# Structural Analysis on the Effect of Base-Catalysed Delignification Process Parameters on Palm Oil Empty Fruit Bunches Fibres using Glycome Profiling

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**Abstract.** Malaysian Oil Palm Empty Fruit Bunches (EFB) fibre was used as a model substrate and was subjected to base-catalysed delignification technique as a function of different temperature, time and base concentration. The resultant substrates were analysed using glycome profiling technique to ascertain the degree of delignification effectiveness and position of the residual lignin in the structure of the pre-treated biomass. The study indicated that the base-catalysed delignification technique removes all the outer lignin structure of the fibre due to the accessibility of the base to the surface of the biomass. Base wash concentration using sodium hydroxide (NaOH) and temperature are the two main factors that determine the effectiveness of lignin removal and increment of surface area accessibility (SAA). The pre-treated biomass that contains the lowest amount of residual lignin content of 6.19 wt.% which corresponds to 74.47% lignin removal shows complete removal of the outer layer of lignin structure but leaves the inner lignin structure intact. This study suggests that the complex interaction between different cell wall constituents plays a much bigger role in order to achieve efficient delignification.

## 1. Introduction

Biomass resources, in particular, agricultural wastes or forestry residues posed significant challenges due to their recalcitrant nature, varying degree of constituents' content and complex intermolecular hydrogen-bond networks. In the review made by Zhao *et al*., pretreatment techniques shall exert influence on accessible surface area, biomass relevant-factors such as pore size, chemical composition (such as lignin and carbohydrate content) and cellulose crystallinity [1]. Reviews made by Zhao *et al*. and Kumar *et al*. provide an extensive body of knowledge on the various physico-chemical techniques of treating and conditioning such feedstock prior to its valorisation to fuels or chemicals [1, 2]. Martin *et al.* provides a review which summarises the limitation of such techniques as it contributes to the formation of non-beneficial side products that could render subsequent process to be less effective such as short-chained mono and di acids, ferulic acid and even monosaccharides [3].

Biomass recalcitrance is a term that is being used to describe the interaction between cell-wall microstructure which essentially provide the strength and structure to any plant [4-6]. Cell-wall is essentially a microfibril structure that consists of vascular bundles built from polymeric cyclodextrin (or cellulose) and surrounded by lignin-hemicellulose matrix. The same hemicellulose contains ferulated functionality which provides site for cross-bonding between the lignin and carbohydrates, also known as lignin-carbohydrate complex [7-9]. Due to this structural heterogeneity and intertwining of different polymeric chemical compositions, it is fairly difficult to deconstruct any biomass.

Glycome profiling is a high-throughput technique that employs glycan-directed monoclonal antibodies (mAbs) to monitor structural changes of plant cell wall [10-11]. The technique allows in situ visualisation of specific substrates, or epitopes as it is called, using specific molecular probes post selective chemical extractions with increasing severity. This essentially allows large and wide range monoclonal antibodies to be utilised to detect and identify diverse features of cell wall polysaccharides [12].

Interactions between lignin, hemicellulose and pectin with cellulose and their contribution to overall biomass recalcitrance has been widely illustrated using Poplar or *populus sp*. as model biomass. One of the striking features of poplar, as observed by DeMartini *et al*., is the existence of cross-linked pectin, arabinogalactan and various loosely-bounded xylans [13-14]. Removal of gene controlling pectin synthesis in poplar has been proved to contribute to reduced recalcitrance of the plant cell wall whilst maintaining the amount of polysaccharide as per the native species [15]. Variation of lignin content, as measured in part with syringyl and guaicol ratio (S/G ratio) was exemplified in poplar, of which lower lignin content would have favourable effect on the sugar hydrolysis [16].

Using Poplar as model biomass, the effect of pretreatment techniques such as hydrothermal, dilute acid and ammonia explosion (AFEX™) on plant cell wall deconstruction can be semi-quantified using glycome profiling. Hydrothermal technique has shown that outer-layer xylans is more soluble in this condition with additional benefit of incremental average pore diameter [17-20]. More closely-bounded xylans in the macrofibrils is much harder to remove, as exemplified by removal of xylan epitopes or subsets with harsher extraction techniques. Similar observations were made by Singh *et al*. using corn stover as substrate [6].

This work is a continuation of the work first published herein [21]. The work shall describe the use of glycome profiling to investigate the changes on the plant cell wall and structural modification induced by base catalysed delignification process using palm oil biomass as substrate. As far as we are concern, no work of similar nature has been done on palm oil biomass. Three (3) independent parameters, namely, temperature, base concentration and time were varied with final lignin content of the cellulose-rich material as the dependent parameter.

## 2. Materials and Methodologies

### *2.1 Materials and Chemicals*

Empty Fruit Bunches (EFB) fibres were sourced from a local mill located in Selangor, Malaysia. The fibres were already processed and pre-cut to 2 mm average fibre length. Further grinding was done on lab scale grinder (IKA Labortechnik) with sieve of 1 mm to get the desired fibre length. All chemicals used, sodium hydroxide (NaOH, Fisher Chemical), ammonium oxalate ($C_2H_8N_2O_4$, Acros Organics), sodium carbonate ($Na_2CO_3$, Acros Organics), potassium hydroxide (KOH, J.T. Baker) and sodium chlorite ($NaClO_2$, Acros Organics) were used as received unless stated otherwise.

### *2.2 Brunauer-Emmett-Teller (BET) Gas Adsorption Method*

Method described by Sing *et al.* (1985) was used to determine surface area of the biomass [22]. Adsorbed materials from the surface of our samples were eliminated by heating the samples under vacuum at a temperature of 353.15K for 10 hours before analysis. The adsorption/desorption experiments were carried out at liquid nitrogen temperature of 77.15 K and were measured on a Micromeritics ASAP 2020 automatic surface area and pore radius distribution analyzer. Quantity of adsorbed and desorbed gas was calculated from changes in pressure using pressure transducers. BET analysis was carried out to determine total surface area of our samples.

*2.3 Sodium Hydroxide (NaOH) Pretreatment of Empty Fruit Bunches (EFB) Fibres*

The NaOH pretreatment or 'based-catalyst delignification' of EFB was followed the procedure published by Zawawi *et al.* [21]. NaOH is one of the strongest base catalyst and it is capable to break the linkage between lignin and hemicellulose in lignin-carbohydrate complexes (LCC). The NaOH pretreatment of untreated EFB fibres was carried out in a 1.5 L PARR reactor (PARR USA). The concentrations of NaOH used was 0.5 wt.% to 5.5 wt.%, residence time was 60 to 120 min and stirring rate was 500 rpm. These experiments were conducted at a temperature range of 373 K to 473 K.The composition of the pre-treated and untreated EFB samples were then analysed using Van Soest method and ELISA (enzyme-linked immunosorbent assay). The methodology for Van Soest method was adopted from work by Goering and Van Soest (1970), Van Soest *et al.*(1991) and Chaves *et al.*(2002) [23-25].

*2.4 Total Sugar Estimation and Glycome Profiling using ELISA*

Sequential extraction of the cell walls (AIR) samples and glycome profiling was carried out as described by Pattathil *et al.* [12] to isolate fractions enriched in various cell wall components. Sequential extraction involved different reagents (50 mM ammonium oxalate, 50 mM sodium carbonate, 1 M KOH, 4 M KOH, three additions of 0.125 g of sodium chlorite and 4 M KOH post chlorite). The supernatants were labelled as 'Ammonium Oxalate Extract', 'Carbonate Extract', '1 M KOH Extract', '4 M KOH Extract' and post chlorite – '4 M KOH PC fraction'. All extracts were dialyzed (using 3,500 Da molecular weight cut-off tubing, spectrum Laboratories Inc. CA, USA) against four changes of deionized water (with an approximate sample to water ratio of 1:60) for 48 h at room temperature and freeze-dried individually. All extracts were dissolved in deionized water at a concentration of 0.2 mg/mL in a 15 mL centrifuge tube. The total sugar estimation was done using a phenol-sulphuric acid colorimetric assay [26, 27]. The extract samples were diluted to a sugar concentration of 0.2 mg/mL. ELISA plates (Costar 3598) were coated with 50 µL per well of extract and allowed to evaporate to dryness overnight in a ventilated incubator at a temperature of 310 K until dry. The glycome profiling analyses involved ELISA-based screening of EFB biomass materials against seven plant cell wall glycan-directed monoclonal antibodies (mAbs). Negative controls consisting of water blanks without samples and antigen were included in all assays and their absorbance subtracted from all samples. The ELISA responses of the monoclonal antibodies binding intensities to each extract were presented as heat maps. Monoclonal antibodies were obtained as hybridoma cell culture supernatants from the main suppliers of plant cell wall glycan-directed mAbs such as CarboSource, PlantProbes and BioSupplies. There were seven mAbs used in the ELISA including xylan (LM10, CCRC-M113), xyloglucan (LM15, CCRC-M96, CCRC- M102), (1→3)-β-glucan (400-2) and (1→3, 1→4)-β-glucan (400-3).

## 3. Results and Discussion

*3.1 Surface Area Measurement (BET) and Compositional Analysis*

Table 1 and Table 2 summarize the BET results and compositional analysis of six (6) different samples subjected to increasing severity of delignification process. BET surface area measurements revealed that EFB pretreated with high concentrated NaOH solution resulted in a more porous material with higher surface area compared to those untreated materials or those treated in less concentrated NaOH solutions. The average pore diameter of sample E is 1.6 times larger compared to sample UN (untreated). Surface area of sample E further confirmed this observation as it is 2.37 times larger than sample UN. Rezende *et al.* confirmed that when sugarcanes bagasse samples were pretreated with NaOH concentrations between 1-4%, no significant morphological differences were observed between the samples [28]. Therefore, in order to increase assessable surface area as well as larger average pore

diameter of EFB samples with low lignin content, it is crucial to subject the EFB fibers to harsh pretreatment conditions of 5.5 wt.% NaOH solutions at 473K for 1 hour.

The lower BET surface area of sample A to D as compared to untreated sample is due to the dehydration step of which it removes water and collapse the open pores. The same observation was made by Kaewprasit *et al.* [29].

**Table 1.** BET results for pretreated and untreated EFB samples

| Sample code | Pretreatment | BET surface area ($m^2/g$) | Adsorption average pore diameter (Å) |
|---|---|---|---|
| **UN** | Untreated EFB | 1.2795 | 67.0955 |
| **A** | Base 0.5 wt.%, 423 K, 120 min | 1.2152 | 61.6034 |
| **B** | Base 1.0 wt.%, 423 K, 120 min | 0.9755 | 36.0061 |
| **C** | Base 1.75 wt.%, 423 K, 90 min | 0.8016 | 45.4848 |
| **D** | Base 3.0 wt.%, 423 K, 120 min | 1.7422 | 46.8362 |
| **E** | Base 5.5 wt.%, 473 K, 60 min | 3.0393 | 108.0183 |

Table 2 shows the composition of hemicellulose, cellulose and lignin by using the Van Soest method. The hemicellulose content for all pre-treated samples varied between 8.83 wt.% to 18.41 wt.% while untreated samples gave hemicellulose content at 26.76 wt.%. Sample E that was pretreated with the highest base concentration gave the highest cellulose content at 84.53 wt.% and followed by sample D and C at 71.41 wt.% and 70.86 wt.%, respectively. The cellulose content for other pretreatments including untreated EFB samples were varied in the range of 41.26 wt.% to 66.40 wt.%. Sample E showed the lowest lignin content of 6.19 wt%. This is followed by samples D, C, B and A at 8.69 wt.%, 9.55 wt.%, 14.06 wt.% and 18.20 wt.%, respectively. Untreated EFB samples gave the highest lignin content at 24.25 wt.%. Based on the overall results, sample E with a pretreatment parameters of NaOH concentration 5.5 wt.% at 473 K for 60 min showed the best performance with highest cellulose content and lowest lignin content as compared with other pretreatment parameters. Study by Lima *et al*. reported that higher NaOH concentration at 4.0 wt.% is adequate to keep lignin dispersed in the pretreatment liquor but with lower NaOH concentration of less than 1.0 wt.%, it is not sufficient to open the cellulose microfibrils and to expose the crystalline surfaces [30]. The study conducted by Rezende *et al.* using sugarcane bagasse samples further confirmed the effectiveness of higher NaOH concentration in lowering the lignin content of biomass [28].

**Table 2.** Analysis of EFB samples using Van Soest method

| Sample code | Pretreatment | Van Soest method (wt.%) | | | |
|---|---|---|---|---|---|
| | | Hemicellulose | Cellulose | Lignin (AIL) | Grand total |
| **UN** | Untreated EFB | 26.76 | 41.26 | 24.25 | 92.27 |
| **A** | Base 0.5 wt.%, 423 K, 120 min | 5.60 | 66.40 | 18.20 | 90.20 |
| **B** | Base 1.0 wt.%, 423 K, 120 min | 18.41 | 62.04 | 14.06 | 94.51 |
| **C** | Base 1.75 wt.%, 423 K, 90 min | 10.15 | 70.86 | 9.55 | 90.56 |
| **D** | Base 3.0 wt.%, 423 K, 120 min | 16.55 | 71.41 | 8.69 | 96.65 |
| **E** | Base 5.5 wt.%, 473 K, 60 min | 8.83 | 84.53 | 6.19 | 99.55 |

*AIL = Acid Insoluble Lignin

*3.2 Glycome Profiling of EFB Fibres*

Recent studies on the usage of glycome profiling technique as a primary semi-quantitative tool to analyse the plant biomass and its components have been published [10, 12-13]. Glycome profiling technique uses a set of extractions to solubilize the different carbohydrate materials from the plant cell wall. It depends on how these components are bound into the walls. All untreated and pre-treated EFB samples were further investigated for the changes on the plant cell wall and structural modification especially on lignin distribution using this technique. The extracted samples released from each untreated and pretreated EFB samples by reagents were loaded onto the ELISA plates and were screened against the monoclonal antibodies. Several plant cell wall glycan-directed monoclonal antibodies (mAbs) were selected to recognize epitopes (specific glycan subcultures) present in the extracts. Figure 1 shows the overall heat maps for pretreated and untreated EFB samples.

Sequential extraction using ammonium oxalate, sodium carbonate, 1 M KOH, 4 M KOH, chlorite and 4 M KOH post chlorite are selected since they have the capability to separate plant cell wall components [31]. These extracts are selectively enriched with various classes of cell wall glycans based on the tightness with which they are integrated into the cell walls [10]. Based on Figure 1, xylan epitopes represent the most abundant recognizable hemicellulosic epitopes in all pretreated and untreated EFB samples. Xylans were extracted out under all extraction conditions as indicated by the binding patterns with strong significant binding of mAbs such as LM 10 with the sample extracts. This observation was supported by the work done by Simmons *et al*. which reported that xylan was the most dominant non-cellulosic polysaccharides that binds to cellulose in the cell wall. The interaction of xylan with cellulose is expected to have the most profound effect on the cell wall characteristics with higher xylan folded onto cellulose surfaces [32] which later increased the cellulose content. Based on our study, this ELISA results rationalize the Van Soest analysis results which showed higher cellulose content in samples A to E and untreated EFB sample as compared to their respective hemicellulose and lignin content. The study conducted by Pattathil *et al.* further confirmed that the plant cell wall is comprised largely of cellulose microfibers that are embedded in a macromolecular matrix of glycans [17].

Figure 1 shows other hemicellulosic epitopes, such as fucosylated xyloglucan (CCRC-M102) and non-fucosylated (CCRC-M96) were less abundantly present based on weak/moderate binding with samples extracts of pretreated and untreated EFB, indicating that xyloglucans are not completely solubilized during the pretreatment even at the high base concentration of NaOH (sample E). Both fucosylated and non-fucosylated xyloglucans were found to be released more in the 4 M KOH treatment for pretreated and untreated EFB samples (Figure 1). Among these xyloglucans, the presence of fucosylated xyloglucan epitopes were in higher proportions compared to non-fucosylated xyloglucans epitopes. The relative distribution of solubilized xylans and xyloglucans were found to be comparable among the pretreatments (from lower to higher base concentration of NaOH), indicating that all pretreatments solubilize similar pools of hemicellulosic glycans. β-glucan were also present in less abundance based on weak/moderate binding of this mAbs with the sample extracts of pretreated and untreated EFB (Figure 1).

This study explains the composition, extractability and relative proportions of cell wall components such as hemicellulose, cellulose, pectin and lignin in EFB fibres. This is to ensure that our hypothesis on alkaline pretreatment is accurate especially on lignin removal. More tightly bound lignin components of the cell wall materials are removed by using a chlorite treatment, leaving polysaccharides into the generated extract. Based on heat maps for chlorite extraction treatment in Figure 1, the removal of lignin in the EFB pretreatment will give the strong binding between the mAbs and sample extracts. However, glycome profiling results shows differently with weak and moderate binding occurred between the mAbs and samples A to E including untreated EFB extracts, indicating that these lignin-associated cell wall glycans were still trapped in the extract of samples after the chlorite treatment. Chlorite extract of pretreated and untreated EFB contained xylans and β-glucan as

denoted by the weak binding of mAbs with sample extracts. No xyloglucans were present in the chlorite treatment (Figure 1). The ELISA results of lignin content were comparable with the Van Soest analysis of lignin content at 6.19 wt.% to 18.20 wt.% that shows considerable amount of lignin still remained tightly bound in the samples even after the harsh alkaline pretreatment (e.g 5.5 wt.% of base concentration). The post chlorite alkaline extract (4 M KOH PC) was used to further solubilize the remaining polysaccharides after lignin removal to make it more extractable. Based on Figure 1, the extracts produced in 4 M KOH PC was found comparable in composition to other alkaline extracts (4 M KOH) with proportions of hemicellulosic polysaccharides, xyloglucans and xylans. The study done by DeMartini *et al.* and Venketachalam *et al.* confirmed that the results were similar for the post chlorite extracts with the significant amounts of various epitopes in the cell wall [10, 33].

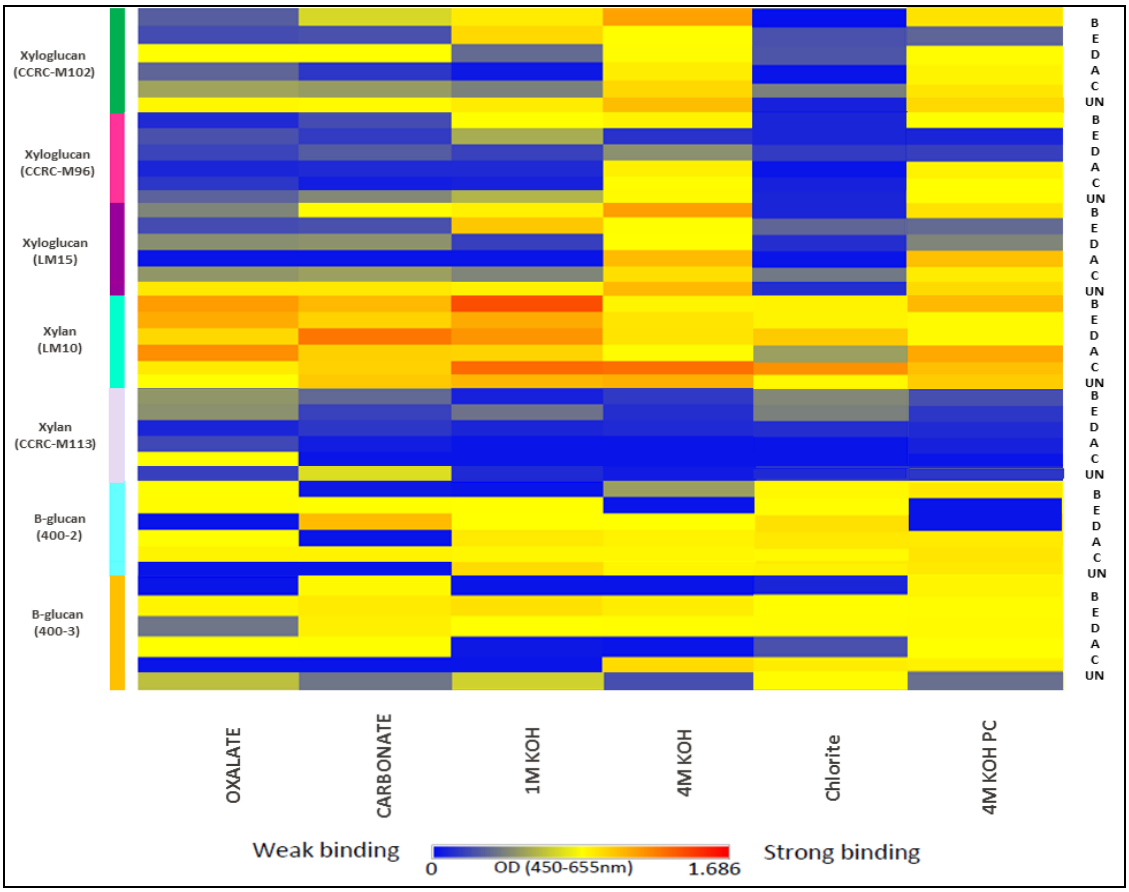


**Figure 1.** Glycome profiling of sequential cell wall extracts of pretreated EFB and untreated EFB. Binding intensities of antibodies to the extracted cell wall glycans, as measured in ELISAs, are depicted as heat maps with blue to red colours representing no binding to strongest binding, respectively. The reagents used for extraction are labelled at the bottom of the heat map.

## 4. Conclusion

BET surface area measurement further confirmed that EFB samples that undergone harsh alkaline pretreatment has the highest surface area among all 6 samples tested. The changes in the cell wall structure of pretreated EFB especially for higher alkaline pretreatment was due to the solubilisation and removal of lignin and lignin-associated carbohydrates during the pretreatment. Glycome profiling technique further confirmed our observations made throughout the chemical analysis using Van Soest method especially on delignification efficiency. It showed that lignin is still present in the structure of EFB fibres whilst most of the outer lignin layer of the EFB sample was removed after harsh alkaline

pretreatment, indicating a tightly bound secondary lignin surrounding inner fibrils of cellulose. Sodium hydroxide pretreatment with base concentration more than 1.0 wt.% was able to reduce lignin content to less than 10.0 wt.%. This pretreatment can be improved by using combined pretreatments such as physical (mechanical)-chemical, physico-chemical or biological-chemical processes.

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