Microwave Pretreatment of Oil Palm Fronds for Enzymatic Saccharification

By

Izyan Farhana binti A.Kaher

Dissertation submitted to in partial fulfilment of the requirement for

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Universiti Teknologi PETRONAS

32610 Bandar Seri Iskandar

Perak Darul Ridzuan

Malaysia

CERTIFICATION OF APPROVAL

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Approved by,

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(Prof. Dr. Yoshimitsu Uemura)

UNIVERSITI TEKNOLOGI PETRONAS

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CERTIFICATION OF ORIGINALITTY

This is to certify that I am responsible for the work submitted in this project, that the original work is my own except as specified in the references and acknowledgements that the original work contained herein have not been undertaken or used by unspecified sources or persons.

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IZYAN FARHANA BINTI A.KAHER

ABSTRACT

Oil palm frond (OPF) is distinguished to be one of the biomass resources in producing the alternative energy. Hence, in this paper, the practicability of the OPF to be used as biomass resource is studied by examining the reducing sugars composition available inside treated and untreated OPF biomass. Prepared sample of OPF have first gone under microwave and conventional pre-treatment followed by chemical treatment using NaOH. Chemical concentrations of 0 and 0.25N for the pre-treatment time of 2, 4, 8 and 12 minutes have been used. The morphological structure study is performed by using Scanning Electron Microscopy (SEM) and it certified that the surface treated with microwave assisted NaOH is more ruptured. Conventional pre-treatment is the most efficient compared to microwave pre-treatment at "low" setting with a temperature of less than 100°C by being able to liberate the highest amount of sugar yield of 32.31%. However, the reducing sugar yield still can be considered as low, even the filter paper can only liberate up to 28.94% of reducing sugar yield. Nevertheless, the pre-treated OPF still managed to yield more reducing sugars compared to untreated OPF which can only managed to yield sugars of 23.40%. Maximum saccharification yield of 32.31% for conventional heating and 26.20% for microwave pre-treatment is observed at optimal conditions of 0.5 g of dry biomass loading, 97.63 FPU/mL of enzyme loading, *T.Reesei* and 72 hour of incubation time.

Keywords: *Conventional pre-treatment; lignocellulosic; oil palm fronds;TricodermaReesei, Scanning Electron Microscopy (SEM)*

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CHAPTER 1

INTRODUCTION

1.1 Background Study

In recent past few years, the world witnessed a dramatic increase in world oil consumption demand and the demand is constantly increasing up to the moment this report is written. A huge percentage of transportationssector whole over the world is entirely depending on petroleum-fuels-based. However,this never ending demands situation and excessive dependent on fossil fuelshas leads to the depletion of fossil fuels and in return, its initiate extensiveresearchand development for a new alternative way from renewable resources in order to disentangle this problem and meeting the worldwide energy demand at the same time. And today, a numerous biomass-based-fuels such as bio-ethanol seem to be emerging. The type of biomass consists of lignocellulosic materials have caughtthe world attention and turns out to be the most potential and compromising biomass feed stocks due to its cheaper price and abundant availability on the earth. Other reason is because of these materials ability increating completely zero competition with food crop biomass feed-stock such as corn and sugarcane (Lee et. al., 2007). Malaysia is known worldwideas the most important agricultural countries. As one of the largest palm oil producer and exporter, Malaysia has generated for approximately 51 million tonnes of OPF by the year 2008 alone (Goh et. al., 2010; MPOB, 2009). By other means, Malaysia creates a long-lasting and notable amount of lignocellulosic materials waste from agricultural sector.

The lignocellulosic biomass materials have to be saccharified through saccharification reaction process in order to yield the fermentable sugars which will later be used as a feedstock in bio-ethanol production through a fermentation process.

By definition, saccharification is a process of breaking down the complex structure of cellulose (known as hydrolysis) in the lignocellulosic materials into simple reducing sugars with the presence of certain types of enzyme such as cellulase. Compared to other hydrolysis process, enzymatic saccharification process has the potential to offer a number of positive effects such as can yield high pure glucose, it only needs mild reaction condition and gives out low environmental impact.In the context of saccharification process, typically before undergoes that particular process, it is compulsory for the biomass to be pre-treated. There are immensenumbers of biomass pretreatment available such as physical pretreatment, chemical pretreatment, physiochemical pretreatment and biological pretreatment (Claassen, 1999). However, optimization is required so that the overall cost of pretreatment can be minimized. In this research study, the methods of pretreatment between microwave (MW) heating and conventional (Hot Plate – HP) heating will be studied and compared. The impact of each pretreatment heating method on the yield of reducing sugars (RS) will be discussed. The influence of NaOHon the percentage yield of RS will also be overviewed.

1.2 Problem Statement

The recalcitrant structure of lignocellulosic biomass which is made of three major types of polymer – cellulose, hemicellulose and lignin makes the hydrolysis to reducing sugars more difficult (Dumitriu S., 1998). The presence of hemicellulose and lignin make the polymer rigid and difficult to break. By other means, this condition might block the penetration of cellulose enzyme to the targeted cellulose. Hence, a pretreatment study prior to saccharification process is conducted to identify the very possible and reliablemethodto enhance the enzymes accessibility and hydrolysis rate. Hence, the yield of reducing sugars from OPF can be maximized.

1.3 Objectives and Scope of Study

In determining the very possible pretreatment method prior to saccharification process towards maximizing the amount of reducing sugars yield, a few objectives have been identified:

i. To study the amount of glucose available inside OPF.
ii. To differentiate between conventional heating and microwave heating in biomass pretreatment and itseffectson the crystalline structure of lignocellulosic materials.
iii. To investigate the influence of different concentration of alkaline solution on the performance of the saccharification process.

Among the variables that have been set to carry out this study are:

i. Type of pretreatment method – hot plate heating and microwave heating
ii. Concentration of each alkaline solution used – 0 N and 0.25 Nconc.
iii. Irradiation and heating time
iv. Changes in crystallinity of biomass material.
v. The yield of reducing sugar after saccharification

CHAPTER 2

LITERATURE REVIEW AND THEORY

2.1 Biofuels

In the current situation, the world is still in the phase of depending on the petroleum as the main energy source. Hence, in concern with the diminishing supply of fossil fuels, it forced the search for alternative and renewable source of energy in order to solve this particular issue. Besides, a continuous usage of fossil fuels has worsen the global warming problem and it creates the necessities to replace fossil fuels with another alternatives energy which are renewable and clean in order to reduce the emissions of CO_2 and greenhouse gas.Biofuel offers several advantages to the environment and sustainability (Pupan, 2002). Based on recent studies made on the advantages of using biofuels such as bio-ethanol produced by lignocellulosic materials, these materials are believed to have lower life cycle fossil energy use and emitted lower amount of green-house gas (GHG) than other conventional petroleum-based-fuels such as gasoline and diesel (Larsen et. al., 2009)

The term of biofuel is referred to liquid or gaseous fuels for the transport sector that are produced from renewable sources such as vegetable oil and biomass. Biofuels include bio-ethanol, bio-methanol, vegetable oils, biodiesel, biogas, biosynthesis gas, bio-oil, bio-char, and bio-hydrogen (Demirbas, 2007).

2.1.1 Feedstocks of Biofuels

Biofuels are easily available since they are various types of feedstock that can be used for biofuels production. Choosing an appropriate feedstock is necessary in order to optimize the economical biofuels production cost. Specifically, there are

three groups (generations) of biofuels which will be mentioned later in this paper. However, they are a numbers of arguments in determining which generation is the most promising source of renewable energy.

2.1.1.1 First Generation of Biofuels

The common feedstocks used for first generation of biofuels are vegetable oils, starch and animal fats. First generation of biofuels has the ability to offer CO_2 benefits and can help to enhance the domestic energy security. However, there are issues concerning about environmental impacts and carbon balances when it comes to the production of biofuels of first generation. The main disadvantages of first generation biofuels which is the food-versus-fuel debatehappened to be one of the main reasons for rising food prices due to thepriceincrease in producing the first generation biofuels (Laursen W., 2006). On the other hands, it also can cause an imbalance on the especially on the biodiversity and use of the land.

2.1.1.2 Second Generations of Biofuels

The second generations of biofuels is largely refers to lignocellulosic materials. Ligncellulosic biomass has caught the worldwide attention due to its ability in creating zero competition with the food crop residues, cheaper price and abundantly available on the Earth. Considering the massive amount of carbohydrates available in lignocellulosic materials, it is also observed as one of the promising biomass resources in alternative energy production (Lee at. al., 2007).A lot of literature is written mentioning on the different pretreatments methods used to improve the production yield of biofuels derived from lignocellulosic materials. However, there are arguments due to the presence of certain technical barriers in producing fuels by using this second generation feedstock which makes it not cost effective since to carry out the process, numbers of expensive equipment with high energy demand will be needed (Xu C. et. al., 2010).

2.1.1.3 Third Generations of Biofuels

Inefficiency and unsustainability for the first and second generation feedstock are among the significant concerns raised in most of written literature. According to M.Balat in 2010, although biofuels from oil crops has been produced in increasing amounts as a clean-burning alternative fuel, its production in large quantities is not sustainable. Hence, another alternative called "third generation feedstock" derived from microalgae and bacteria have emerged. The idea of producing biofuels by using microalgae and bacteria as feed stocks have seemed to be potential and viable for biofuels production process in which currently is dominated by palm oil as the main feedstock. To date, there have been many attempts to investigate the viability of microalgae in producing biofuels for industrial scale process (Slade. R., 2012).

2.2 Palm Oil Residues

For the past few decades in Malaysia, palm oil industry is happened to be one of very well grown agricultural-based industry. Currently, Malaysia has become the world's largest producer and exporter of palm oil, replacing Nigeriaas the chief producer since 1971 (Yusof, 2006). Over the last 25 years, Malaysia has produces for approximately 40 – 60% of world palm oil production. Besides, Malaysia also generates a huge quantity of palm oil biomass including oil palm trunks (OPT), oil palm fronds (OPF), empty palm fruit bunches (EPFB), shells and fibres (Chew and Bhatia, 2008). Of the whole palm tree, it has the ability to form up to 90% of biomass and the remaining of 10% can be used to produce palm oil.

According to *National Biomass Strategy 2020: New Wealth Creation for Malaysia's Palm Oil Industry* report in the year 2011, the amount of oil palm fronds alone account for about 75% of the biomass volume. MPOB (2010) also reported that the most generated oil palm waste is OPF, which amounted to 83 million tonnes (wet weight) annually as per the year 2010. However, as for now, OPF is under-utilized since majority of the palm oil plantation developers believe that OPF is crucial for soil conservation and nutrient recycling, so the developers tend to leave the pruned fronds in their plantation (Wan Zahari et al., 2002). Therefore, OPF has been selected to be the subject of study in this experiment to investigate the possibility of using oil palm fronds (OPF) as biomass resource in producing fermentable sugars that can be used for second generation of bio-ethanol production.

Mainly, in this context of study, only the lignocellulosic materials from oil palm fronds (OPF) pre-treated with microwave and conventional heating will be studied.

2.3 Lignocellulosic Materials

They are abundant of lignocellulosic materials waste materials available worldwide. Lignocellulosic material consists of mainly three different types of polymers, namely cellulose, hemicelluloses and lignin which are associated with each other (Fengel and Wegener, 1984). Each of these polymers carries out different purposes; almost entirely half of the biomass materials comprises of cellulose. According to Laureano-Perez et al. in 2005, the cellulose in a plant consists of parts with crystalline (organized) structure and parts with amorphous (not well organized) structure. Usually, the cellulose will be associated together with other compound such as lignin to prevent degradation on its structure.

Of cellulose and hemicelluloses, lignin is one of the most abundant polymers present in the cellular wall. It functions as to provide a structural support and increase the physical strength of the plant. The lignin is also a non-water soluble and optically inactive since the lignin itself is an amorphous heteropolymer and exists in irregular arrangement, hence it makes the enzymatic and chemical degradation of lignin very tough (Ohkuma et al., 2001).

Meanwhile, for hemicellulose, it is the second most abundant natural polymer available on Earth (Agbor et al., 2011) and hemicellulose is refers to a complex carbohydrate structure that consists of different polymers consists of pentoses (D-xylose, D-arabinose), hexoses (D-glucose, D-mannose and D-galactose) and sugar acids. Hemicellulose is made up of branches with short lateral chains that consist of different sugars, which can be considered as easy hydrolyzable polymers. Also, hemicellulose serves as a connector between the lignin and the cellulose and it helps to enhance the rigidity for the whole cellulose-hemicellulose-lignin network.

The structure of the lignocellulosic materials itself caused the process of breaking down the complex structure getting more difficult. Hence, a very good assessment on the very suitable and possible pretreatment involving modes of

heating used must be held to ensure an effective and practical cellulose conversion process (saccharification).

2.4 Biomass Pretreatment Method

Pretreatment is one of great importance tool for practical cellulose conversion process, in which at this stage, the structure of cellulose will be altered to make it more accessible to the enzymes that convert carbohydrate polymers into fermentable sugars (Mosier N., 2005). There are various kinds of pretreatment technologies available worldwide. They can categorized into physical pretreatment, chemical pretreatment, physio-chemical pretreatment and biological pretreatment. Physical pretreatment involving the process of drying, size reduction (through grinding, milling etc) and granulometric separation (Woiciechowski AL, et al., 2013)

2.4.1 Alkaline Pretreatment Method

Alkaline pretreatment has been recognized lately due to its aptness in removing lignin from biomass (Chen et al., 2011). It comprises various types of bases such as calcium hydroxide (lime), sodium hydroxide, potassium hydroxide and others (Chang VS, 2000).Sodium, calcium, ammonium hydroxide and potassium are amongst the most suitable bases. Of all four types, NaOH has been studied the most. Meanwhile $Ca(OH)_2$has been indicated to an effective bases used for pretreatment and is the least expensive (MacDonald DG. et al., 1983). The potential for alkaline pretreatment for achieving desired results varies relying on the treatment conditions and substrate used. Normally, this pretreatment is more operative on herbaceous wood, hard wood and agricultural remains with low content of lignin (Kumar R. et al., 2009). The major effect of using alkaline pretreatment in enhancing the penetration rate on enzyme is by decreasing the degree of polymerization (DP) of cellulose and caused the cellulose to swell and leads to the increment of internal surface area, thus it will be more accessible for enzymes to pass through during the hydrolysis stage (Kumar R. et al., 2009).

2.4.2 Significant of Heat Supply during Pretreatment

Alkaline pretreatment has been used worldwide due its effectiveness and cheap price (Sun Y. et al., 2008). However, to achieve better pretreatment performance, thermal energy is crucial in facilitating the bond breaking between biomass molecules. By other means, different heating devices or different modes of heating will give out difference efficacy on lignocellulosic materials. A plenty of literatures have showed the significance of using different kind thermal supply in term of heating device in pretreatinglignocellulosic feed stocks. But alas, only few literatures address the comparison in term of the heating device effectiveness in lignocellulosicpretreatment.

2.4.3 Microwave Heating

Over the years, microwave (MW) pretreatment has been used globally due to its great ability in facilitating the lignocellulosic disruption by using its thermal irradiation generated through dielectric heating. The statement is agreed by Azuma et al. (1984) who mentioned in their literature microwave irradiation has been used in pretreatinglignocelullosic biomass since long ago. Microwave irradiation has the ability to change the ultra structure of cellulose, degrade the lignin structure and also enhance the enzymic susceptibility of reducing sugars produced.

In microwave heating, the energy is introduced without the needs of energy source to be in contact with the reaction mixture. For microwave, the method in facilitating the cellulosic breakdown is mainly via molecular collision caused by dielectric polarization. The electromagnetic field generated by microwave has the capacity to interact with the samples directly and produce heat, thus the chemical, physical and biological process can be accelerated. In contrary with hot plate heating whereby the cellulosic breakdown is done through the coil heating before it gets transferred to the mixture via convective mode (Guo GL. et al., 2008).

2.4.4 Conventional Heating

Pretreatment through a hot plate (HP) heating is one of the accepted and preferred method conventional heating. It is requires less cost compared to other pretreatment. HP heating method has been recognized as a result of localized over hot plate surface heating which enables fast and effective means of lignocelulosicdiscruption. However, compared to other pretreatments method, HP heating is rather slow and has the ability to cause a temperature gradient within the sample (Ruiz E. et al., 2008).

2.4.5 Enzymatic Saccharification

According to Eriksson T. et al. (2002), in order to get an efficient hydrolysis of cellulose, a number of enzymes will be required. All these enzymes work synergistically to hydrolyse the cellulose by creating new accessible sites for each other, removing obstacles and relieving product inhabitation. In saccharification process, the sugar polymers such as hemicelluloses and cellulose will be depolymerised by degrading the glycosidic bond with the presence of microbial enzyme. Subsequently, the process of fermentation will take place to convert the sugar to fuels and chemicals (Menon and Rao, 2012).

Due to extensive research in determining the enzymes performance, it caused the enzyme price to be lower and cheaper. However, the amount of enzyme loading is suggested to be as low as possible in order to reduce the production cost. In return, it might increase the time taken to complete the hydrolysis process.

Besides, the use of high concentration of substrate has the ability to increase the product inhabitation problem and consequently will slow down the enzyme performance. Another major obstacle towards achieving an efficient hydrolysis is the presence of lignin itself which can shield the cellulose chains and adsorbs the enzyme. Hence, a proper pretreatment is needed prior to hydrolysis. Without a proper treatment, it might cause a great effect on the saccharification and cost of ethanol production in future.

CHAPTER 3

METHODOLOGY AND PROJECT WORK

3.1 Experiment Methodology

This is an experimental project hence the analysis will be focusing on the results obtained from the laboratory work. The result trend will be analyzed and a proper justification will be made for this experimental project.

3.1.1 Research from the literature

The first phase of this project is started by selecting the related literatures mainly on lignocellulosic biomass materials (i.e: oil palm fronds, oil palm trunk, empty fruit bunches, rice straw, maize etc), enzymaticsaccharification, thermal pretreatment, conventional heating, microwave heating and others.

3.1.2 Preparation of substrate, OPF

The oil palm fronds (OPF) used for this experiment will be obtained from KampungFelcraNasaruddin in Bota, Perak. The OPF collected is washed, cut into uniform particle size, dried and grinded before used in order to sustain the consistency of the results.

3.1.3 Pretreatment Methods Used

Two types of pretreatment methods are being used in this project which are; (1) conventional heating method, and (2) microwave heating method. Then, the amount of compound loss will be determined for each method.

3.1.4 Alkaline Used

Two different bases are being used in this study which are; (1) NaOH, and (2) $Ca(OH)_2$. The effect of using different concentration of NaOH at different time interval and type of bases used is overviewed and studied at the end of the studies.

3.1.5 Enzymatic Saccharification

The enzymatic saccharification in incubator shaker will be conducted for all the pretreated samples for 72 hours. The optimal incubation time and the amount of recovered biomass will be determined. From here, the methods that yield more reducing sugars will be identified.

3.1.5 Documentation and Report

All the results obtained will be documented and result trending will be analyzed. A brief justification and comparison with other research paper will be made in order to come out with a proper deduction for this project.

3.2 Procedure

Below are the listed procedures in conducting the research.

3.2.1 Raw Material (OPF) Preparation

OPF is collected from FELCRA (Federal Land Consolidation and Rehabilitation Authority) Nasaruddin oil palm mill at Bota, Perak, Malaysia. The raw OPF was dried at temperature 105°C for 30 hours. Drying is an important step in determining the densification process of the materials with moisture. There are several factors to be considered while performing drying step such as the type of dryer, drying conditions, drying medium and characteristics of biomass because these are the factors that will affect the final quality of the feedstock (Lam PS et al, 2013)

Figure 7: The photo of OPF raw material at FELCRA Nasaruddin, BotaKanan, Perak

Figure 8: The photo of washed OPF which has been cut and ready to be dried at drying oven

Afterwards, the OPF was grinded into uniformly small particle size in order to maintain the consistency of the results and sieved to four distinctive group of particle size range which are;

- 0.10 mm – 0.25 mm
- 0.25mm – 0.50 mm
- 0.50 mm – 1.00mm
- 1.00 mm – 2.00mm

The OPF sample size within the range of 0.10mm – 0.25mm is selected to be used throughout the experiment since it is the smallest in size and can provide a better contact during pretreatment and saccharification experiments.

3.2.2 Raw Material Characterization

Several means of characterization has been taken into consideration throughout the experiment. This includes: (1) moisture content, (2) % component composition, (3) elemental analysis.

3.2.2.1 Moisture Content Determination

The procedure in determining moisture content is conducted by referring the ASTM method, E 871 – 82(1998). The procedure is as mentioned below:

1. The sample is dried for 30 min on oven at 105°C in the drying oven, then cooled in the desiccators to room temperature.
2. The weight of crucibles are measured and labelled as container weight, W_c
3. 2 g of grinded OPF sample with range of 0.1 mm – 0.25 mm is placed in the crucibles and be recorded as initial weight, W_i
4. The sample and the crucibles is placed in the drying oven for 24 hour at 105°C.
5. After 24 hours, the sample and the container is removed from the oven and cooled in the desiccator to room temperature.

6. After it reached room temperature, the sample and the container is removed from desiccators and weighted immediately. The weight is recorded.
7. The sample and the container is later returned to the oven at be dried at 105°C for 1 hour. Step 6 is repeated continuously until the total weight change between weighing varies less than 0.2%. The reading taken is recorded as final weight, W_f.
8. The percentage of moisture content is calculated by using this formula:

$$\textbf{Moisture Content in analysis sample}, \% = \frac{\mathbf{Wi - Wf}}{\mathbf{Wi - Wc}} \times \mathbf{100}\%$$

Where,

W_i : initial weight (g)

W_f : final weight (g)

W_c : container weight (g)

3.2.2.2 Determination of Percent Component Composition of OPF

The raw material (untreated OPF) solid samples will undergoes qualitative analysis characterization by using Thermo-Gravimetric Analysis (TGA) in order to examine the weight % of their lignin, cellulose, hemicelluloses, ash and moisture content of the treated and untreated OPF. It should be possible to predict the yield and compositions of the saccharification product of OPF feedstock when its composition is known. Negligible interactions among the three biomass compositions are observed in their study when using TG analysis (Yang et al., 2006). The experiment is carried out with sample masses of about 10mg using a linear heating rate of 10°C/min within the range of temperature between 28°C - 840°C and a steady nitrogen flow rate of 100cm^3/min. The weight loss and derivative weight loss curves on the separate biomass will obtained in a graph of derivative weight loss (%/°C) versus time (min). From the graph obtained, the graph pattern will be overviewed and discussed further.

3.2.2.3 Solid Characterization

The solids characterization of the wet cakes (solid residues) is verified by using the method of elemental analysis and scanning electron microscopy (SEM).

Meanwhile, the elemental analysis is conducted by using CHNS analyzer to examine the elemental value of carbon, hydrogen, nitrogen, oxygen and sulphur, meanwhileHPLC analyzerwill be used to test the reducing sugars presence such as glucose. SEM is used in determining the surface morphology of the untreated and pre-treated OPF biomass.

3.2.3 Microwave Power Determination

3.2.3.1 Variation of Microwave Power Setting

The microwave oven used in this experiment has the ability to produce up to 800W output. The irradiation power output was set at different level, however the exact value is unknown since the power setting available are termed only as “LOW”, “MEDIUM” and “HIGH”. Hence, an experiment was conducted to study the amount of radiation being absorbed by OPF sample at different microwave power setting. The estimation of power absorbed is based on 100ml of distilled water since the pre-treatment method used in future will be using OPF sample immersed in 100ml aqueous solution which has close heat capacity with distilled water. The procedure is as below:

1. After the initial temperature of distilled water is taken using thermometer, 100ml of distilled water was added into 250mL Schott beaker.
2. The Schott beaker was put on the centre point of the rotating dish inside the microwave oven.
3. With “LOW” power setting, the bottle was microwaved for t = 2, 4, 8 and 12 minutes. After that, the temperature of the distilled water is recorded immediately. The temperature change will be used to determine the total irradiation energy absorbed in one minute.

3.2.4 Conventional Heating Temperature Determination

For conventional heating, the estimation of heat absorbed by the sample is based on 100ml of distilled water since the pre treatment method used in future will be using OPF sample immersed in 100ml aqueous solution which has close

heatcapacity with distilled water. Adding on to that, hot plate will be used as an alternative to the Bunsen burner. Typical operating temperatures for hotplate usually are varied from 100°C to 380°C. Hence, experiment is conducted to study the amount of heat absorbed by OPF at different interval settings. The temperature recorded at interval of t = 2, 4, 8 and 12 minutes for conventional heating is compared with the temperature reading produced by MW. The experiment set-up which produced the closest and adjacent temperature value to MW is selected as the permanent set-up for conventional heating. To achieve that, the level of distilled water and also the size of beaker used are varies.

3.2.4.1 Conventional Heating Temperature Determination

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3.2.5 Pre-treatment

In order to pre-treat the OPF sample, 2 experiments were conducted. The procedures were discussed as below:

3.2.5.1 Experiment 1: The effect of conventional-alkali pre-treatment at different pre-treatment time

In this experiment, the pretreatment time becomes the manipulated variable. The chosen time is t= 2, 4, 8 and 12 minutes. The procedure is as follows:

1. 3g of OPF is put into 250mL Schott beaker with 100mL of 0 N ofNaOH aqueous solutions (water only) for pre-treatment.
2. The sample was heated by subjecting to conventional treatment on the hotplate for 2 minutes at desired temperature.
3. After 2 minutes, the mixture is removed immediately from the hot plate (HP) and weighed. The mixture and beaker is filtered using vacuum pump to separate the wet cake (solid residues) and filtrate.
4. The pre-treated solid residue is washed using sieve using neutral pH with tap water and weighed before being transferred into 100mL sample bottle. Meanwhile, the filtrate will be weighed immediately. Both wet cake and filtrate are kept in refrigerator.
5. Step 1 to 4 was repeated for different concentration of NaOHwhich is at – 0.25N concentration.

3.2.5.2 Experiment 2: The effect of microwave-alkali pre-treatment at different pre-treatment time

In this experiment, the pretreatment time becomes the manipulated variable. The chosen time is t= 2, 4, 8 and 12 minutes. The power setting is fixed at "LOW" settings at desired temperature. The procedure is as follows:

1. 3g of OPF is put into 250mL Schott beaker with 100mL of 0 N ofNaOH aqueous solutions (water only) for pre-treatment.
2. The sample was heated by subjecting to microwave treatment for 2 minutes at desired temperature.
3. After 2 minutes, the mixture is removed immediately from the MW and weighed. The mixture and beaker is filtered using vacuum pump to separate the wet cake (solid residues) and filtrate.
4. The pre-treated solid residue is washed using sieve using neutral pH with tap water and weighed before being transferred into 100mL sample bottle. Meanwhile, the filtrate will be weighed immediately. Both wet cake and filtrate are kept in refrigerator.

5. Step 1 to 4 was repeated for different concentration of NaOHwhich is at – 0.25N concentration.

3.2.6 Measurement of Cellulase Activities

The cellulose activities are determined by using the method designed by International Union of Pure and Applied Chemistry (IUPAC). The cellulose activities are determined by measuring the filter paper unit (FPU) per millilitre of enzyme solution that releasing 2.0mg of reducing sugar. The FPU will later be calculated by using the following equation:

$$\mathbf{FPU} = \frac{\mathbf{0.37}}{[\mathbf{enzyme}]\mathbf{releasing\ 2.0mg\ of\ glucose}}\ \mathbf{units/ml}$$

Below are the listed procedures in determining the cellulose activities:

1. DNS Reagent and 0.05M of citrate buffer at pH 4.8 is prepared by using the method designed by NREL.
2. Filter paper assay for saccharifying cellulose is conducted by preparing three categories of experimental tubes (assay mixtures, blanks and controls, and glucose standard). The substrate is a 50 mg of Whatman No. 1 filter paper strip (1.0 × 6.0 cm).
3. Enzyme assay tube is prepared by using following procedure:
    a. A rolled of filter paper is placed into each 13 × 100m test tube.
    b. 1.0 mL of 0.05M citrate buffer with pH 4.8 is added to the tube.
    c. 0.5mL of appropriately diluted enzyme is added in citrate buffer. Two dilutions are made for each enzyme sample.
4. Blank and controls experimental tubes are prepared by following the listed procedures:
    a. 1.5mL of citrate buffer is added into 100mL of test tube and a filter paper strip is placed inside the tube which acts as substrate control.
    b. To prepare reagent blank, 1.5mL of citrate buffer is added in 100mL test tube
    c. Meanwhile, for enzyme control, 1.0mL of citrate buffer is added in together with 0.5mL of each enzyme dilution.

5. Glucose standard preparation procedures:
    a. A working stock solution of anhydrous glucose (10mg/ml) is prepared. The aliquot of this working stock solution is tightly sealed and stored frozen.
    b. The glucose dilutions are made from the working stock solution in the following manner:
        i. 1.0mL + 0.5mL buffer = 3.35mg/0.5mL
        ii. 1.0mL + 1.0mL buffer = 2.50mg/0.5mL
        iii. 1.0mL + 2.0mL buffer = 1.65mg/0.5mL
        iv. 1.0mL + 4.0mL buffer = 1.00mg/0.5mL
    c. The glucose standard tubes are prepared by adding in 0.5mL of each of the above glucose dilution to 1.0mL citrate buffer in 13 × 100mm of test tube
6. Blank and controls, glucose standard and enzymatic assay is later be incubated at 50°C for 60 minutes. DNS reagent is added afterwards.
7. After that, each of the experimental tubes is vigorously boiled in water bath for exactly 5 minutes for color development. The test tube is later be cooled by transferring all the experimental tubes to cold ice water bath.
8. 0.2mL of color-developed mixture for each tube together with 2.5mL of water is mixed by using the pipettor into the spectrophotometer cuvette. The absorbance at 540nm against reagent blank is measured.
9. After that, a linear glucose standard curve is constructed by plotting the absorbance at 540nm against absolute amounts of glucose (mg/0.5ml). Using this standard curve, the amount of glucose released for each sample tube after enzyme blank subtraction is determined.
10. The concentration of enzyme which would have released exactly 2.0mg of glucose is determined by plotting glucose liberated against enzyme concentration.
11. From there, the FPU is calculated by using the above formula.

3.2.7 Enzymatic Saccharification

The enzymatic sachharification of pre-treated OPF was based on the method devised by NREL. In the method, it was mentioned that the total volume of the mixture used was 25mL.

For saccharification, 1g of OPF was used as dry basis. The enzymes used were 10mg/mL of *TricodermaReesei, T.Reesei*. The enzyme is diluted to 0.01mg/mL concentration, before being mixed with the pretreatedOPF sample. For every 25mL sample solution, 2mL of cellulose solution and 1mL of 0.002mg/mL of β-glucosidase is used. 2% of biocide made from sodium azide is added in to prevent the growth of organisms during the digestion process. Most of the time, enzymatic hydrolysis requires buffer solution, therefore a proper pH can be obtained and maintained throughout the process. In this experiment, 0.05M citrate buffer solutions with pH 4.8 were used. The amount of buffer solution required c determined by using the equation below:

V_T = 25mL = Mass of OPF on wet basis (g=mL) + Volume of citrate buffer + Volume of enzymes + Volume of biocide

V_T = *total sample volume*

Volume of enzymes = *2mL of cellulose + 1mL of β-glucosidase*

Volume of biocide = *1mL*

Below are the procedures for saccharification of the pretreated samples.

1. 0.5 g on dry basis of pre-treated OPF is loaded into 100mL Erlenmeyer flask with cap.
2. The citrate buffer is loaded into the flask (based on the amount calculated from the equation aforementioned above).
3. 2mL of *T.Reesei*enzyme together with 1mL of β-glucosidase and 1mL of sodium azide is loaded into the mixture.

4. The concoction is placed into the incubator shaker set at rotation speed of 150rpm and temperature of 50°C.
5. The sample is later be incubated for 72 hours (3 days). 1mL of the sample aliquot was taken out for every 12 hours and inserted in vial for HPLC analysis.

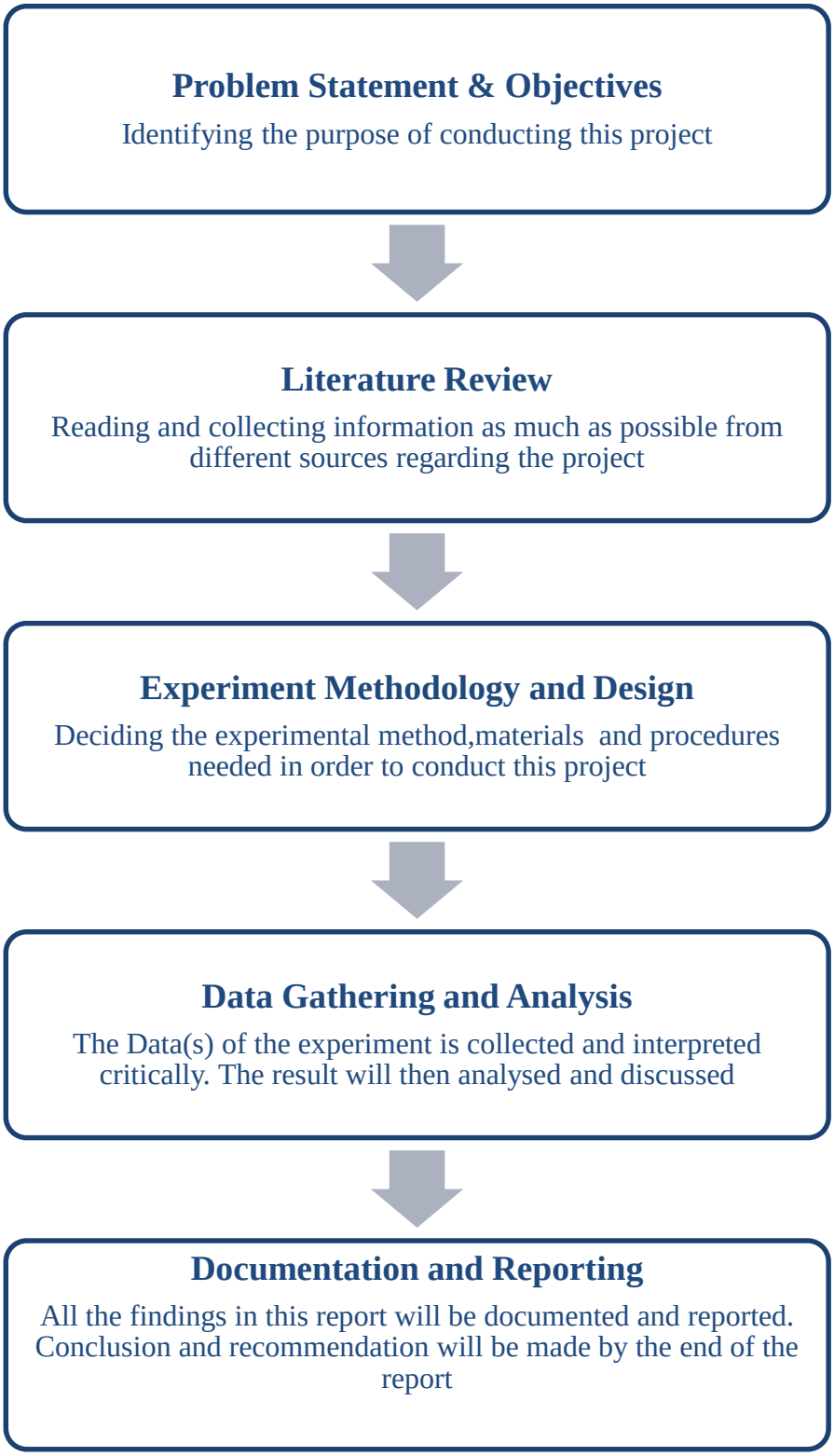
3.2.8 Calibration Curve

Calibration curve are required to pinpoint the retention time of the reducing sugar and also to translate the peak area from HPLC graph into a concentration unit. In this experiment, only fructose and glucose will be taking into account since it can be easily liberated from cellulose. Below are the procedures in generating the calibration curve:

1. Five solutions of known sugar with a concentration of 1.0, 2.5, 5, 7.5 and 10g/l respectively were prepared.
2. The sample was later analyzed by using HPLC.
3. Using the data obtained from the HPLC, graph of peak area against concentration was plotted.

3.3 Project Process Flow

This is the process flow for this research project that must be so that the objectives of the study can be successfully achieved.



3.4 Gantt Chart and Key Milestone

No	Details	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1.	FYPII Project Work														
2.	FYPII Activities: Experimental Work/Simulation Work														
3.	Progress report submission														
4.	FYPII Activities: Experimental Work/Simulation Work														
5.	Pre-EDX														
6.	Draft Submission														
7.	Project Work Continue: Analysis and reporting														
8.	Softbound submission														
9.	Technical Paper Submission														
10.	Oral Presentation														
11.	Submission of Hardbound														

Gantt chart

Key Milestone

CHAPTER 4

RESULT AND DISCUSSION

4.1 Raw Material Characterization

4.1.1 Moisture Content of OPF Raw Material

The OPF collected is washed and dried to avoid the fungus growth on the OPF which might degrade the raw material. OPF is later grinded and sieved into range size of:

- 0.10 mm – 0.25 mm
- 0.25 mm – 0.50 mm
- 0.50 mm – 1.00 mm
- 1.00 mm – 2.00 mm

The smallest size range of 0.10 mm – 0.25 mm has been selected.

Subsequently, around 2.00 g (±0.05) of OPF is taken out from the raw material for moisture content determination purposes. The weight of the sample before and after drying is recorded. The weight loss difference calculation is performed by using the formula:

$$\Delta \mathbf{m} = \mathbf{m_{t=24+n}} - \mathbf{m_{t=24}}$$

The sample is repeatedly dried for another 1 hour until the weight is constant (less than 0.2%). Once the weight difference is less than 0.2%, the moisture content, MC for the sample is conducted by using the formula of:

$$\textbf{Moisture Content in analysis sample}, \% = \frac{\mathbf{Wi - Wf}}{\mathbf{Wi - Wc}} \times \mathbf{100}\%$$

Sample	W_i OPF (g)	W_i cru + OPF (g)	W_f= 24 hrs (g)	W_f = 25 hrs (g)	W_f = 26 hrs (g)	W_f = 27 hrs (g)	MC (%)
1	2.0009	18.8389	18.7200	18.7301	18.7300	18.7300	5.4198
2	2.0093	16.8296	17.0100	17.0001	17.0200	17.0200	5.4057

Table 3: Moisture Content Determination of OPF Raw Materials

$$\text{Average MC\%} = \frac{5.4198 + 5.4057}{2} = 5.41275$$

From the result obtained, it can be seen that, after drying at t = 25 hours for Sample 1, it gives higher value compared to sample weight at t = 24 hours. Meanwhile, for Sample 2, after drying at t = 26 hours, it gives higher value compared to previous reading. This error might occur due to moisture absorbance. The sample might absorb some moisture from the surrounding after drying process takes place. However, throughout the experiment, a uniform distribution of moisture content is achieved and the drying activity is stopped at t = 27 hours.In the end, the average moisture content of raw OPF obtained from the test is 5.4127%. It is crucial to determine the moisture content of the OPF biomass for the benefits of effective thermal treatment due to the decreasing amount of its calorific value (Demirbas A., 2005)

4.1.2 Moisture Content in Prepared Sample

The moisture content of prepared sample is calculated after drying process takes place. The weight of the OPF before and after pretreatment is measured.After done with the pretreatment, all pretreated samples are washed vigorously by using tap water until it reach neutral pH (6.90 < pH < 7.10) before being used in enzymatic saccharification process later on. Variation of pH in pretreated sample may distract the enzymes and cause denaturation. After it reached neutral pH, the samples are later being put in sample bottles. 1.0 g from the OPF solid residues we are dried for 24 hours in 105℃ oven temperature to determine the moisture content.

Sample	Before drying			After drying 24 hrs	MC %	Average MC %
	Petri dish	OPF sample	Total			
HP NaOH 0.0 N T2	54.3357	1.0056	55.3413	54.7398	59.8150	60.8124
HP NaOH 0.0 N T2	57.0286	1.0178	58.0464	57.4173	61.8098	
HP NaOH 0.0 N T4	54.7022	1.0264	55.7286	54.9294	77.8644	78.3087
HP NaOH 0.0 N T4	34.6250	1.0185	35.6435	34.8414	78.7531	
HP NaOH 0.0 N T8	44.4112	1.0063	45.4175	44.5661	84.6070	84.1521
HP NaOH 0.0 N T8	56.9201	1.0029	57.9230	57.0836	83.6973	
HP NaOH 0.0 N T12	57.2133	1.0058	58.2191	57.3726	84.1619	84.6305
HP NaOH 0.0 N T12	55.6470	1.0033	56.6503	55.7965	85.0992	
HP NaOH 0.25 N T2	31.4171	1.0051	32.4222	31.5867	83.1261	83.2446
HP NaOH 0.25 N T2	30.9156	1.0050	31.9206	31.0828	83.3632	
HP NaOH 0.25 N T4	1.2672	1.0030	2.2702	1.4291	83.8584	83.8253
HP NaOH 0.25 N T4	2.2081	1.0063	3.2144	2.3712	83.7921	
HP NaOH 0.25 N T8	2.2101	1.0080	3.2181	2.3787	83.2738	83.2920
HP NaOH 0.25 N T8	2.1655	1.0060	3.1715	2.3334	83.3101	
HP NaOH 0.25 N T12	26.8625	1.0083	27.8708	27.0401	82.3862	82.3312
HP NaOH 0.25 N T12	31.3857	1.0043	32.3900	31.5637	82.2762	

Table 4 (a): Moisture Content in Prepared Sample for Conventional Heating

HP : Hot Plate (Conventional Heating)

Sample	Before drying			After drying 24 Hours	MC %	Avg MC %
	Petri dish	OPF sample	Total			
MW NaOH 0.0 N T2	46.5774	1.0400	47.6162	46.7262	85.5769	86.1560
MW NaOH 0.0 N T2	52.4501	1.0147	53.8115	52.9314	86.7350	
MW NaOH 0.0 N T4	55.1938	1.0365	56.2295	55.3178	87.9595	86.6378
MW NaOH 0.0 N T4	53.9324	1.0120	54.9435	54.0801	85.3162	
MW NaOH 0.0 N T8	41.3805	1.0043	42.3841	41.5922	78.8509	79.2947
MW NaOH 0.0 N T8	48.8051	1.0172	49.8225	49.0114	79.7385	
MW NaOH 0.0 N T12	53.3769	1.0088	54.3854	53.5713	80.6998	80.7281
MW NaOH 0.0 N T12	50.2263	1.0523	51.2792	50.4294	80.7564	
MW NaOH 0.25 N T2	53.9460	1.0361	54.9810	54.0696	87.9645	87.6171
MW NaOH 0.25 N T2	52.7874	1.0149	53.8023	52.9166	87.2697	
MW NaOH 0.25 N T4	50.2367	1.0108	51.2508	50.3574	88.3854	88.3678
MW NaOH 0.25 N T4	46.5972	1.0498	47.6470	46.7195	88.3502	
MW NaOH 0.25 N T8	53.3889	1.0733	54.4636	53.5348	86.5368	84.8780
MW NaOH 0.25 N T8	40.4411	1.0810	41.5287	40.6291	83.2192	
MW NaOH 0.25 N T12	48.8190	1.0560	49.8733	48.9595	86.5341	86.4919
MW NaOH 0.25 N T12	55.2076	1.0354	56.2484	55.3533	86.4497	

Table 2 (b): Moisture Content in Prepared Sample for Microwave Heating

MW : Microwave Heating

4.1.3. Proximate Analysis of Lignocellulosic Components of OPF

The findings from Thermo-Gravimetric Analysis (TGA) reveal the weight loss% of the lignin, cellulose, hemicelluloses, ash and moisture content of OPF sample under nitrogen atmosphere at heating rate of 10°C min^{-1}.

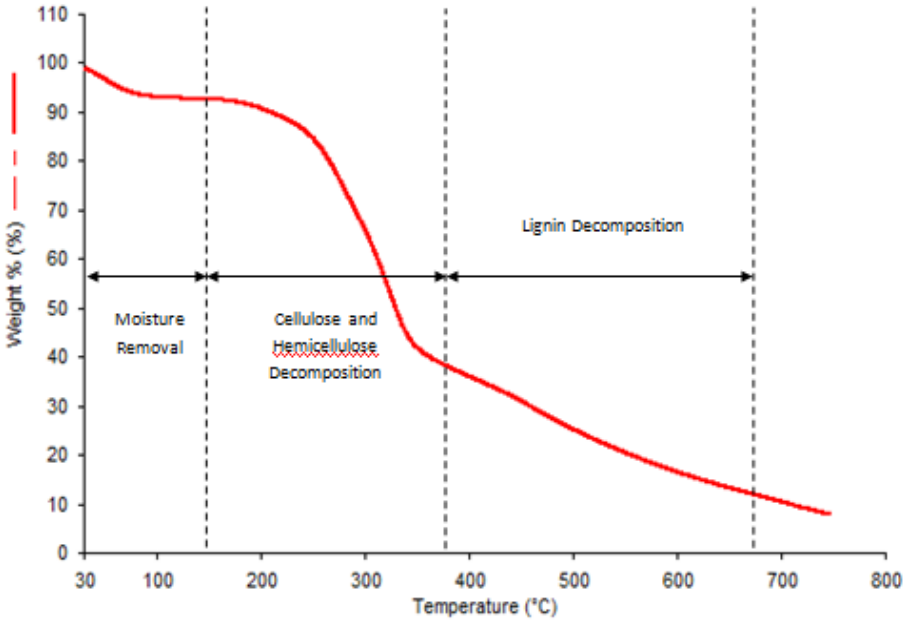


Figure 9(a): Dried OPF decomposition - Thermogravimetric curve

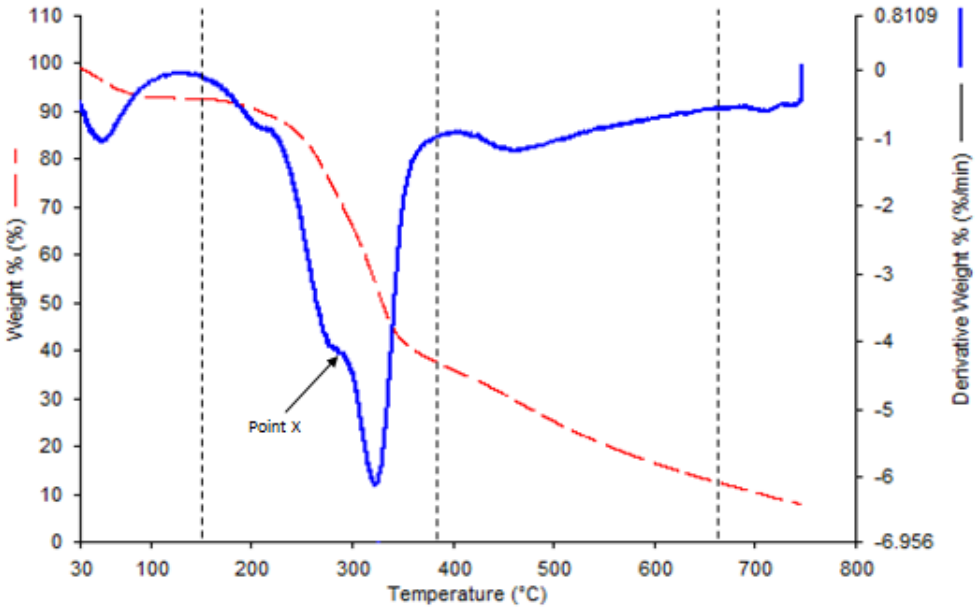


Figure 3(b): Dried decomposition of OPF - Derivative Thermogravimetric (DTG) curve

From the findings in Figure 3a, the thermal decomposition takes place at lower temperature approximately at 303K (30°C). The first and largest lost can be observed and it is due to the decomposition of cellulose and hemicelluloses. Meanwhile, the second larger lost seems to be equivalent to the decomposition of lignin. The decomposition pattern of cellulose and hemicelluloses is not fully comprehended however, according to Vamvuka D et al (2003), it reveals that hemicelluloses is usually broke down at lower temperature in contrast with cellulose that break down at higher temperature. The statement was in agreement with other researchers such as Cozzani V et al (1995), Werther J et al (2000) and Williams P. T et al (1991) that mentioned the lower temperature peak indicated the hemicelluloses decomposition and the higher temperature peak exhibits the cellulose decomposition. Apparently, the point X in DTG curve might have been the start point of cellulose break down. From DTG curve, the ignition temperature for OPF dried sample is identified at 423K (150°C), meanwhile the peak temperature is obvious at 595K (322°C). Notably, the ignition temperature is a temperature where the burning profile experienced a sudden rise and the peak temperature is a temperature where maximum rate of weight loss during thermal combustion takes place. Seemingly, these values are comparably lower compared to coal fuels studied by W.A Wan AbKarimGhani et al (2004) which are 623K (350°C) for ignition temperature. The almost constant flat curves at higher temperature represent the lignin which happened to be slowly decomposed within a wider temperature range (Williams P T et al., 1991).

4.1.4 CHNS Analysis

CHNS analysis is an important tool in the process of characterizing the carbon content and carbon to oxygen ratio in relation to calorific value inside the biomass. From the analysis, the amount of carbon (C), hydrogen (H), nitrogen (N) and oxygen (O) will be determined. The analysis will be replicate twice and from there, the average value of each sample is recorded in the table below.

No of sample	Weight of sample (g)	Results, %					
		C	H	N	S	O	C/O Ratio
1	2.121	43.14	5.99	0.72	0.31	49.84	0.8656
2	2.152	43.78	6.12	0.62	0.41	49.07	0.8922
Average	2.137	43.46	6.06	0.67	0.36	49.46	0.8787

Table 4: CHNS Analysis Result

Table 4 summarizes the results from CHNS analysis of raw oil palm fronds carried out by using PerkinElmer 2400 CHNS Analyzer. The average carbon content value obtained from CHNS analysis shows 43.46%. The carbon contents value of OPF is compared to another literature reported by other researcher, whereby almost similar value of carbon contents is obtained by M.A.K.M Zahari et al (2012) which is49%. The high amount of carbon content indicates the suitability of lignocellulosic biomass to be use as renewable carbon source in producing the value-added products through fermentation process later. In comparison with solid fossil fuels, biomass is considered to have much less carbon content and more oxygen content (Demirbas, 2004)

4.2 Microwave and Conventional Heating Temperature Setting

The setting for conventional microwave heating is adjusted to make the heating process more precise hence can produce greater yields and higher quality of products. This is due to different mechanism of heat transfer takes place between conventional and microwave heating. Heat is transferred to the surface of the beaker and biomass via conduction or convection in conventional heating. On the contrary, microwave is not considered as another form of heat but rather form of energy that are represented as heat via their interaction with the biomass materials (www.chemicalprocessing.com)

In determining the temperature setting for both heating method, the temperature of microwave at "low" setting and hot plate at each intervals of t = 2, 4, 8 and 12 minutes is recorded. The final temperature, T_2 of both heating method is compared. The conventional setting that gives out the closest T_2 with MW at low setting is being selected as the conventional heating set-up throughout the project research. From the table below it can be seen that the conventional settings of 250ml Schott beaker filled with 100ml of distilled water gives out the closest T_2 compared to the settings of 250 ml beaker filled with 50ml of distilled water. The summary settings are shown in table below.

Heating Method	Time (min)	Trial	Mass, m (g)			Temperature (˚C)			Time, t (s)	Heat Energy, Q (kJ)	Power, P (W)
			Beaker	Beaker + water	water	T_1	T_2	ΔT			
MW (**Low** Setting)	t = 2	1	105.62	203.49	97.87	25.40	54.40	29.00	120	11.86	0.10
	t = 4		97.51	188.73	91.22	25.40	70.50	45.10	240	17.19	0.07
	t = 8		127.72	216.65	88.93	25.20	94.60	69.40	480	25.79	0.05
	t = 12		105.58	206.35	100.77	25.10	101.8	76.70	720	32.30	0.04
HP (250ml beaker &**100ml** water)	t = 2	1	97.31	190.10	92.79	24.10	56.30	32.20	120	12.49	0.10
	t = 4		105.37	202.43	97.06	24.20	72.40	48.20	240	19.55	0.08
	t = 8		105.37	208.59	103.22	22.50	97.20	74.70	480	32.22	0.07
	t = 12		105.35	211.25	105.90	22.50	97.10	74.60	720	33.01	0.05
	t = 2	2	97.31	190.10	92.79	24.10	56.30	32.20	120	12.49	0.10
	t = 4		105.37	202.43	97.06	24.20	72.40	48.20	240	19.55	0.08
	t = 8		105.33	210.93	105.60	22.40	97.30	74.90	480	33.05	0.07
	t = 12		105.36	210.85	105.49	22.30	97.10	74.80	720	32.98	0.05

Table 5: Microwave and Conventional Heating Temperature Settings

Heat energy, Q (kJ) is calculated by using the formula of:

$$\mathbf{Q = mC_p\theta}$$

Where : m – Mass of the distilled water, g

Cp – Heat capacity of water, 0.004186 kJ/gram °C

θ – Temperature changes, °C

Meanwhile, the power, P (W) irradiated/absorbed is calculated by dividing respective heat energy, Q (kJ) with time (s).

$$\mathbf{P = {}^{Q}/_{s}}$$

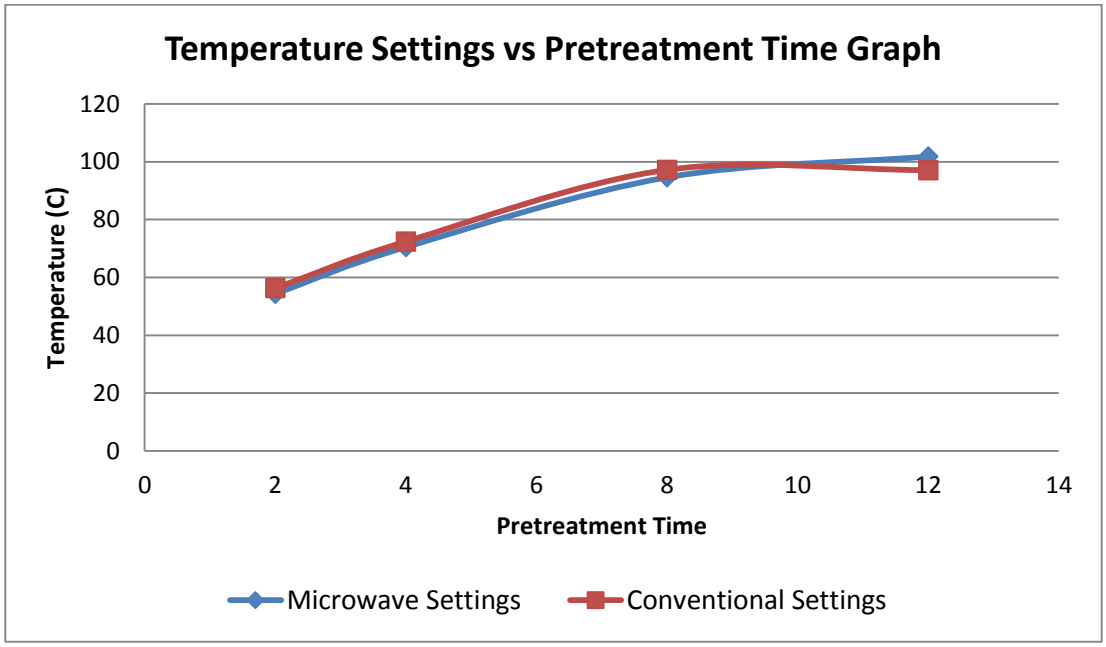


Figure 10: A graph of temperature against pre-treatment time

From Table 5, the graph of temperature recorded against pretreatment time is plotted as in Figure 4 to observe the consistency of temperature setting between microwave temperature and conventional temperature settings.

4.3 Pre-treatment

4.3.1 Effect of pre-treatment on the mixture solution thickness

The pre-treatment step for OPF biomass by using 0 N and 0.25 NofNaOHforboth microwave and conventional heating is conducted. The total mass of OPF in 100ml of solution is measured before and after pre-treatment. Later, the filtrate and the wet cake are separated by using the vacuum pump and the weight of both filtrate and wet cake is measured. However, during the separation process, some of the OPF tend to stick to the filter paper. It caused difficulties to remove the OPF solid residues from the filter paper.

The effect of pre-treatment is evaluated by observing the thickness of the mixture solution (supernatant) taken after the pre-treatment took place. From the observation made in Figure 3, the color of supernatants is getting thicker when the pre-treatment time is increase from 2 to 12 minutes. And also, the amount of solution is decreased as the pre-treatment time increase. This is due to the loss of liquid phase due to the vaporization activity which may indirectly affect the solution amount and the alkaline concentration. This statement is also agreed by Rashid et al. (2011).

Figure 11: The photo of pre-treated sample (filtrate) at 0 N ofNaOH

4.3.2 Effect of pre-treatment on OPF weight loss

The total weight loss of OPF is crucial in determining the effectiveness of pre-treatment used. To study that, the weight value for each biomass sample is measured before and after heating and a graph of weight loss against pre-treatment time for both heating method is plotted.

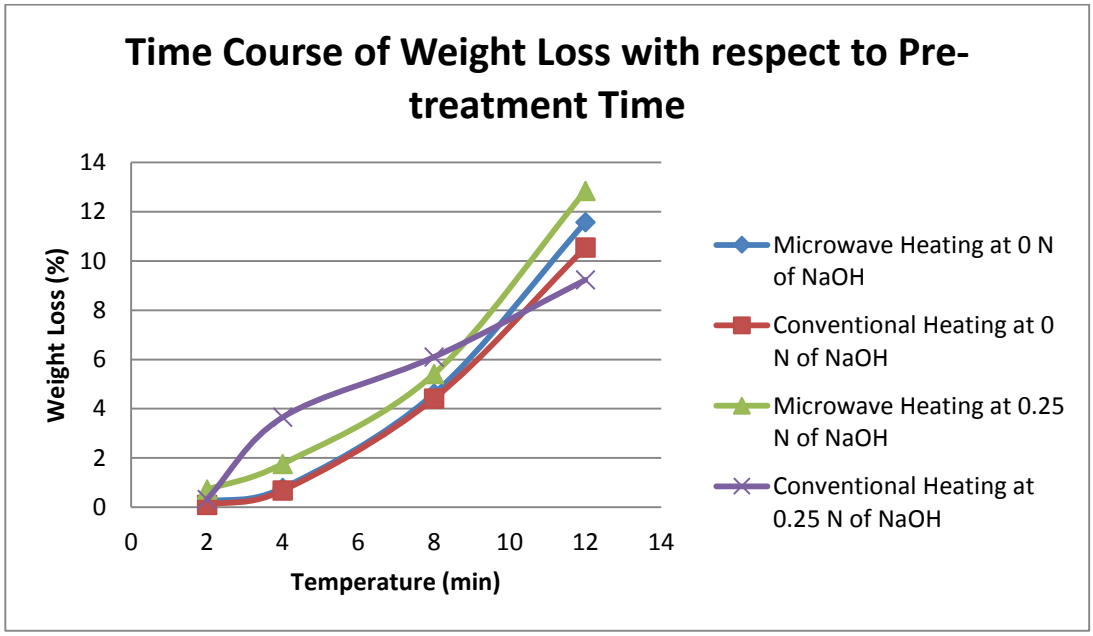


Figure 12: Time Course of OPF Weight Loss for Two Heating Method

Figure 6 shows that, the final weight loss is higher when it is pre-treated by using microwave assisted alkali pre-treatment compared to conventional alkali pre-treatment. One of the reasons that might contribute to this factor is might be due to the ability of microwave that has the ability to enhance some kind of reactions during the pre-treatment (S. Zhu et al, 2006).The ability of microwave in producing a very rapid drying is very convincing even without the need to overheat the atmosphere temperature compared to conventional heating, hence it caused the materials heated to vaporize faster and eventually increase the weight loss.

4.3.3 Effect of pre-treatment on morphology structure

On the other hand, the effect of pretreatment is studied by comparing the morphology structure on the surface of pretreated OPF biomass with 0 N and 0.25 N of NaOH at pretreatment time of 12 minutes and non treated OPF biomass by using SEM (Scanning Electron Microscopy) method.

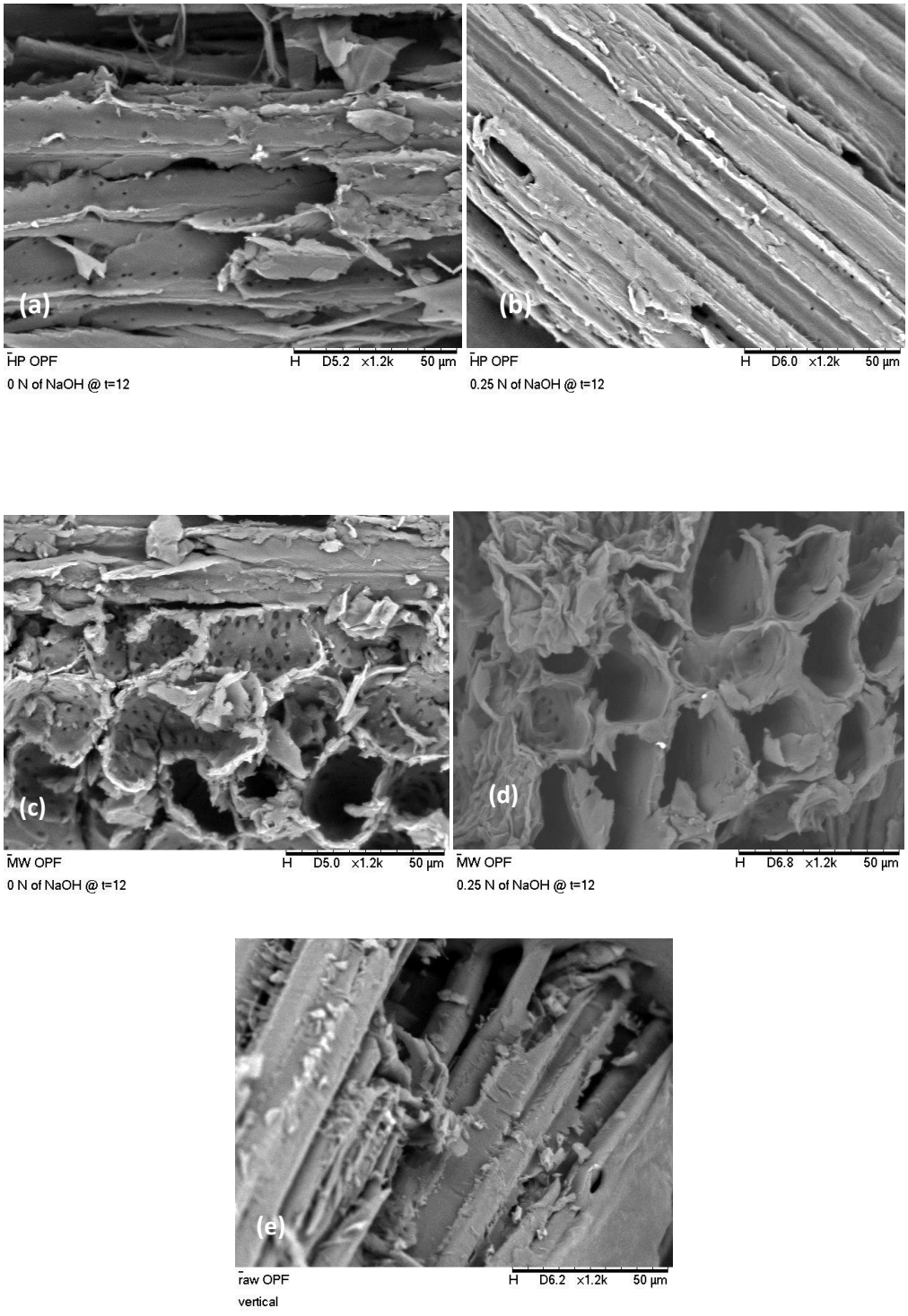


Figure 7: SEM image of OPF analyzed at optimum condition (Magnification = 1200x)

From the washed solid residue, 1.0(±0.01) gram of OPF solid residue is dried in dried oven under 105℃ for 24 hours. After 24 hours of drying, the samples are taken out and put inside a desiccator for few minutes until it reach room temperature. Figure 7 shows the morphology structure of OPF biomass from SEM analysis before and after pretreated with NaOH solution. The findings revealed the effect ofpretreatment on lignin structure and crystalline structure of biomass. Figure 7(e) shows the structure of raw untreated OPF is hard and rigid. The crystalline structure is still in densely-packed condition. The effect of conventional heating is represents by Figure 7(a) and (b) whereby conventional heating do not give too much different in altering the crystalline structure of OPF biomass. However, from Figure 7(c) and (d) which shows the effect of microwave pretreatment on crystalline structure, it can be observed that the porosity of OPF lignocellulosic biomass is higher compared to conventional heating and microwave pretreatment is aggressively reduce the cellulose crystallinity.

4.4 Enzymatic Activity Determination

From the enzymatic activity determination, the absorbance at 540 nm for all *TricodermaReesei* assay mixtures, blanks and controls, and glucose standards is being considered. The value obtained from the spectrophotometer is recorded and summarized as below.

Sample	Conc.	Rep.	Content	Absorbance		
				1	2	Average
Blank	-	-	1.5 mL buffer	-	-	-
	-	-		-	-	-
Substrate control	-	1	1.5 mL buffer + paper strip	0.010	0.010	0.010
	-	2		0.007	0.007	0.007
Glucose standard (mg/mL)	6.70	1	1.0 mL buffer + 0.5 mL glucose dilution	0.763	0.766	0.765
	5.00	1		0.229	0.229	0.229
	3.30	1		0.468	0.469	0.469
	2.00	1		0.271	0.271	0.271
Enzyme control	Dilution 1	1	1.0 mL buffer + 0.5 mL enzyme dilution	0.007	0.733	0.370
	Dilution 2	1		0.552	0.553	0.553
	Dilution 3	1		0.391	0.392	0.392
	Dilution 4	1		0.308	0.308	0.308
	Dilution 5	1		0.212	0.212	0.212
Mixture assay	Dilution 1	1	1.0 mL buffer + 0.5 mL enzyme dilution + paper strip	0.398	0.391	0.395
	Dilution 1	2		0.411	0.411	0.411
	Dilution 2	1		0.379	0.379	0.379
	Dilution 2	2		0.394	0.394	0.394
	Dilution 3	1		0.294	0.294	0.294
	Dilution 3	2		0.247	0.247	0.247
	Dilution 4	1		0.285	0.285	0.285
	Dilution 4	2		0.285	0.240	0.263
	Dilution 5	1		0.154	0.154	0.154
	Dilution 5	2		0.151	0.152	0.152

Table 6: Summary of Enzymatic Activity Determination

From the data obtained, the graph of absorbance at 540nm against glucose concentrations (mg/0.5 mL) is plotted in order to get the correlation coefficient.

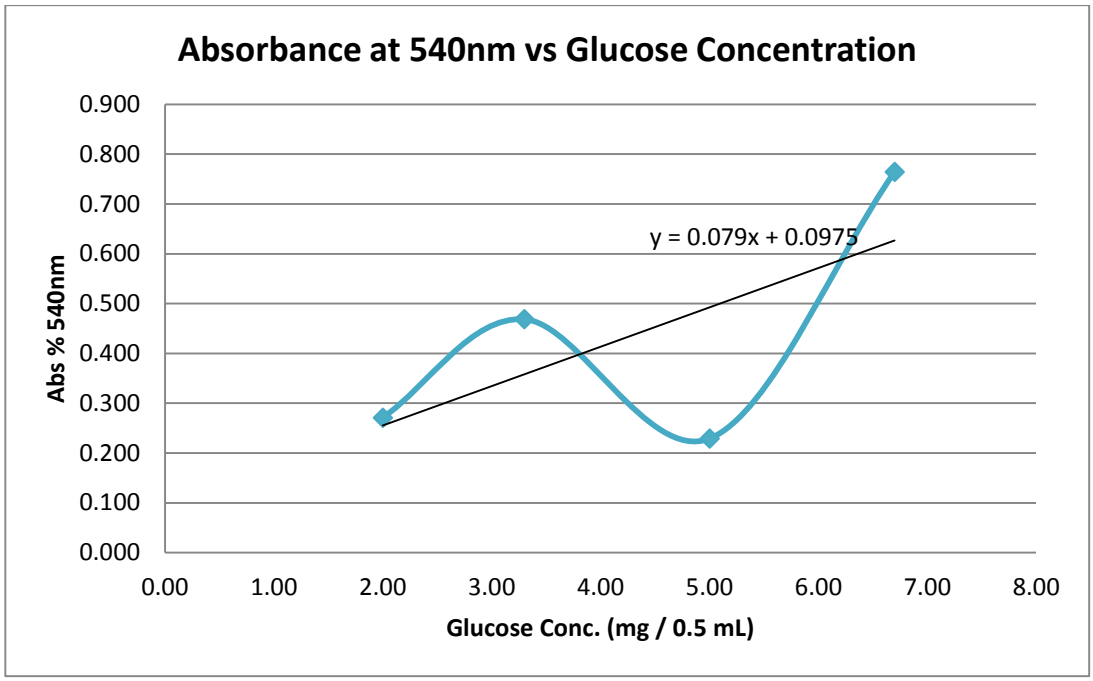


Figure 8: Graph of Absorbance at 540nm vs Glucose Concentration

Referring to the graph in Figure 8, by using the standard curve equation,

$$\mathbf{Y = 0.079X + 0.097}$$

The glucose amount released by each sample tube after enzyme blank subtraction is calculated. For the example,

Glucose concentration determination for enzyme control at Dilution 1,

Abs_{540nm}, Y = 0.730

Hence, substituting the value Y of into the standard curve equation will give the amount of glucose released, X. In this case, X = 16.025 mg/0.5 mL.

Abs at 540nm	Glucose Conc. (mg/0.5mL)	Enzyme Concentration
0.730	16.025	0.013
0.553	11.532	0.010
0.392	7.456	0.008
0.308	5.342	0.005
0.212	2.911	0.003

Table 7: Glucose Concentration for Enzyme Control at Abs 540nm

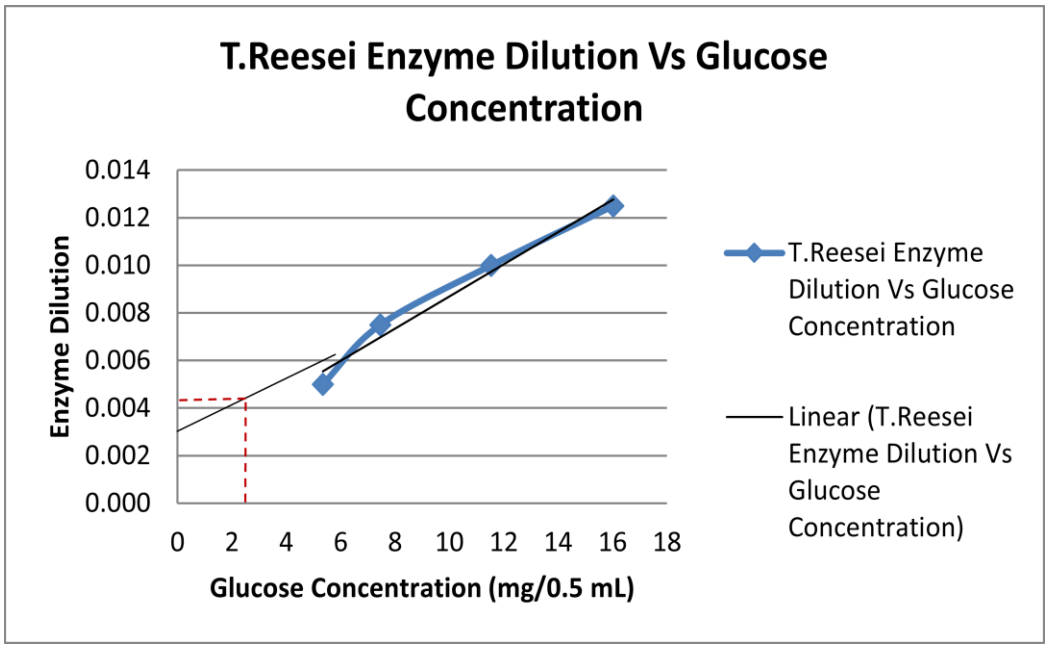


Figure 9: Graph of enzyme dilution vs glucose

From Figure 9, the concentration of enzymes which have released exactly 2.0 mg of glucose is obtained by plotting the enzyme concentration against amount of glucose released. In this case, concentration of *T.Reesei*enzyme required to produce 2.0 mg of glucose is approximately 0.0038 mg/mL. The value will later be used in preparing the saccharification process.

Filter Paper Unit (FPU) is calculated from the value of enzyme concentration above. According to NREL (2008), FPU is actually referring to the amount of enzyme activity that when assayed, will produce an equivalent amount sugars equal to 2.0 mg of glucose.

$$\text{FPU} = \frac{0.37}{0.0038} = 97.36\ \text{FPU/mL}$$

4.5 Enzymatic Saccharification

Saccharification is performed in incubator shaker under temperature of 50°C for 72 hours with shaking rotation of 150 rpm. 0.1 mL of aliquot is removed for every 12 hours of time interval after the flask contents are well mixed. The sample is later be subjected to HPLC analysis to study the amount of glucose released by analysing the peak area obtained from HPLC. Table 6 shows the summary of peak area from HPLC.

Pre-treatment Time (min)	Peak Area After 72 hours of Saccharification			
	HP 0 N NaOH	HP 0.25 N NaOH	MW 0 N NaOH	MW 0.25 N NaOH
2	867	5234	1382	1992
4	2483	1809	1424	1836
8	2074	3544	1671	1824
12	2191	2012	2014	1438
Untreated OPF	2768			

Table 8: Peak Area from HPLC for all samples

To measure the amount of glucose released, a calibration curve from the standard glucose calibration is needed to convert the peak area to concentration unit. Figure 10 reveals the calibration curve obtained for glucose.

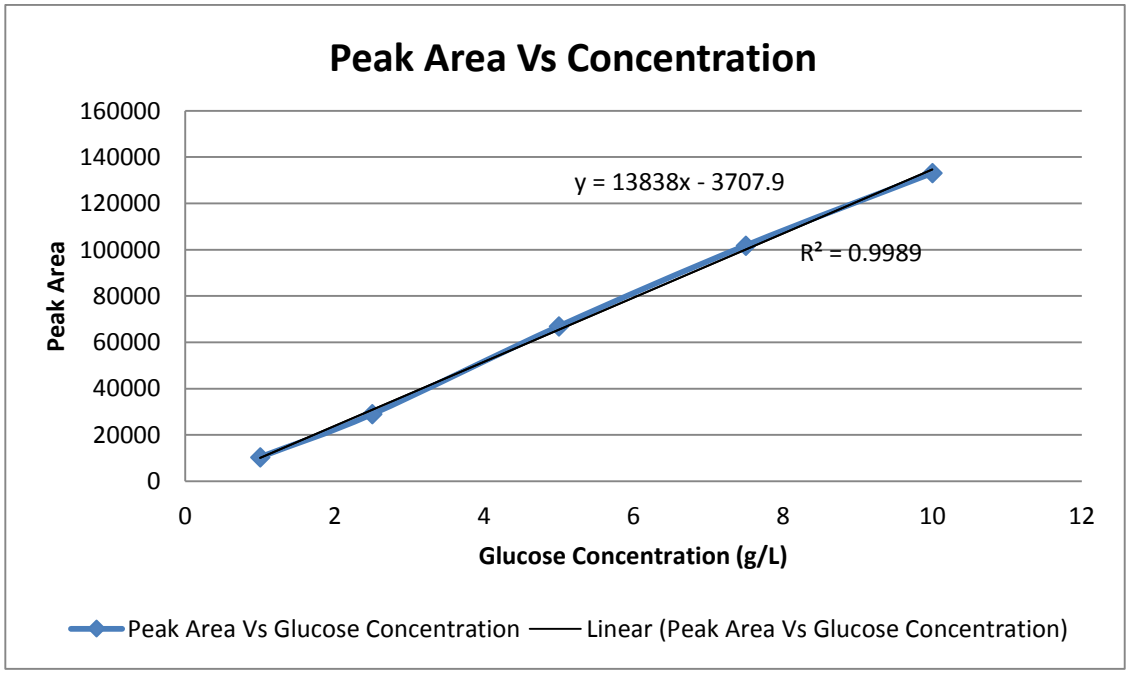


Figure 10: Glucose Calibration Curve

After that, the peak area data is later translated into concentration unit by using the standard curve equation obtained from the calibration curve graph above. Table 9 shows the summary of glucose yield concentration calculated.The graph of reducing sugars (glucose) yield versus pre-treatment time is plotted**.**

Pre-treatment Time (min)	Reducing Sugar Concentration after 72 hours Saccharification (g/L)			
	OPF Treated with HP 0 N NaOH	OPF Treated with HP 0.25 N NaOH	OPF Treated with MW 0 N NaOH	OPF Treated with MW 0.25 N NaOH
2	0.331	0.646	0.368	0.412
4	0.447	0.399	0.371	0.401
8	0.418	0.524	0.389	0.399
12	0.426	0.413	0.413	0.371
Untreated OPF	0.467			

Table 9: Summary of Reducing Sugar Concentration

From the data obtained, the glucose concentration is further calculated to get the percentage reducing sugar yield by using the formula provided by Beszedes et al. (2012). The summary of the glucose yield percentage calculated is shows in Table 8:

$$\mathbf{Y_G} = \frac{\mathbf{Glucose\ Concentration}\ (\frac{\mathbf{g}}{\mathbf{L}}) \times \mathbf{Volume_{hydrolysis(L)}}}{\mathbf{OPF\ biomass\ loading_{dry\ (g)}}} \times \mathbf{100}\%$$

Where,

$Volume_{hydrolysis}$ = 0.025 L (25 mL)

$OPF\ biomass\ loading_{dry}$ = 0.5 g

Pre-treatment Time (min)	Glucose Yield Percentage After Saccharification at 72 Hours (%)			
	OPF Treated with HP 0 N NaOH	OPF Treated with HP 0.25 N NaOH	OPF Treated with MW 0 N NaOH	OPF Treated with MW 0.25 N NaOH
2	16.527	32.306	18.388	20.591
4	22.366	19.931	18.539	20.028
8	20.888	26.199	19.432	19.985
12	21.311	20.664	20.671	18.590
Untreated OPF	23.395			

Table 10: Glucose Yield Percentage

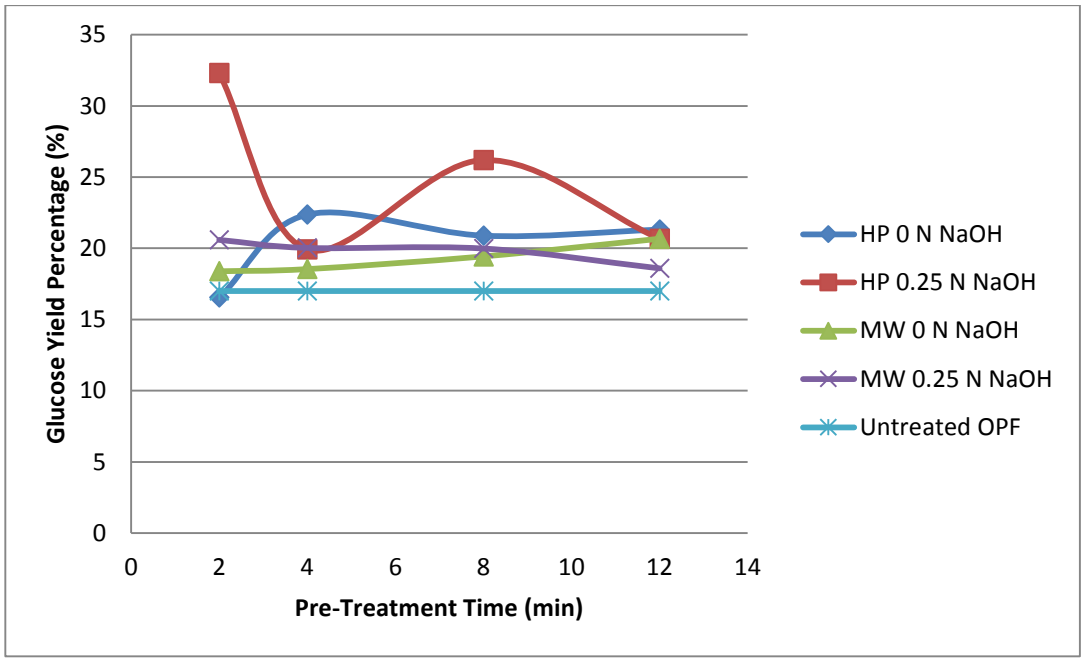


Figure 11: Graph of Glucose Yield Concentration vsPretreatment Time at 72 Hours of Saccharification

Figure 11 reveals the influence of microwave pre-treatment and conventional pre-treatment on reducing sugar yield from the pre-treated OPF biomass. After the period of 72 hours of incubation, the highest reducing sugar yield for microwave-alkali-assisted pre-treatment and conventional-alkali-assisted pre-treatment is at the lowest pre-treatment time, t = 2 min which are 0.646 g/L (32.31%) and 0.412 g/L(20.59%) respectively. However, the amount of reducing yield is decreasing as pre-treatment time increases for microwave-assisted-alkali and conventional-assisted-alkali pre-treatment. S. M. Nomanbhay (2013) mentioned in his literatures that extended period of pre-treatment may be resulted in the loss carbohydrates of the pre-treatment liquor which can reduce the amount of reducing yields at the moment hydrolysis process is conducted. That may be one of the reason on why the amount of reducing sugar yield by microwave-assisted-alkali (MW with 0.25 N of NaOH) shows the lowest concentration amount of reducing sugar yield at t = 12 min which is 0.372 g/L. this is eventually makes the method of using microwave at low setting in combination with dilute 0.25 N of NaOH gives least efficiency in pre-treating the OPF biomass. This is contradicts with the results of microwave-assisted-alkali pretreatment which has more capacity to increase the yield of reducing sugar compared to conventional-alkali-assisted pre-treatment reported by other literatures. S. Zhu et al (2005) reported that the amount of reducing sugar yield concentration

from the enzymatic hydrolysis of pre-treated wheat straw is the highest at higher concentration which is 42.9 g/L. Same case study were also reported by Md S Umikalsum et al (1998) in the treatment of OPEFB. However, there were cases where the low concentration of NaOH is found to be more effective such as the one reported by Soto et al (1994) where 0.5% of NaOH is found to be more effective that 3% of NaOH in the treatment of sunflower hull and Latif et al (1994) in the treatment of grass straw.

Nevertheless, different pattern of graph is observed for reducing sugar yield from both conventional and microwave without alkali pre-treatment whereby the amount of reducing sugar produced is increasing as pre-treatment time increases. Notably, the amount of reducing yield is higher for conventional pre-treatment compared to microwave pre-treatment at each time of interval.

Based on the study made by Gabhane et al. (2011), it shows that microwave heating can only be efficient at the temperature more than 200°C. The statement is agreed by Hu et al. (2008) who mentioned in his literature, the reducing sugar yield is higher after undergoes microwave pre-treatment compared to conventional pre-treatment only when the temperature reached 190°C. The reason is explained by Budarin et al (2010) whereby at temperature below 180°C, the polar molecules in cellulose will experience less freedom hence it prevents them to rotate and move, which resulting in poorer interaction in the end.

On the other hand, the difference observed on the rate of reducing sugar yield may be due to the changes take place in the cellulose structure which led to the increment of susceptibility to enzyme attack (Cowling, 1975). The changes take places is involving the pore structure, lignin and hemicelluloses removal, particle size, crystallinity and also degree of polymerization (Fan et al, 1980). Other reason is maybe due to higher free water content during hydrolysis (Maurya et al., 2013).

From overall observation, it can be noticed that the enzymatic hydrolysis is less efficient based on the inconsistency of reducing sugar yields for both microwave and conventional pre-treatment. Major factors that contribute to this distraction is may be due to low biomass loading. The low amount of biomass loading lead to the less yield of reducing sugar since the hemicelluloses and cellulose available is less (Maurya et al., 2013).

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

A large amount of unexploited OPF could serve a great benefit in producing bio-ethanol. From this study, characterization of OPF found that it contains high amount of carbon element of 43.46% hence make it suitable to be used as the feedstock for bio-ethanol production. SEM analysis reveals that microwave and conventional pre-treatment are reliable in altering and reducing the crystalline structure of OPF biomass for further penetration of enzyme. For without-alkali-assisted pre-treatment, the highest glucose yield reading is recorded at the highest pre-treatment time, 0.426 g/L for conventional heating and 0.413 g/L for microwave heating. Meanwhile, notably, at the lowest pre-treatment time of 2 minutes of alkali-assisted pre-treatment with 02.5 N of NaOH gives the highest yield of reducing sugar concentration of 0.646g/L for conventional heating compared to microwave heating which only yield 0.412 g/L. Optimization of saccharification conditions managed to produce high percentage reducing sugar yield of 32.31% for conventional pre-treatment and 26.20% for microwave pre-treatment.

5.2 Recommendation

It is recommended that other type of enzymes other than *TricodermaReesei* should be tested to maximize the reducing sugar yield during hydrolysis. Other recommendation is that to get and analyse the reading of glucose concentration during saccharification (enzymatic hydrolysis) from HPLC for every 24 hours instead of by analysing the glucose concentration at 72 hours only. Hence, the efficiency of enzymatic hydrolysis at different incubation time can be optimized and compared. Besides, the level of biomass loading should be taken into consideration before conducting the enzymatic hydrolysis in order to maximize the yield of reducing sugar.

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APPENDICES

Appendix 1

Appendix 1: The photo taken during enzymatic activity determination

Appendix 2

Appendix 2: The photo taken during saccharification process of OPF