng 2002).

From Fig. 1(c) it can be clearly seen that the OPEFB surface was covered with many circular craters perforating to the bottom, showing that the silica bodies were highly disrupted after the combination of chemical and thermal treatment. Compared to Fig. 1(b), the combined thermal and NaOH treatment resulted in a greater loss in silica, whereby fewer silica bodies were observed on the surface. Fan *et al.* (1987) reported that treatment of lignocellulosic material with dilute NaOH caused swelling, leading to an increase in the internal surface area, and a reduction in the degree of polymerization and

crystallinity, causing structural separation between lignin and carbohydrates and a disruption of lignin. The treated OPEFB fibres were clearly separated and entirely exposed. Cracks were also visible on the OPEFB surface, which is most probably due to increasing porosity and surface area. The surface of the treated OPEFB fibres appeared smoother in comparison to the untreated fibres. Based on this analysis, it can be concluded that the pretreatment, especially the combination of chemical and thermal treatment, alters the morphology of the OPEFB which may enhance accessibility to enzymatic attack.

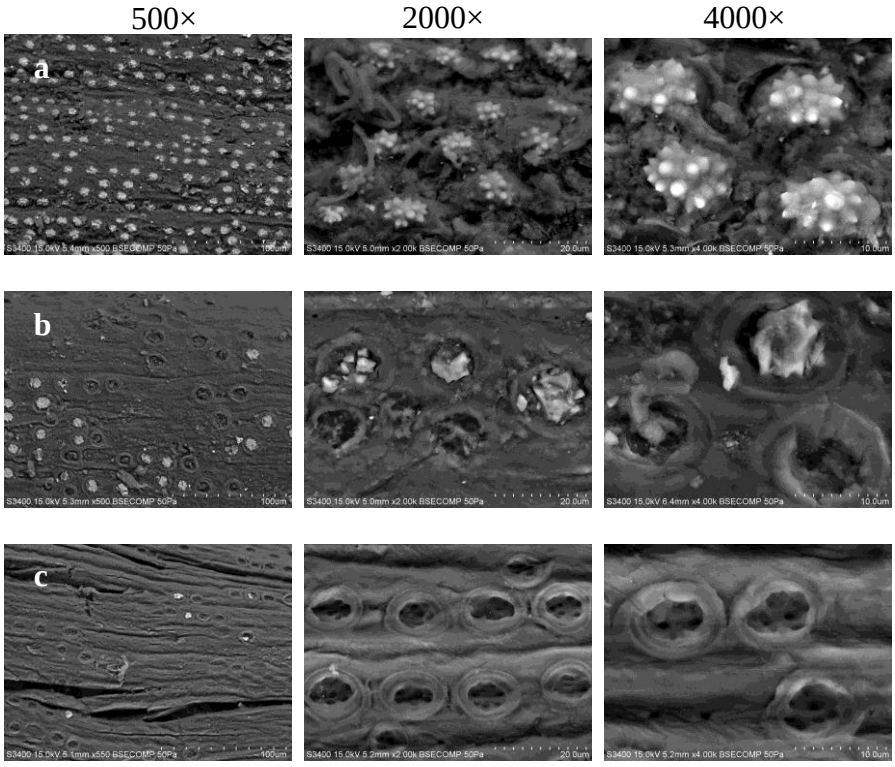


Fig. 1. SEM images of (a) untreated OPEFB, (b) OPEFB treated by autoclaving at 121 °C for 5 minutes, and (c) OPEFB treated with 0.5% NaOH followed by autoclaving at 121 °C for 5 minutes

X-ray diffraction (XRD) analysis

Figure 2 presents the X-ray diffraction analysis that was conducted to identify crystallinity changes of the treated OPEFB samples. The peaks that appeared at 2θ ranging from 20° to 25° indicate the crystalline nature of the carbon-based material. The presence of a broad peak within that range reflects the fact that the OPEFB comprised both amorphous and crystalline structures. The peak intensity of untreated OPEFB was lower than treated OPEFB for both methods, indicating that the crystalline fraction of untreated OPEFB was lower than that of treated OPEFB. In lignocellulosic material, hemicelluloses and lignin are the amorphous substances, whereas cellulose is composed of crystalline and amorphous regions (Reith *et al.* 2009). The increase in peak intensity of treated OPEFB was most likely due to the removal of hemicelluloses and to a slight extent due to the removal of lignin. The pretreatment processes can also cause cellulose to depolymerize, removing a fraction of the amorphous region and leaving the crystalline cellulose (Kumar *et al.* 2009). In our initial expectation, the combined thermal- and

chemical-treated OPEFB should give more significant crystalline changes than the thermal treated OPEFB due to the additional disruption of hemicellulose and lignin by the alkaline hydrolysis. However, the peak intensity of the combined thermal- and chemical-treated OPEFB was slightly lower than thermal treated OPEFB, indicating that the crystallinity region of cellulose from the combined thermal-chemical-treated OPEFB was lower than that of thermal-treated OPEFB. Fan *et al.* (1987) reported that dilute NaOH treatment of lignocellulosic materials causes swelling, resulting in an increase in internal surface area and a decrease in the crystallinity index. Hence, the unexpected result obtained is possibly due to the hydrolysis and swelling of the cellulose fibres by NaOH, which leads to a reduction in the amorphous-to-crystalline ratio.

The 2θ value at 44° corresponds to SiO_2 and was identified in all samples. The existence of a high crystallinity SiO_2 peak was also reported by Law *et al.* (2007), who claimed that the strong bonding of silica bodies (SiO_2) with the plant cell matrix protects and gives mechanical strength to the OPEFB structure. Although the peak intensity indicated some changes, prediction of SiO_2 crystal size using the Scherrer equation resulted in inconsistent values for all the samples (data is not provided in this work). In case of thermal chemically-treated OPEFB, the process swells the cellulose fibres and leads to the removal of large SiO_2 particles. However, this removal could not be distinguished by the XRD analysis. It is hypothesized that the detached SiO_2 crystals (indicated in SEM image) were scattered on the sample surface and interfere with the analysis results. On the other hand, complete removal of large SiO_2 particles from the sample surface would leave only fine SiO_2 particles beneath. These fine SiO_2 particles were unable to be removed because of their presence within the siliceous pathway. The existence of SiO_2 in the siliceous pathway inside the OPEFB strands has been described well by Law *et al.* (2007).

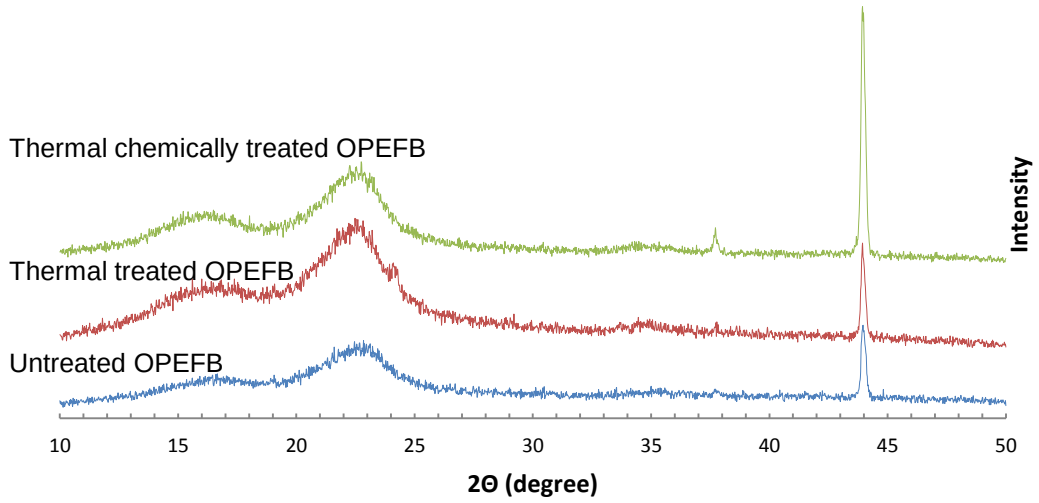


Fig. 2. XRD analysis of untreated and treated OPEFB

Fourier transform infrared (FTIR) analysis

The structural composition in OPEFB' matrix was analyzed using Fourier transform infrared (FTIR) spectroscopy. Figure 3 shows the FTIR results for several functional groups in the OPEFB matrix. The discussion in this work is limited to the

hemicelluloses, cellulose, and lignin molecular structure of polysaccharides. The disappearance of some absorption bands might be contributed by the degradation of structure in the OPEFB matrix or the transformation of some functional group into a more stable substance. The appearance of some absorption bands is assumed to come from the restructuring of unstable functional groups into a more stable structure.

The FTIR spectra of all samples indicated that the most significant changes of strong bands were those between 2930 cm^{-1} and 2856 cm^{-1}. The peak at 2930 cm^{-1} belongs to the C-H stretching vibration of methyl, methylene, and methoxy groups (Meligy *et al.* 2004). Ray and Sarkar (2001) also reported that the vibrations in the region between 3000 cm^{-1} and 2850 cm^{-1} associated with C-H stretching of lignin, hemicelluloses, and cellulose all decreased after alkalization. Based on these, we conclude that some of the functional groups were affected by the physical treatment and chemical addition, and the stronger changes occurred when both methods were applied. Here it was assumed that the effect of thermal treatment probably caused partial degradation of the hemicellulose structure, as it consists to a large extent of a weak polysaccharides matrix in amorphous form. However, there were no significant changes in the other bands or peaks, which generally may indicate that, the rest of the functional groups which are attributed to cellulose, hemicelluloses, and lignin were still very strong and were not affected by both treatment methods. Some significant changes of peaks between 1800 cm^{-1} and 1000 cm^{-1} were also noticed in terms of the IR absorbance or amplitude. However, the qualitative analyses used in the present study were unable to quantify the differences between the samples.

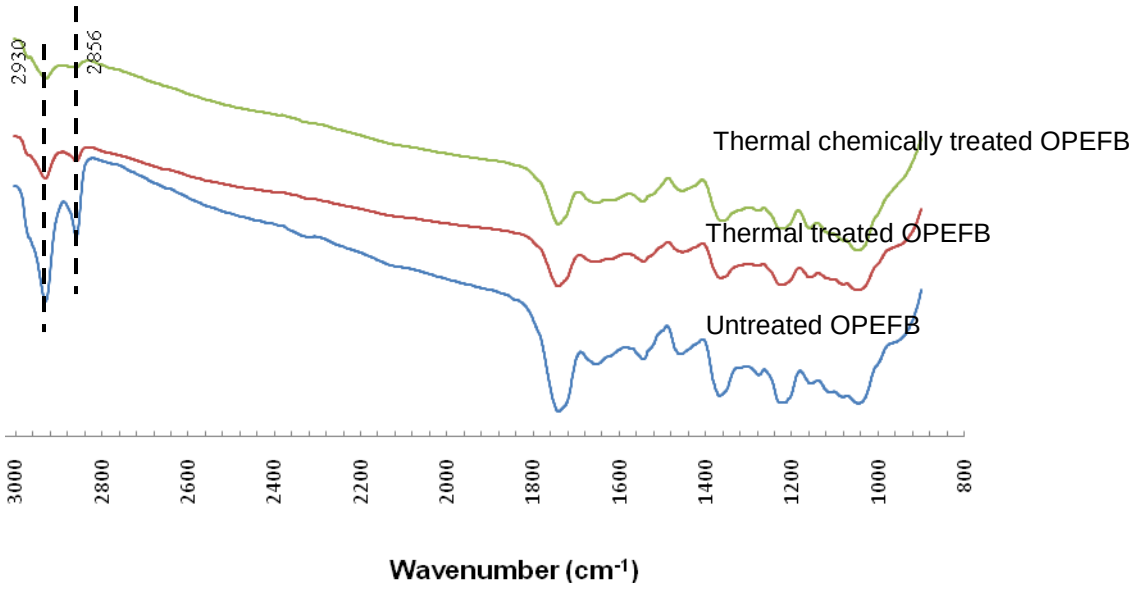


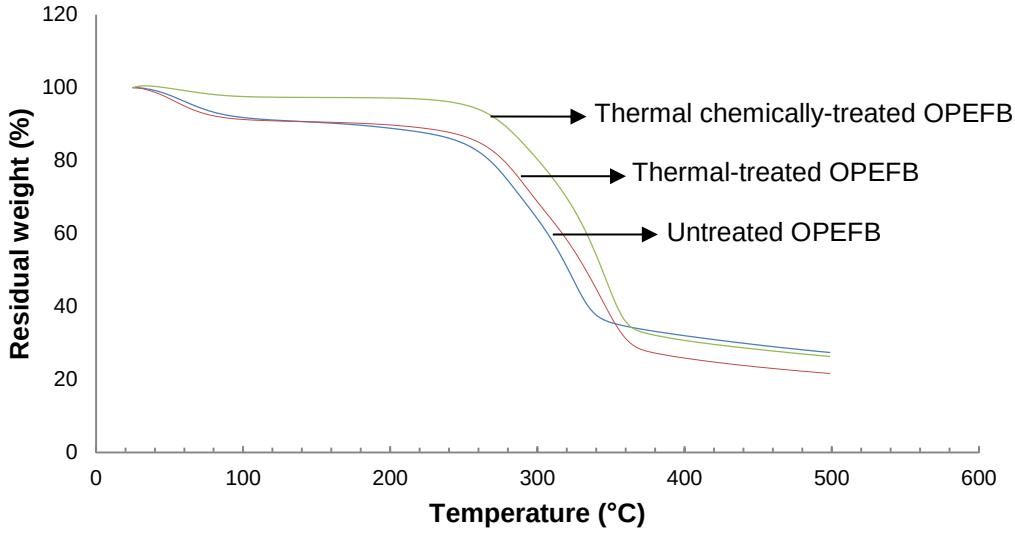
Fig. 3. FTIR spectra of treated and untreated OPEFB

Thermogravimetric and derivative thermogravimetric analysis (TG/DTG)

The TG analysis in Fig. 4 shows the thermal degradation of untreated and treated OPEFB. The thermal degradation can be demonstrated by a decrease in the residual weight and an increase in the temperature difference due to the exothermic combustion reaction. The thermal degradation of untreated OPEFB began at a lower temperature in comparison to the treated OPEFB, which was around 200 °C. This is mainly attributed to

the degradation of hemicelluloses. Sagehashi *et al.* (2006) reported that hemicellulose is enriched with many polyose chains and branched structures, which are susceptible to decomposition at a low temperature (200 to 260 °C). The thermal-treated OPEFB and thermal-chemically treated OPEFB degradation starts at around 250 °C to 270 °C, respectively. Degradation of untreated fiber started at a lower temperature due to the presence of thermally unstable fiber compounds such as hemicelluloses and lignin, whereas the treated fiber is more stable due to the partial removal of these compounds (Beckermann and Pickering 2008). The fact that the untreated OPEFB had a lower residual weight loss compared to the treated ones demonstrated that the treatment was successful in the removal of hemicelluloses and some part of the less-stable lignin components.

(a)



(b)

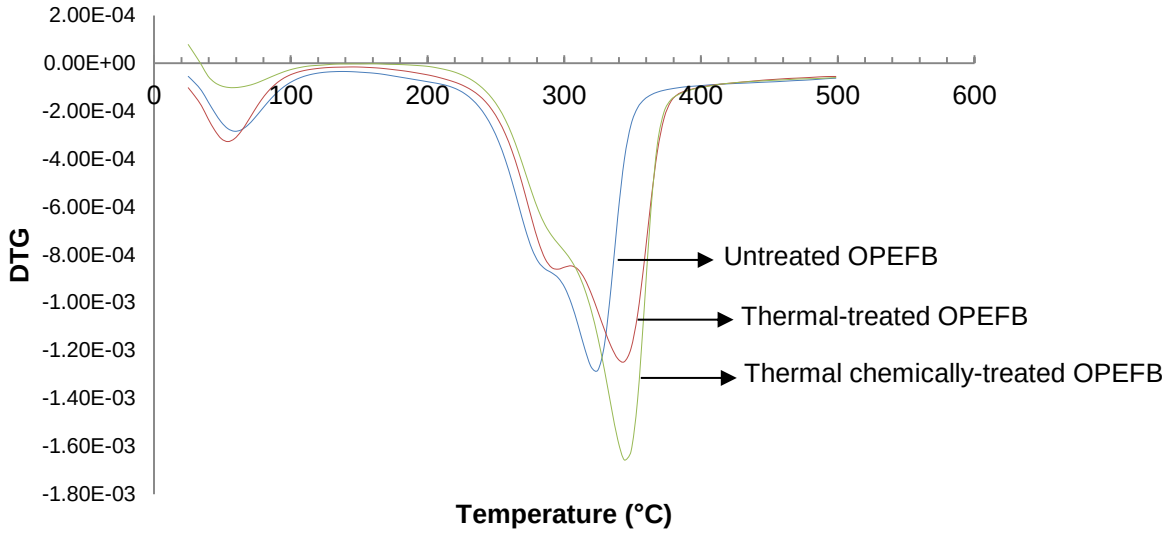


Fig. 4. TG (a) and DTG (b) curve of untreated and treated OPEFB samples

From the DTG pattern, it was also noticed in the case of the thermal chemically-treated OPEFB that there was a shift in the main peak from 320 °C to 350 °C. This significant change demonstrated an increase of the material's thermal resistance to much higher temperature. Furthermore, it was noticed that the hemicellulose shoulder disappeared in the range 270 to 300 °C, which might be caused by depolymerization of hemicelluloses during the pretreatment process. Thus, we can conclude that the treatment process actually turned the OPEFB into more stable crystalline type components as indicated in the discussion of the XRD analysis.

Cellulase Production with Various Carbon Sources

The production of cellulases is one of the main factors in cellulosic component hydrolysis. However, the cost of substrate is the main challenge in the economics of enzymes production. The utilization of OPEFB as a substrate in cellulases production may improve its economics. Haltrich *et al.* (1996) reported that the carbon source acts not only as a substrate for microorganisms but also produces the necessary inducing compounds. Hence, cellulase production from treated and untreated OPEFB was analyzed and compared to corresponding experiments using microcrystalline cellulose.

In this study, it was found that when untreated OPEFB was used as a carbon source, very little production of β-1-4-exoglucanase (FPase), β-1-4-endoglucanase (CMCase), and β-glucosidase from *T. fusca* were detected with activity of 0.08, 0.07, and 0.02 U mL^{-1}, respectively. High amounts of cellulases were produced with activity of about 0.43, 0.33, and 0.04 U mL^{-1} when microcrystalline cellulose was used as a carbon source. However, the cellulases activity obtained from thermal chemically-treated OPEFB was only about 27% lower than that of microcrystalline cellulose. OPEFB treated using both methods proved to be a potent inducer of cellulases with more than 2-fold increase compared to untreated OPEFB. A summary of cellulase activities is presented in Table. 1. The highest activity of Fpase, CMCase, and β-glucosidase from thermal chemically-treated OPEFB was found to be 0.34, 0.24, and 0.04 U mL^{-1}, respectively. In comparison, under optimized condition, Madhanraj *et al.* (2010) reported that FPase activity by *Aspergillus niger* is 0.058 U/mL when using saw dust as a substrate.

Table 1. The Maximum Production of Cellulase by *T. fusca* with Different Treatment of OPEFB

Carbon source	Cellulase enzyme activity (U mL^{-1})		
	CMCase	FPase	β-glucosidase
Microcrystalline cellulose	0.33 ± 0.002	0.43 ± 0.003	0.04 ± 0.005
Thermal chemically treated OPEFB	0.24 ± 0.002	0.34 ± 0.001	0.04 ± 0.004
Thermal treated OPEFB	0.18 ± 0.008	0.29 ± 0.006	0.02 ± 0.008
Untreated OPEFB	0.07 ± 0.009	0.08 ± 0.009	0.02 ± 0.011

The enzyme activity of β-glucosidase is low compared to CMCase and FPase activities. In general, there is a synergistic effect between CMCase and FPase, whereby FPase acts on insoluble cellulose degradation while CMCase is not very active against

crystalline cellulose. For a complete hydrolysis of cellulose, these enzymes complement each other. On the other hand, β-glucosidase activity can be related to the amount of cellobiose produced from the action of FPase (Baharuddin *et al.* 2009). This can explain the lower production rate of β-glucosidase activity.

From this study, it can be found that the combination of chemical and thermal treatments of OPEFB improved the production of all three major cellulases. Generally, the susceptibility of cellulose to microbial attacks increased after pretreatment due to the lower lignin and hemicellulose content, contributing to higher cellulases production. This result was also in accordance with a previous study done by Umikalsom *et al.* (1997), who reported that NaOH had the greatest effect on OPEFB structure alteration compared to other chemicals that were considered.

CONCLUSIONS

1. The local strain, *T. fusca* was dominant as a cellulase producer, indicating that it has potential to degrade the cellulosic component of OPEFB.
2. Analysis on surface morphology of OPEFB samples showed that the most significant change is the removal of silica bodies after a combination of thermal and chemical pretreatment, and this observation was supported by the changes in the hemicelluloses, cellulose, and lignin structures throughout the pretreatment process.
3. The cellulases activity obtained from thermal chemically-treated OPEFB was comparable to microcrystalline cellulose, where the activity of microcrystalline cellulose was only about 27% higher than thermal chemically-treated OPEFB. OPEFB treated by thermal method only also proved to be a strong inducer of cellulases with more than 2-fold higher production in comparison to untreated OPEFB.

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