Combined pretreatment using alkaline hydrothermal and ball milling to enhance enzymatic hydrolysis of oil palm mesocarp fiber

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1 ABSTRACT

2 Hydrothermal pretreatment of oil palm mesocarp fiber was conducted in tube reactor
3 at treatment severity ranges of $\log R_o = 3.66$ -4.83 and partial removal of
4 hemicellulose with migration of lignin was obtained. Concerning maximal recovery
5 of glucose and xylose, 1.5% NaOH was impregnated in the system and subsequent
6 ball milling treatment was employed to improve the conversion yield. The effects of
7 combined hydrothermal and ball milling pretreatments were evaluated by chemical
8 composition changes by using FT-IR, WAXD and morphological alterations by SEM.
9 The successful of pretreatments were assessed by the degree of enzymatic
10 digestibility of treated samples. The highest xylose and glucose yields obtained were
11 63.2% and 97.3% respectively at cellulase loadings of 10 FPU/g-substrate which is
12 the highest conversion from OPMF ever reported.

13
14 **Keywords:** Oil palm mesocarp fiber, ball mill, alkaline hydrothermal, xylose, glucose

16 1. Introduction

17 Bioethanol derived from biomass has been recognized as a potential substitute to
18 fossil fuel since biomass is abundant in nature, non-food competitive and sustainable.
19 Oil palm industry has contributed the highest percentage of biomass generated from oil
20 palm processing. It was estimated approximately 96.0 Mt of fresh fruit bunches (FFB)
21 has been processed and amounted 19.2 Mt crude palm oil (CPO) production (MPOB,
22 2014). Oil palm mesocarp fiber (OPMF) is one of lignocellulosic biomass generated
23 from oil palm processing and consist mixtures of exocarp (outer skin), mesocarp (pulp)
24 and crushed endocarp (shell). One ton of FFB could generates 0.12 ton of OPMF and
25 by this calculation, it is projected about of 11.5 Mt was generated in 2013 (MPOB,

2014). In normal practice this materials were used as a source of fuel in a boiler system to produce energy for the mill`s internal use. Under Clean Development Mechanism (CDM), OPMF has been used as a source of carbon in composting process and in anaerobic digester to improve methane production. OPMF are lignocellulosic complex composites of cellulose, hemicellulose and lignin like other plant biomass. Natural recalcitrance of lignocellulosic biomass complex hinders the accessibility of enzyme in hydrolysis process thus limit the production of pentose and hexose sugars. Four steps are involves in biochemical routes of bioconversion of biomass to ethanol production; 1) pretreatment of lignocellulosic biomass, 2) enzymatic hydrolysis, 3) fermentation and 4) separation of bioethanol and purification (Mosier and Wyman, 2005; Nitsos et al., 2013). The successful of pretreatment step is crucial in determining the fate of future processes.

Different pretreatment methods have been adopted with the aim to reduce and remove the natural recalcitrance of lignocelulosic biomass to get maximum access of cellulase to cellulose. The available methods may vary from physical, chemical, and thermochemical depending on the types and nature of biomass (Mosier and Wyman, 2005). Communion process such as planetary/ attrition ball milling and wet disc milling has resulted in reduction of particle size, increased surface area, pore volume and reduced crystallinity index (CrI) of cellulose, thus enhancing the enzymatic digestibility of biomass (Hideno et al., 2009; Silva et al., 2010; Liao et al., 2011). Concerning maximal accessibility of cellulase to cellulose, a large portion of hemicellulose and lignin have to be removed from the cellulose-hemicellulose-lignin matrix (Mosier and Wyman, 2005; Musatto et al., 2008). Hemicellulose can be partially removed through hydrothermal pretreatment such as steam and aqueous (autohydrolysis) methods since these treatments use only water as natural reactant and

catalyst under a wide range of temperature and residence time (Möller et al., 2011; Nitsos et al., 2013). The progress of fractionation of lignocellulosic materials are heavily dependent on the intensity of pretreatment, normally expressed as severity factor, $\log R_o$ (Overend and Chornet, 1989). Precise control of treatment severities may avoid the production of fermentative inhibitors such as acetic acid, furfural and 5-hydroxy-methyl-furfural (HMF) (Overend and Chornet, 1989; Möller et al., 2011; Nitsos et al., 2013). Hydrothermal pretreatment is always associated with the release/migration and re-condensation of lignin in the form of spherical droplets on the surface of pretreated biomass (Donohoe et al., 2008). Formation of pseudolignin from hemicellulose degradation increased the amount of lignin droplets, resulting in `traffic jam` effect and non-specific binding of lignin on cellulose, which may have a detrimental effect of enzymatic hydrolysis, thus reducing the yield of glucose in cellulose conversion (Donohoe et al., 2008; Selig et al., 2007; Pu et al., 2013).

Recently, combined hydrothermal and subsequent NaOH treatment was conducted to dissolve hemicellulose components and remove the lignin from the pretreated biomass, thus enhancing the enzymatic digestibility of pretreated biomass (Musatto et al., 2008; Gao et al., 2013; Ishiguro et al., 2014). In the present study, a combination of pretreatment methods were performed with the aim to disrupt the organized polymeric structure of OPMF. The treatment efficiency was demonstrated by the high xylose and glucose yields from enzymatic hydrolysis of treated OPMF.

2. Materials and methods

2.1 Preparation of raw materials and componential analysis

Oil palm mesocarp fiber (OPMF) was collected from Serting Hilir Palm Oil Mill, Jempol, Negeri Sembilan, Malaysia. The collected OPMF consisted of mixtures

of exocarp (outer skin), mesocarp (pulp), crushed kernel and endocarp (shell) (Fig. S1). The crushed kernels and shells were manually separated from OPMF fibers prior to componential analysis and other experimental work in order to avoid error in the data analysis. Unless otherwise stated, the sample used in this study was in its original size as collected from the mill (20-30 mm). The compositions of extractives, cellulose, hemicellulose, acid soluble lignin, acid insoluble lignin and ash content were determined by a method recommended by Teramoto et al. (2008).

2.2 Mechanochemical activation- ball milling pretreatment

Ball milling (BM) pretreatment was performed according to a method reported by Inoue et al. (2008). Untreated and hydrothermally treated OPMF were treated using the planetary ball mill Pulverisette 5 (Fritsch, Germany). The sample (20 g), was milled at 250 rpm in a 500 mL milling cup with 25 spheres ($\psi = 20$ mm). Planetary ball mill Pulverisette 7 (Fritsch, Germany) was used for lower amount of treated OPMF. The sample (0.5 g), was milled at 250 rpm in a 45 mL milling cup with 6 spheres ($\psi = 5$ mm). Milling was carried out for a total time of 60 - 240 min (with a cycle of 10 min run and 10 min pause) at room temperature. The experiments were performed in duplicate. The BM time indicated in this study refers to the actual milling time, excluding the paused time. Samples were kept *in vacuo* at room temperature prior to enzymatic hydrolysis.

2.3 Alkaline pretreatment

Four gram (4 g) of oven dried OPMF samples were placed in a laboratory bottle with stopper (NEG, Japan) and then mixed with 100 mL of NaOH solution (1.0%, 1.5% and 2.5%). The mixture was incubated at 50°C for 3 h with stirring. After

pretreatment, the solid residue was separated by filtering (Filter paper No. 2, Advantec, Japan) and wash with distilled water until neutral pH. The solid residue was oven dried at 90°C for 24 h prior to chemical analysis and enzymatic saccharification.

2.4 Hydrothermal pretreatment

Hydrothermal pretreatment of OPMF was conducted in a stainless steel tube reactor (outside diameter, 25 mm; wall thickness, 2 mm; and length, 100 mm). In general, 3 g of oven dried OPMF and 30 ml of distilled water was used to fill the reactor. Solid to liquid ratio (S:L) of 1:10 was used in this study. Fully tightened reactor filled with biomass sample and water was then carefully emerged into a sand bath, which was maintained at temperature range from 180-220°C governed by automatic temperature controller. The reactor was agitated at 60 rpm in order to provide homogenize mixing of samples in the tube reactor. After completion at 20 min residence time the reactor was transferred from sand bath into water reservoir and cooled down to 30°C. The slurry was withdrawn from the reactor and transferred into 65 mL glass bottles with cap (NEG, Japan) and stored prior to enzymatic hydrolysis. The pH value of the hydrothermally treated OPMF samples was measured using a digital pH meter (D-53, Horiba, Japan).

Alkaline hydrothermal pretreatment was performed by addition of diluted NaOH solution (0.5-2.5%) into a tube reactor at the same S: L ratio as mentioned above. The slurry was filtered using filter paper No. 2 (Advantec, Japan) and washed with distilled water until neutral pH. The neutralized solid was oven dried at 90°C for 24 h prior to enzymatic hydrolysis. The intensity of the hydrothermal treatment was expressed as severity factor ($\log R_o$). The severity parameters corresponding to different hydrothermal pretreatment conditions are calculated as in Eq. (1).

1

$$R_o = t_{\text{exp}} [(T-100)/14.75] \quad (1)$$

3 In which t is the reaction time (min), and T is the hydrolysis temperature (°C) (Overend
4 and Chornet, 1989).

5

6 **2.5 Sieving procedure**

7 Ball milled-treated OPMF samples was separated by using an Analysette 3
8 vibratory sieve shaker (Fritsch, Germany) with three different sieves size were selected;
9 2 mm, 500 μm and 250 μm and operated for 10 minutes at an amplitude of 0.5.

10

11 **2.6 Fourier transform infrared (FT-IR)**

12 Fourier transform infrared (FT-IR) spectra were recorded on a Perkin-Elmer
13 Spectrum One FT-IR spectrometer over a range of 4000 to 450 cm^{-1} with 128 scans. A
14 resolution of 4 cm^{-1} was taken for each sample. Solid samples were pelleted with KBr
15 containing a sample mass fraction of 0.01. The vibration transition frequencies of each
16 spectrum were baseline corrected and the absorbance was normalized between 0 and 1.

17

18 **2.7 Wide angle X-ray diffraction (WAXD)**

19 WAXD patterns were obtained using a Rigaku RINT-TTR III X-ray
20 diffractometer (Tokyo, Japan) equipped with nickel filtered Cu Ka radiation ($k =$
21 0.1542 nm) at 50 kV and 300 mA. The disk pellets were prepared by compacting
22 oven-dried samples at 2 ton using a KBr disk apparatus. The diffractograms were
23 detected in the range $2\theta = 2-60^\circ$ at a scan rate of $2^\circ/\text{min}$. The crystallinity index (CrI)
24 was calculated using Eq. (2) based on the method of Segal et al. (1959).

25

$$1 \quad \text{Crystallinity index (\%)} = [(I_{002} - I_{am})/I_{002}] \times 100 \quad (2)$$

2 I_{002} : The intensity at about $2\theta = 22.2^\circ$

3 I_{am} : The intensity at $2\theta = 17.6^\circ$

4

5 **2.8 Scanning electron microscopy (SEM) analysis**

6 The untreated and treated OPMF samples were sputtered with Pt-Pd for 100 s
7 (Ion sputter; Hitachi, Japan). The coated samples were examined by field emission
8 scanning electron microscopy (S-3400N, Hitachi, Japan) at 1 kV.

9

10 **2.9 Enzymatic hydrolysis**

11 Unless otherwise stated, enzymatic hydrolysis was performed using an
12 enzyme cocktail constituting 40 FPU/ mL *Acremonium* cellulase (Meiji Seika Co,
13 Japan), and 10% Optimash BG (Genencor International, California, USA). In a
14 standard assay, 0.75 mL (10 FPU/g substrate) of *Acremonium* cellulase, 2.5 mL of 1.0
15 M acetate buffer, pH 5.0, and 0.6 mL of 10% Optimash BG were added to treated
16 samples (3g of dry weight) in 65 mL tube (NEG, Japan). The reaction mixture was
17 added with distilled water to a total volume of 50 mL. In a smaller reaction, about 0.05
18 g of treated samples with total hydrolysis mixtures (1 mL) were placed in 2 mL
19 Eppendorf tubes. The enzymatic hydrolysis was performed at 50°C for 72 h with
20 stirring/shaking. The experiment was performed in **triplicate** and the results are
21 presented as the average values. The enzymatic digestibility was represented by the
22 obtained sugars (g sugars/ g materials) or sugar yield as calculated in equation (3):

23

$$24 \quad \text{Sugar yield (\%)} = [\text{weight of monomeric sugars after enzymatic hydrolysis/ weight of} \\ 25 \quad \text{potential total monomeric sugars after hydrolysis using H}_2\text{SO}_4] \times 100 \quad (3)$$

2.10 HPLC analysis

Detection of sugars before and after enzymatic hydrolysis was performed using high-performance liquid chromatography (HPLC) equipped with a refractive index detector (RID-10A, Shimadzu, Japan) using an Aminex HPX-87P column (7.8 mm I.D. × 30 cm, BioRad, USA) with a Carbo-P micro-guard cartridge. The column oven was set at 80°C and samples were eluted at 0.60 mL/min with water. Acetic acid, furfural, hydroxymethylfurfural, and other chemical compounds were prepared and analyzed as reported earlier (Inoue et al., 2008).

3. Results and discussion

3.1 Component analysis of OPMF

As shown in Table 1, the dry basis of OPMF compositions used in this study was determined to be 25.0% cellulose, followed by 25.7% hemicellulose, and 25.5% lignin. The content of OPMF was comparable to other studies (Iberahim et al., 2013), however all compositions were relatively lower than that reported by Nordin et al. (2013). The extractive was detected higher in this study due to difference in preparation of raw material whereas samples were sun dried prior cutter milled instead of washing and cleaning that removed excess oil from oil palm extraction process (Nordin et al., 2013). The differences of the values presented here may be due to the different methods employed in the determination of OPMF composition. The presence of crushed endocarp or palm kernel shell in the OPMF sample may also influence the results. The high lignin content and equal composition of cellulose and hemicellulose probably makes OPMF a difficult biomass to be treated, therefore several pretreatment methods are tested in this study.

3.2 Pretreatment of oil palm mesocarp fiber

3.2.1 Effect of ball milling pretreatment

OPMF samples were ground to a size under 2 mm by cutter mill prior to ball milling experiment. Ball milling pretreatment of OPMF by an appropriate milling period was aimed to reduce the particle size, increase its surface area and reduce the crystallinity index of cellulose thus enhancing its enzymatic digestibility and biosugar conversion. As shown in [Table 2](#), reduction of particle size was recorded about 42.6% for a ball milling time of 240 min. The enzymatic hydrolysis of ball-milled treated OPMF was performed to observe the efficiency of ball milling pretreatment and the yield of xylose and glucose were recorded ([Table 2](#)). Even though xylose and glucose conversion yield increased with ball milling time, the conversion yields obtained from this study were low compared to other studies ([Hideno et al., 2009](#); [Silva et al., 2010](#); [Liao et al., 2011](#)). It was postulated that minimal reduction of CrI of cellulose and low sugar conversion obtained from this process might due to strong OPMF structure that limit the enzyme penetration. To add weight to **this** hypothesis, fractionation of ball-milled treated OPMF samples for 240 min was carried out using an Analysette 3 vibratory sieve shaker (Fritsch, Germany) as shown in [Fig. S2a](#). Three different fiber sizes were recorded and approximately 58-60% of samples collected from sieving $\leq$ 250 μ m were mainly from the degradation of mesocarp (pulp) fibers ([Fig. S2b](#)). The rest of the samples were exocarp fibers that remained unaffected by the ball milling pretreatment. This finding may suggest that exocarp fibers have solid surface and strong cellulose-hemicellulose-lignin network and recalcitrance to enzymatic penetration. There have been conflicting reports regarding correlation between direct and indirect conversion of lignocellulose into glucose from particle size reduction alone

(Vidal et al., 2011). Besides reduction of particle size, it was reported that greater enzymatic hydrolysis efficiency could be achieved by increasing the pore size and volume by removing a larger percentage of hemicellulose and lignin (Musatto et al., 2008; Vidal et al., 2011; Palonen et al., 2004). From the results, OPMF appeared to be not amenable to degradation by BM pretreatment giving a low reduction in particle size, CrI and sugar yields, indicating a small effect on the chains and minimal damage to the cellulose-hemicellulose-lignin network. Other pretreatment methods should be explored to alter and unravel the rigid structure of OPMF.

3.2.2 Effect of NaOH pretreatment

Chemical pretreatment of OPMF was performed by addition of NaOH at various concentrations and incubated at 50°C for 3 h. This experiment was performed to clarify whether the addition of alkali would improve the conversion yield of sugars. The yield of xylose and glucose gradually increased over higher NaOH concentrations and reached the highest conversion at 20.5% and 46.5% respectively when 2.5% NaOH was used (Table 3). Improved sugar conversion yields from NaOH- treated OPMF samples can be explained by saponification of ester bonds that link lignin to structural carbohydrate (Ishiguro and Endo, 2014) and dissolution of lignin that acts as shield of plant cell walls, thus loosening up and weakening the tough OPMF structures (Ibrahim et al., 2013). Sun et al. (1995), reported that alkali pretreatment on wheat straw has resulted in swelling of cellulose and reduction of CrI of cellulose and increased the conversion of glucose. Higher conversion of xylose and glucose can be obtained at higher NaOH concentrations, however that is not the main objective of this study as pretreatment of OPMF by NaOH has been reported earlier (Ibrahim et al., 2013). Even though alkali pretreatment has advantage over mechanochemical treatment in terms of

cost and treatment efficiency, higher loading of chemicals may limit its large scale applications and downstream processing, as well as giving negative impact to the environment. Nevertheless, the results confirmed that addition of NaOH affected the rigid structure of cellulose-hemicellulose-lignin network and provides useful insights in designing an efficient OPMF pretreatment.

3.2.3 Effect of hydrothermal pretreatment

Hydrothermal pretreatment has been employed in the pretreatment of many lignocellulosic biomass, as the process only used water as a reaction medium (Overend and Chornet, 1989; Mosier and Wyman, 2005; Möller et al., 2011; Nitsos et al., 2013). Furthermore, *in situ* acetic acid production during hemicellulose degradation will further enhanced the hydrothermal process performance. It has been reported that the types and concentration of biosugars are greatly influenced by the holding temperature and residence time (Overend and Chornet, 1989; Möller et al., 2011; Nitsos et al., 2013). In this study, temperature in the range from 180-220°C and residence time for 20 min were selected to monitor the combined effect of treatment severities ($\log Ro$), chemical composition changes, trend of sugars and by-products released during the treatment process as shown in Table 4. The hydrothermal pretreatment of OPMF resulted in the solubilization of a fraction solids biomass and changes of pH. At moderate reaction temperature, $\log Ro = 3.66$, 21.7% of OPMF solubilization was recorded. The solubilization correlated well with the treatment severity as higher solubilization was achieved at higher temperature, indicating that partial removal of hemicellulose. At the harshest treatment, $\log Ro = 4.83$ about 49.2% of biomass solubilization was obtained.

1 The pH of the treated OPMF slurries was recorded to be in the range from 3.3
2 to 4.1 at treatment severity of $\log Ro = 3.66$ - 4.83. The reduction of pH was due to
3 acetic acid accumulation caused by cleavage of acetyl group in hemicellulose
4 degradation (Möller et al., 2011; Xiao et al., 2013). Hemicellulose content in solid
5 phase was dramatically decreased (51.8%) when OPMF samples were
6 hydrothermal-treated at $\log Ro = 3.66$ and reduced further over higher treatment
7 conditions. The hydrothermal treatment also resulted in higher recovery of cellulose
8 due to hemicellulose removal and the highest cellulose content of 46.3% was recorded
9 when OPMF samples was pretreated at $\log Ro = 3.94$. The hydrothermal pretreatment
10 employed beyond this value has resulted in cellulose degradation indicating `over
11 cooking` of the biomass. Hydrothermal pretreatment has always associated with the
12 release and migration of lignin from cell wall and re-condensation of lignin in the form
13 of spherical droplets on the surface of treated biomass samples (Donohoe et al., 2008;
14 Selig et al., 2007; Pu et al., 2013). About two-fold of Klason lignin was recorded when
15 OPMF was hydrothermal-treated at $\log Ro = 3.66$ compared to untreated sample.
16 Increased in Klason lignin over treatment severities indirectly indicated the successful
17 breakdown of natural recalcitrant polymer matrix of OPMF samples. Our results are in
18 agreement with the findings reported earlier (Donohoe et al., 2008; Selig et al., 2007;
19 Pu et al., 2013; Xiao et al., 2013) whereas Klason lignin was found in accumulation
20 percentage after dilute acid and hydrothermal treatment of cellulose and lignocellulosic
21 biomass.

22 Besides partial degradation of hemicellulose and Klason lignin accumulation,
23 hydrothermal pretreatment of OPMF also caused the released of free monomeric
24 sugars. The major monomeric sugar detected was xylose and others were detected in
25 lower amounts, < 10 mg/ g of substrates (Table 4). The degradation of hemicellulose

1 increased with higher treatment severities until reaching the highest xylose production
2 of 22.7 mg/ g at $\log Ro = 4.25$. Further increased in treatment severities has resulted in
3 the generation of inhibitory products such as furfural and HMF from pentose and
4 hexose which may inhibit enzymatic hydrolysis and fermentation process. Three major
5 by-products namely acetic acids, furfural and HMF were recorded in this study with
6 traces of formic acids during hydrothermal pretreatment of OPMF, indicates a
7 negligible degradation of furfural and HMF. Treatment severities beyond $\log Ro = 4.25$
8 has resulted higher conversion of xylose into furfural. Precise control of treatment
9 parameters (reaction temperature and time) are necessary in the hydrothermal
10 pretreatment to avoid the degradation of exposed cellulose resulted from hemicellulose
11 removal (Xiao et al., 2013; Inoue et al., 2008). The hydrothermal pretreatment of
12 OPMF met the optimal conditions at treatment severity, $\log Ro = 4.25$ by considering
13 maximum xylose released in the treated liquids with moderate conversion of
14 degradation by-products.

15 Treated OPMF samples at different treatment severities was subjected to
16 enzymatic hydrolysis as previously explained in methodology section to observe the
17 effect of hydrothermal pretreatment on enzymatic hydrolysis and sugars conversion.
18 The hydrolysis result was expressed as yield of xylose and glucose obtained as shown
19 in Fig. 1. Xylose yield decreased at higher treatment conditions probably due to the
20 degradation of xylose to furfural. In contrast, glucose conversion increased, reaching
21 the highest conversion at $\log Ro = 4.54$ (53.4%) in line with the higher hemicellulose
22 removal and cellulose recovery. Glucose yield decreased at higher treatment severity
23 due to degradation of hexoses into HMF as explained earlier. Besides degradation of
24 glucose to HMF, other by-products such as acetic acid and furfural might influenced
25 the efficiency of enzymatic digestibility. Acetic acids and furfural might inhibit the

cellulase activities however the values of by-products obtained herewith are considered low (Xiao et al., 2013; Gong et al., 1999). Other possible inhibitory effects on cellulase activities might be by non-specific binding of lignin as higher lignin released was observed at higher treatment severities (Mosier and Wyman, 2005; Musatto et al., 2008; Selig et al., 2007). Enzymatic hydrolysis of hydrothermally treated OPMF samples achieved optimum with 53.4% glucose conversion yield at $\log R_o = 4.54$. It was found that higher glucose conversion was obtained when delignification was performed by NaOH after dilute acid and hydrothermal pretreatment process from brewer's spent grain (Musatto et al., 2008), sugarcane bagasse (Gao et al., 2013) and eucalyptus chips (Ishiguro et al., 2014). However, those works was performed in two steps treatment process whereas filtration, washing and drying steps were involved in which all possible inhibitors were removed. In this study, reaction temperature plays a key role in the type and concentration of sugars produced and proved that hemicellulose has been partially removed at certain treatment conditions. However, the cellulose conversion into glucose from OPMF obtained in this study was low compared to previous studies (Nitsos et al., 2013; Gao et al., 2013; Xiao et al., 2013) due to substrate and cellulase loadings applied. The represent results showed an improvement of pretreatment should be done in order to get maximum access of cellulase to the cellulose. In a later section physico-chemical changes of OPMF samples when treated with hydrothermal alone, impregnated with mild NaOH and combined alkaline hydrothermal- ball milling with the aim to obtain maximum conversion of lignocellulosic OPMF into fermentable sugars will be discussed.

3.3 Physicochemical characteristics of combined hydrothermal and ball mill treated OPMF

Physico-chemical characteristics of untreated and pretreated samples at different pretreatment methods were evaluated in order to understand the mechanism involved in every pretreatment. FTIR spectra of untreated, hydrothermal, alkaline hydrothermal and combined alkaline hydrothermal-ball milling treatment are shown in Fig. S3. Bands 1740 cm^{-1} and 1240 cm^{-1} are mainly ascribed to the stretching vibration of the C=O and C-O bonds of the acetyl ester units present in hemicellulose (Nitsos et al., 2013) and the decrease in intensity of those bands was recorded due to partial removal of hemicellulose and deacetylation that took place in hydrothermal reactions (with and without NaOH addition). Band 1513 cm^{-1} corresponds to the stretching vibration of lignin aromatic rings (Nitsos et al., 2013; Nordin et al., 2013). There was an increased in band intensity due to release or migration and relocation of lignin on the surface of hydrothermally and combined hydrothermal with ball- milled treated OPMF sample. Impregnation of 0.5% NaOH in the hydrothermal process has improved solubilisation of lignin and resulted in reduction of band intensity due to delignification. Meanwhile bands intensity at 2850 cm^{-1} and 2918 cm^{-1} corresponded to the stretching vibration of C-H group relating to cellulose and hemicellulose (Nik Mahmud et al., 2013) that affected after treatment processes. From the results obtained it may suggest that hydrothermal pretreatment with and without NaOH impregnation has affected the structural damage of cellulose-hemicellulose-lignin network by cleavage of ester bonds between lignin and carbohydrate complex and solubilisation of hemicellulose, respectively.

A reduction in cellulose crystallinity is necessary in order to maximize the conversion of glucose since the presence of high crystallinity is one of the limiting factors in the enzymatic hydrolysis of cellulose (Mosier and Wyman, 2005; Hendriks and Zeeman, 2009). In general, the WAXD spectra and CrI values confirmed the

1 disruption of the crystalline structure because of the increased milling time (Table 2).
2 The cause of decreasing CrI values can be explained by the fragmentation of crystalline
3 grains, the deformation of crystalline structure, and the increase of amorphization
4 during ball milling pretreatment (Hendriks and Zeeman, 2009; Lin et al., 2010; Liao et
5 al., 2011; Kim et al., 2013). The CrI of cellulose from combination of hydrothermal and
6 ball milling pretreatments are shown in Fig. S4. The increase in CrI values of the
7 hydrothermal and alkaline hydrothermal treatment can be attributed to an increase in
8 cellulose concentration due to partial removal of hemicellulose and migration and
9 relocation of lignin (Nordin et al., 2013; Inoue et al., 2008; Kaparaju et al., 2010) from
10 OPMF treated samples. Scanning electron microscopy (SEM) was used to observe the
11 effect of ball milling and combined hydrothermal-ball milling treatment at structural
12 level. Fig. S5 shows SEM images illustrating changes in surface morphology of
13 unmilled raw materials (< 2 mm) and ball-milled treated OPMF over varying
14 processing time. Under optimal hydrothermal pretreatment conditions (log Ro = 4.25,
15 200°C, 20 min) the effect of hydrothermal and combined hydrothermal-ball milling
16 treatments on OPMF was visualized as shown in Fig. S6. SEM images has confirmed
17 the hydrothermal pretreatment affected on the breakdown of chemical bonds and
18 altered the organized polymer structures and `peeled off` of outer layer of fibers was
19 observed. The presence of higher accumulation of lignin droplets (Fig. S6b-f) were
20 supported by the increased intensity in FT-IR of lignin vibration bands that attributed to
21 the release and migration of lignin on the surface of fibers as discussed earlier (Fig. S3).

22 23 **3.4 Improved enzymatic hydrolysis by combined alkaline hydrothermal-ball** 24 **milling pretreatment**

1 [Table 5](#) shows a comparative analysis results of chemical composition changes
2 and yield of hydrolysed sugars when OPMF samples were treated at different
3 conditions. Hydrothermal treatment affected hemicellulose dissolution up to 64.8%
4 with high percentage of lignin re-deposition on the surface of treated OPMF.
5 Impregnation of NaOH at varying concentrations has reduced the Klason lignin content
6 by 41.2%- 66.1% at 1.5% and 2.5% NaOH respectively. It was obvious from this study
7 that chemical composition of OPMF changed differently at acidic and alkali
8 hydrothermal reaction. In alkaline hydrothermal reaction, only **23.6%** hemicellulose
9 degradation was recorded with higher cellulose percentage, 48.9% at 1.5% NaOH
10 addition compared to untreated and hydrothermally treated samples. There was no
11 lignin droplet found as supported in [Fig.S6g-i](#). The treated biomass solid was subjected
12 to enzymatic hydrolysis and the efficiency of the pretreatment developed was
13 expressed by the yield of hydrolysed sugars produced. There was an increasing xylose
14 and glucose conversion towards higher NaOH dosage, achieving the highest
15 conversion of **46.5%** and **63.9%**, respectively when 1.5% of NaOH was added and this
16 was relatively higher than untreated and whole slurries of hydrothermal- treated OPMF
17 sample. Our results suggested that hydrothermal with and without impregnation of
18 NaOH has improved the xylose and glucose conversion compared to untreated sample.
19 A greater glucose conversion was observed when OPMF was treated under alkaline
20 condition probably due to saponification of ester bonds that links between lignin and
21 carbohydrate complex then loosening up the fibrils and most of lignin was fragmented
22 and dissolved at $\log R_o = 4.25$. Washing step employed compared to pretreated whole
23 slurries may avoid inhibition of cellulase from degradation by-products. In other
24 reports, higher glucose conversion from various biomass was obtained such as brewer`s
25 spent grain (BSG) ([Musatto et al., 2008](#)), sugarcane bagasse ([Gao et al., 2013](#)) and

wheat straw (Govumoni et al., 2013) when alkali pretreatment was employed in the second step of pretreatment processes due to delignification took place.

Since higher percentage of hemicellulose can be retained and partial removal of lignin was obtained in alkaline hydrothermal pretreatment, combined hydrothermal and ball milling treatment was performed to alter the structure and unravel the OPMF polymer matrix further. Hydrothermally treated OPMF samples, without and with impregnation of 1.5% NaOH at $\log R_o = 4.25$ was ball milled for 2 h and enzymatic hydrolysis was performed. About 18- 24- fold increase in glucose yield was obtained from combined hydrothermal and ball milling sample without and with impregnation of 1.5% NaOH respectively compared to untreated sample. About 6-fold increase in xylose was obtained from alkaline hydrothermal process. There was substantial improvement of xylose and glucose yield especially alkaline hydrothermal and ball milling treatment compared to hydrothermal treatment alone because ball milling treatment has been reported to cause reduction of particle size, breaking the cellulose-hemicellulose-lignin network (Silva et al., 2010; Inoue et al., 2008), and reducing the CrI of crystalline cellulose (Kim et al., 2013; Lin et al., 2010) thus enhancing conversion of cellulose into glucose. In contrast, only 2.95% increment of glucose conversion yield was recorded when hydrothermally treated OPMF was ball milled for 2 h compared with only hydrothermal treated samples indicates tough properties of OPMF fibers with no substantial reduction of CrI value as supported by previous analysis (Fig. S4). From data obtained, there is no direct correlation between CrI of cellulose and enzymatic digestibility since both hydrothermal (with and without NaOH addition) and alkaline hydrothermal-ball milling treatment with higher and slightly lower CrI values than untreated sample showed increased in glucose conversion. Our finding was in agreement with studies reported by Park et al. (2010)

1 since CrI of cellulose was one of several other factors influenced the accessibility of
2 cellulase to cellulose. Interestingly, about 31.0% of glucose yield was obtained from
3 alkaline hydrothermal and ball milling treatment compared to alkaline hydrothermal
4 alone (Table 5), indicates significant role of NaOH in alteration of physico-chemical
5 properties of OPMF samples.

6 Substantial improvement of glucose conversion of combined treatment with
7 ball milling was reported earlier (Ishiguro and Endo, 2014; Inoue et al., 2008; Teramoto
8 et al., 2008). About 100% of glucose conversion to glucose was achieved when
9 eucalyptus wood chips was treated by 20% of NaOH per substrate weight in combining
10 hot compressed water (HCW) (170°C, 60 min) and ball milled for 2 h (Ishiguro and
11 Endo, 2014) and combined sulphuric acid-free ethanol cooking and ball milling
12 (Teramoto et al., 2008) . In other study, Inoue and co-workers (2008) reported that, the
13 eucalyptus treated with HCW (160°C, 30 min) followed by ball milling for 20 min had
14 improved the digestibility of both xylan and glucan. They concluded that HCW-ball
15 milling treatment substantially reduced cellulase loadings and ball milling time thus
16 reducing the total ethanol production cost. It was found that removal of hemicellulose
17 and lignin has greater impact than reduction of CrI of cellulose and particle size. This
18 may conclude that the addition of NaOH as low as 1.5%, successfully broke down the
19 ester bond between lignin and carbohydrate linkages, loosening up the fibrils and
20 making OPMF more fragile that eased in the ball milling process.

21 The effect of cellulase loadings on enzymatic hydrolysis of alkaline
22 hydrothermal-mechanochemical treated OPMF is presented in Fig. 2. As widely
23 understood, lower cellulase loading in the enzymatic hydrolysis of biomass will bring
24 down the total production cost, however the rate of sugar conversion was rather low and
25 needed longer treatment time. In this work, several pretreatments and combination of

pretreatments were investigated to obtain minimum cellulase loading in the enzymatic hydrolysis step. The conversion of OPMF into xylose and glucose achieved more than 70% at cellulase loadings 5 and 10 FPU/ g -substrate when incubated for 72 h and exhibited total conversion yield which correlated well with cellulase loadings. The results obtained here are comparable to those reported earlier (Inoue et al., 2008). Faster conversion rates were observed when the samples were loaded with higher enzyme activities at 40 and 80 FPU/g-substrate with more than 85% of total conversion yields obtained after 24 h incubation period. Lower xylose and glucose conversions at 60.7% and 87.0% were reported when the OPMF was hydrolyzed at 60 FPU/ g-substrate using sole NaOH pretreatment (Iberahim et al., 2013). This might be due to low lignin removal that still hindered cellulase attack. The present pretreatment offered several advantages over previous studies (Iberahim et al., 2013; Nik Mahmud et al., 2013) with lower NaOH addition, larger percentage of hemicellulose retained, with higher xylose and glucose conversions.

4. Conclusion

Results obtained showed that alkaline hydrothermal pretreatment improved partial removal of Klason lignin and silica bodies, increased saponification of ester bonds, and detainment of hemicellulose component. Subsequent mechanochemical pretreatment has substantial impact on reduction of particle size, CrI of cellulose and increase surface area of treated OPMF, thus enhancing the enzymatic digestibility. The developed method proved that all possible factors might limit the accessibility of cellulase to cellulose has been reduced. Furthermore this strategy has advantages in terms of maximal bioconversion compared to hydrothermal process alone and showed the highest conversion of xylose and glucose from OPMF ever reported.

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2 enzymatic hydrolysis of *Tamarix ramosissima*: Evaluation of the process as a
3 conversion method in a biorefinery concept. Bioresour. Technol. 135, 73-81.
4

5 **List of Tables**

6

7 **Table 1** Composition of OPMF used in this study and compared from previous reports
8

9 **Table 2** Effect of BM-treated OPMF on particle size and crystallinity index, xylose and
10 glucose

11 ¹Crystallinity index of BM-treated samples determined by wide-angle X-ray diffraction
12 and calculated as described in material and methods section.

13 ²Determined by HPLC
14

15 **Table 3** Effect of NaOH pretreatment on oil palm mesocarp fiber

16 ¹5% (w/v) substrate was hydrolyzed at cellulase loading, 10 FPU/g-substrate, and
17 incubate for 72 h at 50°C with stirring.
18

19 **Table 4** Chemical composition, monomeric sugars and degradation by-products
20 obtained from hydrothermally treated OPMF at varying treatment severity (log Ro)

21 ¹Monomeric sugars were determined by HPLC

22 *Data presented herewith are average from triplicate experiments
23

24 **Table 5** Effect of impregnation of NaOH in hydrothermal system on chemical
25 composition, xylose and glucose yield

- 1 ¹The experiment was performed in duplicate
- 2 ²Monomeric sugars were determined by HPLC
- 3 ³Both pretreated liquid and solid were subjected to enzymatic hydrolysis
- 4 ⁴Pretreated solids were recovered after rinsed with distilled water until neutral pH and
- 5 dried at 90°C
- 6 HT = hydrothermal treatment at 200°C for 20 min (log Ro= 4.25)
- 7 *Hemicellulose dissolution, % = (Hemicellulose_{untreated} – Hemicellulose_{treated})/
- 8 Hemicellulose_{untreated} x 100%

9

10 **Figure Captions**

11 **Figures**

12

13 **Fig. 1.** Yield of xylose and glucose from recovered solids of hydrothermal treatment

14 versus log Ro (● xylose, ■ glucose) at cellulase loading 10 FPU (g/ g- substrates).

15

16 **Fig. 2.** Time course enzymatic hydrolysis of combined alkaline hydrothermal-ball

17 milling treated OPMF (HT + 1.5% NaOH + BM 120 min) samples at varying

18 cellulase loadings. BT (hydrothermal treatment, BM (ball milling).

19

Table 1

Components		Content (wt.%)	
Cellulose	25.0 ± 1.7	28.8 ± 0.48	42.8 ± 0.69
Hemicellulose	25.7 ± 3.3	25.3 ± 0.65	33.1 ± 2.01
Acid insoluble lignin	25.5 ± 0.5	28.9 ± 2.07	20.5 ± 3.44
Extractives	11.4 ± 0.2 ^a	6.3 ± 0.51 ^b	-
Ash	5.8 ± 0.2	2.6 ± 0.34	3.6 ± 0.74
References	This study	Iberahim et al. (2013)	Nordin et al. (2013)

^aAcetone extractives

^bEthanol extractives

‘-‘ Not determined

Table 2

Ball milling time	Geometric mean diameter (μm)	Size reduction (%)	CrI¹ (%)	Glucose (%)²	Xylose (%)²
Unmilled (<2 mm)	407.5	0	38.1	4.1 ± 1.5	10.9 ± 0.6
BM- 60 min	397.8	2.4	36.8	7.3 ± 2.1	12.2 ± 2.2
BM- 120 min	392.5	3.7	35.1	7.4 ± 0.7	12.7 ± 0.4
BM- 240 min	233.8	42.6	29.5	10.3 ± 2.6	14.9 ± 1.8

Table 3

Pretreatment¹	Yield of hydrolyzed sugars (%)	
	Glucose	Xylose
Untreated	4.1 ± 1.5	10.9 ± 0.6
NaOH 1%	28.4 ± 3.4	17.6 ± 1.3
NaOH 1.5%	40.4 ± 1.3	22.4 ± 0.5
NaOH 2.5%	46.5 ± 6.9	20.5 ± 3.9

Table 4

		Severity factor (log <i>Ro</i>)					
		Untreated	3.66 (180°C, 20 min)	3.94 (190°C, 20 min)	4.25 (200°C, 20 min)	4.54 (210°C, 20 min)	4.83 (220°C, 20 min)
pH of pretreated liquid		6.8 ± 0.2	4.1 ± 0.1	3.8 ± 0.3	3.6 ± 0.2	3.5 ± 0.4	3.3 ± 0.2
Solid recovery (w/w %)		100	78.3 ± 0.9	63.8 ± 5.2	56.6 ± 0.8	54.4 ± 0.7	50.8 ± 0.2
Chemical compositions (w/w %)							
Cellulose		25.5 ± 1.7	40.0 ± 0.3	46.3 ± 0.5	43.9 ± 1.1	38.7 ± 1.5	33.6 ± 2.6
Hemicellulose		25.0 ± 3.3	12.4 ± 0.1	7.7 ± 1.8	8.7 ± 1.2	6.2 ± 0.8	7.0 ± 0.1
Klason lignin		25.5 ± 0.5	50.9 ± 6.8	51.6 ± 5.4	54.5 ± 1.3	58.0 ± 1.0	60.9 ± 1.9
Monomeric sugars and degradation by-products formation (mg/g)^{1*}							
Glucose	-		1.8 ± 2.0	2.7 ± 1.3	4.4 ± 3.2	2.8 ± 1.2	1.4 ± 0.2
Xylose	-		3.2 ± 2.4	13.1 ± 2.3	22.7 ± 3.6	5.0 ± 2.0	0.1 ± 0.2
Galactose	-		0.6 ± 0.8	2.5 ± 1.9	4.0 ± 1.0	2.1 ± 1.9	0.8 ± 1.1
Arabinose	-		8.2 ± 1.5	5.5 ± 1.1	4.3 ± 0.5	2.2 ± 0.5	0.8 ± 0.5
Mannose	-		0.0	3.3 ± 2.1	5.1 ± 3.1	2.9 ± 3.0	0.8 ± 1.1
Acetic acids	-		20.9 ± 5.2	38.4 ± 6.0	55.9 ± 7.2	74.6 ± 6.4	81.0 ± 0.9
Furfural	-		2.1 ± 0.9	9.7 ± 1.7	24.9 ± 6.1	35.8 ± 1.2	32.2 ± 1.2
5-HMF	-		0.9 ± 0.8	1.3 ± 0.7	2.1 ± 0.8	4.1 ± 0.2	6.3 ± 0.3

Table 5

Pretreatment ¹	Chemical compositions (w/w %)			Yield of hydrolyzed sugars, % (g/g-substrate) ²	
	Cellulose	Hemicellulose	Lignin	Glucose	Xylose
Untreated OPMF	25.5 ± 1.7	25.0 ± 3.3	25.5 ± 0.5	4.1 ± 1.5	10.9 ± 0.6
HT (whole slurry) ³	45.9 ± 0.7	8.8 ± 2.8	57.5 ± 1.3	48.4 ± 6.2	46.9 ± 9.9
HT + 0.5% NaOH ⁴	44.3 ± 1.9	17.3 ± 0.7	47.4 ± 0.9	53.1 ± 13.3	21.1 ± 3.6
HT + 1.0% NaOH ⁴	46.5 ± 2.7	17.8 ± 2.4	46.5 ± 0.5	39.6 ± 13.8	42.3 ± 2.9
HT + 1.5% NaOH ⁴	48.9 ± 2.3	19.1 ± 1.3	33.8 ± 0.6	63.9 ± 6.2	46.5 ± 1.4
HT + 2.5% NaOH ⁴	51.7 ± 1.6	27.2 ± 0.7	19.5 ± 0.3	52.2 ± 9.5	26.5 ± 8.7

*Hemicellulose dissolution, % = (Hemicellulose_{untreated} – Hemicellulose_{treated})/ Hemicellulose_{untreated} x 100%

Figure 1

Figure 2