

**KINETICS AND OPTIMIZATION STUDIES OF PRESSED PERICARP
FIBERS PRETREATED WITH ORGANOSOLVENT**

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**UNIVERSITI SAINS MALAYSIA
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**KINETICS AND OPTIMIZATION STUDIES OF PRESSED PERICARP
FIBERS PRETREATED WITH ORGANOSOLVENT**

by

HII KIEW LING

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requirements for the degree of
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LIST OF ABBREVIATIONS

Symbol	Description
3D	Three dimensional
AC	Activated carbon
AFEX	Ammonia Fiber Explosion
ANOVA	Analysis of variance
BG	Beta-glucosidase (cellobiase)
BMP	Best management practice
CCD	Central composite design
CMC	Carboxymethyl cellulose
CMCase	Endoglucanase
CPO	Crude palm oil
CS	Combine severity
CSTB	Conventional stirred tank bioreactor
DNS	Dinitrosalicylic acid
DoE	Design of experiment
EE	Ether extract
EFB	Empty fruit bunch
EG	Ethylene glycol
EtOH	Ethanol
FFB	Fresh fruit bunch
FPase	Filter paperase (cellobiohydrolase/exoglucanase)
FPU	Filter paper unit
g	Gram
HPR	High pressure reactor
HPR	High pressurized reactor
hr	Hour
IU	International unit
OFAT	One-factor-at-a-time
OPF	Oil palm frond
OPT	Oil palm trunk

PDA	Potato detroxe agar
PEG	Polyethylene glycol
PKC	Palm kernel cake
PKO	Palm kernel oil
PKS	Palm kernel shell
PL	phospholipids
POME	palm oil mill effluent
PPF	Pressed pericarp fibre
ppm	Part per million
RSM	Response surface methodology
RSPO	Roundtable on Sustainable Palm Oil
SmF	Submerged fermentation
SSF	Solid state fermentation
STBEV	Stirred tank bioreactor with external vessel
v/v	Volume per volume
w/w	Weight per weight

LIST OF SYMBOLS

Symbol	Description	Unit
\bar{k}	Total kinetic constant	1/(min. M)
A	Arrhenius constant	-
C	Cellulose content	g/g
E	Enzyme concentration	g/L
Ea	Activation energy	Kcal/mol
f	Co-factor	$M^{-1} \text{ min}^{-1}$
I	Inhibition factor	-
k	Kinetic constant	1/t @ 1/h
K_e	Equilibrium constant	-
L	Lignin content	g/g
P	Glucose concentration	g/L
R	Gas constant	Kcal/mol/K
R^2	Co-efficient of determination	-
S	Initial substrate concentration for hydrolysis	g/L
t	Time	min @ h
T	Temperature	K
X	Lignin conversion	-
 <i>Subscripts</i>		
0	At time 0	-
a	Enzyme adsorption	-
b	Bulk lignin	-
c	Cellulose	-
D	Enzyme deactivation	-
e	Equilibrium	-
H	Hydrolysis	-
i	Initial lignin	-
j	At stage j of lignin	-
L	Lignin for hydrolysis	-
P	Product formation	-

r	Residual lignin	-
S	Substrate (cellulose) for hydrolysis	-
t	At time t	-
<i>superscript</i>		
m	Kinetic order	-

KAJIAN KINETIK DAN PENGOPTIMUMAN PRA-RAWATAN HAMPAS KELAPA SAWIT DENGAN PELARUT ORGANIK

ABSTRAK

Kehadiran lignin di dalam hampas kelapa sawit (PPF) boleh merencatkan tindakbalas antara mikroorganisma selulolitik dengan selulosa, dan juga boleh merencat aktiviti enzim selulase. Untuk meningkatkan kerentanan selulosa, PPF telah dirawat dengan pelarut organik di bawah tekanan dan suhu yang tinggi dalam reaktor bertekanan tinggi (HPR). Alkohol monohidrat (propanol) menunjukkan penyahlignifikasi dan hidrolisis yang lebih baik pada 38.5% dan 46.1%, masing-masing. Etanol telah dipilih sebagai pelarut pra-rawatan untuk kajian lanjutan bagi penskrinan mangkin sebab ketersediaannya yang banyak serta perspektifnya dari segi ekonomi. Etanol yang dimangkinkan oleh asid nitrik memberikan keputusan yang paling tinggi dengan 48.9% penyahlignifikasi dan 96% hidrolisis PPF, iaitu peningkatan sebanyak 7.4 kali ganda daripada PPF yang tidak dirawat (PPF yang mentah). Dalam kajian ini, keadaan optimum untuk pra-rawatan PPF juga telah dijalan dengan menggunakan cara satu-faktor-pada-satu-masa (OFAT) dan kaedah statistik. Didapati bahawa pra-rawatan optimum PPF telah tercapai pada 180°C, nisbah etanol kepada air 0.65 yang dirangsangkan oleh 0.8% (w/w) asid nitrik. Melalui kaedah sambutan permukaan (RSM) yang digandingkan dengan rekabentuk rencam berpusat (CCD), didapati bahawa nisbah etanol kepada air pada 0.61 yang dimangkinkan oleh 0.69% (w/w) asid nitrik pada 182.84°C dengan masa tindakbalas 92.17 min adalah keadaan yang paling optimum untuk penyahlignifikasi (55.2%) dan hidrolisis PPF (90%) berbanding dengan PPF tanpa rawatan. Model kadar reaksi serentak yang melibatkan 3 spesies lignin telahpun dicadang dan diubahsuaikan, dan

didapati bahawa model ini berpadanan dengan data eksperimen dengan nilai pekali penentuan (R^2) 0.91. Bagi pemuliharaan selulosa dan hidrolisis PPF, model umum kadar reaksi yang telah diubahsuai sedikit dan mekanisme asas reaksi enzim telahpun dicadangkan, masing-masing. Kedua-dua model ini telahpun disahkan, dan ia menunjukkan nilai R^2 yang memuaskan, iaitu 0.87 dan 0.85, masing-masing. Untuk mengkaji kebolehlaksanaan pra-rawatan PPF bagi penghasilan selulase oleh *Pycnoporus sanguineus*, pra-rawatan PPF telah ditambahkan dengan 20-80% (v/v) kumbahan kilang kelapa sawit (POME). Daripada kajian OFAT, 6 g/L pra-rawatan PPF yang ditambahkan dengan 60% (v/v) POME memberikan penghasilan selulase yang optimum pada 5.31 IU/mL FPase, 10.13 IU/mL CMCCase, dan 0.76 IU/mL BG. Melalui kajian RSM, keadaan optimum bagi 6.21 g/L PPF yang ditambahkan dengan 63% (v/v) POME, memberikan aktiviti selulase yang paling tinggi (5.32 IU/mL FPase, 10.28 IU/mL CMCCase, dan 0.81 IU/mL BG) pada 30°C, 250 rpm selepas 7 hari fermentasi. Peningkatan aktiviti selulase dalam lingkungan 1.002 hingga 1.07 kali ganda telah diperolehi bagi kaedah RSM berbanding dengan kaedah OFAT. Nilai-nilai ini adalah 1.6 – 2.2 kali ganda lebih tinggi daripada nilai aktiviti enzim yang dihasil dengan menggunakan POME sahaja, berbanding dengan PPF pra-rawatan yang ditambahkan dengan POME. Dengan menggunakan keadaan optimum ini, penghasilan selulase telah dikaji di dalam bioreaktor tangki teraduk bergelung luar (STBEV). Beberapa teknik tersekatgerak telah dijalankan untuk mencari keadaan yang paling sesuai bagi pengoperasian STBEV, di mana POME bertindak sebagai media untuk pertumbuhan sel manakala PPF bertindak sebagai media atau matrik untuk penyekatan gerak sel. Didapati media kultur dengan PPF terperangkap dalam agar memberikan keputusan dalam lingkungan 1.2 – 1.5 kali ganda penghasilan selulase berbanding dengan fermentasi menggunakan POME sahaja.

KINETICS AND OPTIMIZATION STUDIES OF PRESSED PERICARP FIBERS PRETREATED WITH ORGANOSOLVENT

ABSTRACT

The existence of lignin in pressed pericarp fibres (PPF) could inhibit the cellulolytic microorganisms from reacting with cellulose which directly inhibits the cellulolytic enzyme activities. In order to increase the susceptibility of cellulose, PPF was treated with organic solvent under high pressure and temperature in a high pressurized reactor (HPR). Monohydric alcohols (propanol) showed better delignification and hydrolysis at 38.5% and 46.1%, respectively. Ethanol was chosen as pretreatment solvent for further screening of catalyst due to its wide availability and economical perspective. Results showed that ethanol catalysed by nitric acid gave the highest delignification (48.9%) and hydrolysis (96%) of PPF, an increase of 7.4-times from the untreated PPF (raw PPF). In this study, optimization of conditions for PPF pre-treatment was also carried out using one-factor-at-a-time method (OFAT) and a statistical tool. It was found that an optimal PPF pretreatment was achieved at 180°C, ethanol to water ratio of 0.65 aided with 0.8% (w/w) of nitric acid. For the response surface methodology (RSM) via central composite design (CCD), it was found that ethanol to water ratio 0.61 catalysed by 0.69 % (w/w) nitric acid at 183 °C with reaction time of 92 min was the best condition for delignification (55.2 %) and hydrolysis of PPF (90 %) as compared to the untreated condition. The irreversible simultaneous reaction rate model involving 3 species of lignin was proposed and modified, and found that it fitted well with the experimental data at co-efficient of determination (R^2) 0.91. As for the cellulose retained and PPF hydrolysis, a conventional reaction rate model with slight modification and a basic enzymatic

reaction mechanism were proposed, respectively. Both the models were validated and showed a satisfactory value of R^2 with 0.87 and 0.85, accordingly. To determine the feasibility of pretreated PPF for cellulase production by *Pycnoporus sanguineus*, PPF was then supplemented with 20-80% v/v of palm oil mill effluent (POME). From OFAT study, it was found that 6 g/L of treated PPF incorporated with 60% (v/v) of palm oil mill effluent (POME) gave the optimal cellulase production at 5.31 IU/mL of FPase, 10.13 IU/mL of CMCase, and 0.76 IU/mL of β -glucosidase (BG) activities. Through RSM study, an optimal condition of 6.21 g/L PPF supplemented to 63% v/v of POME was found to give the highest cellulase production (5.32 IU/mL of FPase, 10.28 IU/mL of CMCase, and 0.81 IU/mL of BG activities) at 30°C, 250 rpm after 7 days of the incubation period. An increase of 1.002-times to 1.07-times of the cellulolytic activities were observed for RSM as compared to OFAT method. These values were higher than the activities of enzyme using only POME as a substrate (1.6-times to 2.2-times), compared to treated PPF supplemented with POME, simultaneously. By using the optimum condition, cellulase production was studied in a stirred tank bioreactor attached with external vessel (STBEV). Different method of cell immobilization onto PPF matrix were carried out in order to find a suitable condition for STBEV operation, where POME create a medium for the cell growth while PPF served as a medium or matrix for cell immobilization. It was found that the culture broth with agar entrapped with PPF in POME showed better immobilization effect and cellulase production with a range of 1.2-times to 1.5-times compared to cultivation with POME only.

CHAPTER 1

INTRODUCTION

1.1 Overview of cellulases production from cellulose and its commercial utilization

Cellulases are getting more and more attention now and the demand for cellulases is increasing rapidly. This demand has served as a driving force for researches on cellulose degrading enzymes. They are currently being used in the textile industry for cotton softening and denim finishing; in detergent market for color care, cleaning and antideposition; in food industry for mashing; and in pulp and paper industry for deinking, drainage improvement and fiber modification (Cherry and Fidantsef, 2003; Kirk *et al.*, 2002). The cellulases market is expected to expand dramatically when it is used to hydrolyze pretreated cellulosic materials to sugar, which can be fermented to commodities such as bioethanol and biobased products on a large scale (Cherry and Fidantsef, 2003; Beilen and Li, 2002; Himmel *et al.*, 1999). The large market potential and the important role that cellulases play in the emerging bioenergy and bio-based products industries provide a great motivation to develop better cellulases preparations utilizing a readily available cheap cellulosic substrate, which is seen as a possible way to improve process economics (Himmel *et al.*, 1999; Doppelbauer *et al.*, 1987).

Cellulases are inducible enzymes which are mainly induced by cellulose (Sukumaran *et al.*, 2005). Conventional way of cellulases production from pure cellulose had attributed to the high cost of cellulolytic enzymes and cellulose ethanol (Sticklen, 2008). Realizing this problem, recent researches have been shifted to the utilization of lignocellulosic feedstocks which are lignocellulosic materials from

agricultural wastes. Generally, cellulose is the most abundant biopolymer present in nature (Zhang and Lynd, 2006; Mullings, 1985; Tong, 1984), and in agricultural as well as many industrial wastes (Madarro *et al.*, 1991) including oil palm industry (Kelly-Yong *et al.*, 2007). Unlike other non-sustainable resources such as fossil fuel and minerals, cellulose is the primary product of photosynthesis. It can be constantly replenished through photosynthesis and growth of plants (Osothship, 1984; Tong, 1984) and has served as the most abundant renewable resource produced in the biosphere (~100 billion dry tons/year) (Percival Zhang *et al.*, 2006; Zhang and Lynd, 2004; Jarvis, 2003; Holtzapple *et al.*, 1990). The vast quantities of these cellulosic waste products have been recognised as an important potential resource or substrate, which can be converted to value-added products that can be used for the chemical and biotechnological processes. According to Gaden (1974), the pathways for cellulose utilization was similar to carbon ecology (Mashitah, 1993). Cellulose biodegradation by cellulases and cellulosomes produced by numerous microorganisms represents a major carbon flow from fixed carbon sinks to atmospheric CO₂ (Percival Zhang *et al.*, 2006; Berner, 2003; Melillo *et al.*, 2002; Falkowski *et al.*, 2000). The system consists of three primary intermediates: starch (cereal grain), sugar (cane and beet), and cellulose (e.g. plant); three secondary intermediates: glucose, ethanol and protein; and three products: fuel, food and bulk chemicals (Figure 1.1).

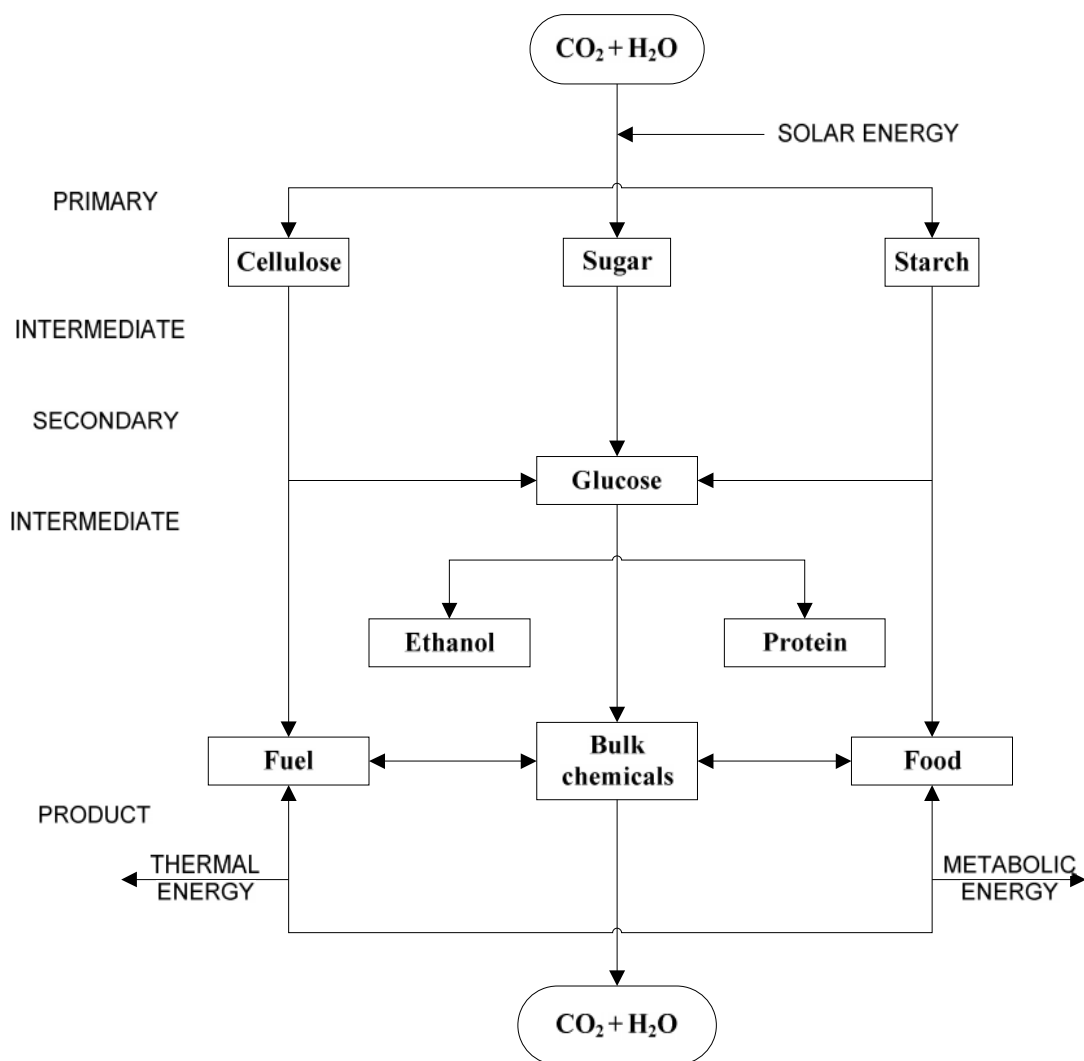


Figure 1.1: The ecology of carbon balance (Mashitah, 1993).

The utilization of these cellulose depend upon its conversion to glucose, which may be regarded as the key intermediate or a chemical feedstock for other micro-organisms in the production of fuel, single cell protein or high-end microbial products such as antibiotics (Madarro *et al.*, 1991; Brown *et al.*, 1987; Turker and Marituna, 1987; Knapp, 1986). Therefore, it can be widely used to produce sustainable bio-based products and bioenergy to replace depleting fossil fuels (Shuit *et al.*, 2009; Kelly-Yong *et al.*, 2007). Furthermore, studies have shown that the use of biobased products and bioenergy can give carbon-free emission (Demain *et al.*,

2005; Demain, 2004; Hoffert *et al.*, 2002; Lynd *et al.*, 1999; Lynd *et al.*, 1991). Development of technologies for effective conversion of agricultural waste and plant residues to fermentable sugars gives a potential partial solution to ensure the sustainable energy resources supply (Hoffert *et al.*, 2002; Lynd *et al.*, 1999) by providing alternative energy sources (Demain *et al.*, 2005). Meanwhile, it also gave positive environmental impacts and promotes economic development (Hoffert *et al.*, 2002; Lynd, 1996; Lynd *et al.*, 1991). This circumstances comply with the green environmental policy and cleaner technology approach (Foo and Hameed, 2010) which is purposed to utilise a diverse range of products, services, and processes that harness renewable materials and energy sources to dramatically reduce the use of natural resources, and minimise or eliminate emissions and wastes.

1.2 Biotechnological potential of pressed pericarp fibres (PPF)

Pressed pericarp fibers or palm pressed fibres (PPF) are by-products or wastes produced in large quantities by the palm oil mill after screw-pressing of palm fruits during the production of crude palm oil (CPO) (Han *et al.*, 2006). In Malaysia, the annual production of PPF as waste materials amounts to approximately 8.56 million metric tons. Despite the bulk density being produced, the utilization of PPF reported was limited. Generally, PPF was used as combustible materials due to the oil retained in its cell wall (Prasertsan and Prasertsan, 1996). Their ash are rich in organic minerals, such as phosphorus (1.7-6.6%), potassium (17-25%) and calcium (7%) which can be used as sources of minerals for plants. In some plantations, all the PPF produced were used as mulching materials to provide organic nutrients to the oil palm trees in plantations or being used as solid fuels to self-supply steam and electricity required (Bakar *et al.*, 2007; Basiron and Simeh, 2005; Prasertsan and

Prasertsan, 1996). Besides, PPF is expected to contain high level of phospholipids (PL) since palm oil is mechanically extracted without solvents, compared to seed oils, which are extracted via solvent extraction (Choo *et al.*, 2004). Few studies have been carried out previously on the extraction of PL by using solvent such as hexane as well as ethanol (Choo *et al.*, 2004) and this technology was further improved through ultrasound-assisted technology (Chua *et al.*, 2009). On the other hand, the recovered fibers from PPF have also been found rich in carotenoid, vitamin E and sterols (Choo *et al.*, 1996). Therefore, researches have been carried out by many researchers to recover and extract carotenes and coenzyme Q10 (Han *et al.*, 2006; Franca and Meireles, 1997; Choo *et al.*, 1996).

Although the characteristics of PPF is similar to the rice straw, its high percentage contents of fiber and lignin made it cannot be digested easily by animals which made it not suitable as food for animals but a good substrate for mushroom cultivation. It was reported that the chemical composition of untreated PPF was estimated to be 39.9 wt% cellulose, 28.9 wt% hemicelluloses, 20.3 wt% lignin, 3.6 wt % ash and 7.2 wt % of extractives (Tong and Hamzah, 1989). Being a lignocellulosic material, PPF could represent a renewable and low-cost raw material for the production of fermentable sugars which can be further transformed into another value-added renewable energy such as bio-hydrogen, bio-ethanol and bio-methane (Lam and Lee, 2011; Karimi *et al.*, 2006). In fact, the cellulose contents found in PPF can be converted to fermented sugar by a simple enzymatic hydrolysis step (Huijgen *et al.*, 2008). Nevertheless, the enzymatic saccharification of insoluble cellulose in natural biomass is always limited due to: (i) decrease susceptibility of substrate to enzymes, (ii) end product inhibition, (iii) reversible inactivation of enzymes adsorbed on the cellulose surfaces, and (iv) deactivation of cellulose that

was surrounded by lignin fragments (Eriksson *et al.*, 2002; Umikalsom *et al.*, 1998; Tong and Hamzah, 1989). The nature of cellulose's crystalline structure was composed of β -1, 4-glycosidic bonds of D-glucose which gave interactive protection to the cellulose by the lignin and hemicelluloses. These properties have caused the lignocellulosic materials difficult to be broken down by the cellulolytic enzymes or microorganisms in any hydrolysis processes due to the macromolecular size owned by the enzymes (Ji *et al.*, 2009; Kuhad and Singh, 2007; Tong and Hamzah, 1989). Thus, pretreatment is necessary to increase the accessibility of cellulose polymer for enzymatic hydrolysis by breaking the lignin seal, partially dissolving hemicellulose, and disrupting the crystalline structure of cellulose into the amorphous form (Jing *et al.*, 2009; Huijgen *et al.*, 2008; Pan *et al.*, 2006; Mosier *et al.*, 2005; Wyman *et al.*, 2005; Sun *et al.*, 2000).

1.3 Problem statement

Hydrolysing cellulose by cellulases has gained considerable interests in the past decades due to the multiple usages of cellulases in industries. However, in Malaysia, there was no attempts have been done to produce enzymes for commercial using local available resources (Lee *et al.*, 2011; Ibrahim, 2008). In this case, Malaysia has spent about US\$ 3.5 million annually just for importing industrial enzymes from Denmark, Netherlands, Belgium, and etc (Ibrahim, 2008). Thus, it would be economic beneficial if Malaysia could self-supply the cellulases for internal uses.

In submerged fermentation process, the cost of carbon source has become a major contribution to overall cellulase production cost (Liming and Xueliang, 2004). According to Domingues *et al.*, (2000), various purified cellulose, such as

Avicel/Sigmacell-100 (Ljunggren, 2008; Rajoka and Malik, 1997), and Solka Floc (Juhász *et al.*, 2005), have been used as carbon source for cellulase production, but their price is too expensive to be applied in large scale processes. Therefore, it is necessary to develop a better cellulases preparation by using a readily available cheap cellulosic substrate in order to further improve the process economics (Mohamed *et al.*, 2010; Himmel *et al.*, 1999; Doppelbauer *et al.*, 1987). Lignocellulosic biomass materials have been a very good option due to their abundance, cheapness and huge potential availability (Kang *et al.*, 2004).

Since the last few decades, many research works have been carried out to investigate the most appropriate pretreatment methods. Among all pretreatment methods, organosolvent pretreatment method has drawn much attention (Küçük and Demirbaş, 1999). It has been reported that delignification with mixtures of aqueous-organic solvents is effective in separating wood into its three main components. The hemicelluloses are soluble in aqueous phase, the lignin is soluble in the organic phase, and the cellulose which cannot dissolve in organic solution is separated as solid (Demirbas, 2000; Bowers and April, 1977). Due to its special characteristics, organosolvent has proved to be a remarkable solvent for delignification pretreatment. Since 2006, researchers investigated treatment using organosolvent under pressurized condition which not only opened an alternative for high temperature/pressure operation (which gave a better result) but also shorten the reaction time significantly (Brosse *et al.*, 2010; El Hage *et al.*, 2010a; El Hage *et al.*, 2010b; Brosse *et al.*, 2009; El Hage *et al.*, 2009; Pan *et al.*, 2007; Pan *et al.*, 2006). Organosolvent pretreatment is gaining more attention when it was reported that the solvents used are always easy to be recovered by distillation and recycled for the next pretreatment operation (Zhao *et al.*, 2009; Kuznetsov *et al.*, 2001).

Many organic solvents have been used to pre-treat lignocellulosic materials but none was reported on PPF. Therefore, in this current study, PPF, an easily and abundantly available palm oil residue in Malaysia will be treated by using organic solvent to investigate its feasibility in glucose recovering followed by cellulases production in submerged fermentation with POME (another liquid oil palm waste). It was estimated that for each tonne of palm oil fresh fruit bunch, around 0.5 – 0.75 tonnes of POME was discharged (Wong *et al.*, 2009). The effluent if let discharged untreated, it will definitely cause heavy environmental problem (Davis and Reilly, 1980) due to its fairly high polluting properties, with high biochemical oxygen demand (25,000 mg/l), chemical oxygen demand (53,630 mg/l), oil and grease (8370 mg/l), total solids (43,635 mg/l) as well as suspended solids (19,020 mg/l) (Ma, 1995). By turning the palm oil wastes (PPF and POME) into cellulases (value-added product), a cost effective process for palm oil waste handling and utilisation can be developed and implied. It is in turn can create a win-win situation whereby a new revenues for economical cellulases production is created and the environmental hazards can be minimized at the same time.

1.4 Research objectives

The main objective of this study is to investigate the potential of pressed pericarp fibre (PPF) as a low-cost feedstock for cellulases production while providing an alternative environmentally benign and cost effective strategy for their treatment.

The measurable objectives are:

- i. To screen the effectiveness of different organosolvents and catalysts for delignification of PPF.

- ii. To determine the optimum operating conditions for catalyzed organosolvent PPF pretreatment using one-factor-at-a-time (OFAT) method, and the design of experiment (DOE).
- iii. To propose kinetic models for PPF pretreatment using organosolvent chosen in objective (i)
- iv. To study the feasibility of cellulases production by *Pycnoporus sanguineus* using pretreated PPF as substrate in shake flask and bioreactor.

1.5 Scope of study

PPF, a lignocellulosic biomass which is rich in cellulose, is a potential feedstock for cellulases production. In this study, both the pressed pericarp fibre (PPF) and palm oil mill effluent (POME), which are oil palm wastes, were utilized and converted into a value-added product, cellulases.

As a lignocellulosic biomass, the significant amount of lignin in PPF has limited the amount of accessible cellulose to be converted into glucose, thus limiting the cellulases production. Therefore, a practical and economical pretreatment is needed in order to remove the lignin as much as possible as well as to increase the susceptibility of cellulose to cellulases. Organosolvent pretreatment was chosen in this study due to its environmentally benign and economical effectiveness. The most suitable organosolvent and catalyst were screened prior to optimization studies of the operating parameters, namely operating temperature, solvent to water ratio, catalyst concentration and incubation time, using one-factor-at-a-time (OFAT) method and a statistical tool. All these parameters were evaluated on their performance of lignin removal, cellulose retained and most importantly, PPF hydrolysis.

Kinetic and modeling of PPF organosolvent pretreatment were carried out to evaluate and predict the lignin removal, cellulose retained and PPF hydrolysis. For both the lignin removal and cellulose retained, and PPF hydrolysis, a conventional degradation reaction and basic enzymatic reaction mechanism has been proposed. The validity of the models was evaluated by determining the co-efficient of determination (R^2).

The PPF treated under optimum condition was preceded into second stage of the thesis, which is cellulases production by using POME as the fermentation medium. The fermentation was carried out in shake flask culture in order to determine the optimum ratio of PPF and POME. Both the OFAT and Response Surface Methodology (RSM) were used to determine the optimum PPF and POME ratio for cellulases production prior to continuous stirred tank bioreactor with external vessel (STBEV) studies. In STBEV study, different types of external loop condition were studied and compared to find the suitable external loop system for cellulases production, thus suggesting the most favorable STBEV system and condition for optimum cellulases production.

1.6 Organization of the thesis

In order to make this study understandable, the thesis was divided into five chapters as follows:

Chapter One presents the overview of cellulase production from cellulose and its commercial application as well as the biotechnological potential of pressed pericarp fibres (PPF). The problem statement, objectives and organization of the thesis were also highlighted in this chapter.

Chapter Two is a literature review that provides a background study on the chemistry of different lignocellulosic materials including PPF as well as various kinds of pretreatment methods coupled with the pretreatment kinetics as reported in the literatures. Besides, the cellulases enzyme systems and their sources, fermentation technologies regarding the fungus applied were also reviewed in this chapter. Subsequently, a statistical analysis which is response surface methodology (RSM), was applied in this study,

Chapter Three describes the materials and methods. Firstly, all the materials and equipments used in this research were listed clearly in a table form. Then, the experimental procedures in preparation of PPF, including the characterization of product and pretreatment, as well as fermentation process, in both shake flask and bioreactor system, and all the analytical procedures were described in detailed. The material and procedures applied during the experimental stage of the study were summarized in the form of flow chart.

Chapter Four discussed the experimental result together with the data analysis at various operating condition and process parameters. The results are organized into five main sections; preliminary study of PPF pretreatment (screening of solvent and catalyst), optimization study of the pretreatment and its kinetics, batch fermentation in shake flask and batch fermentation in bioreactor with external loop. The results were also presented in graphical form for better interpretation with supporting evidence elaborated in details.

Chapter Five gives the overall conclusion based on the findings obtained in Chapter Four. Recommendations for future research are also highlighted.

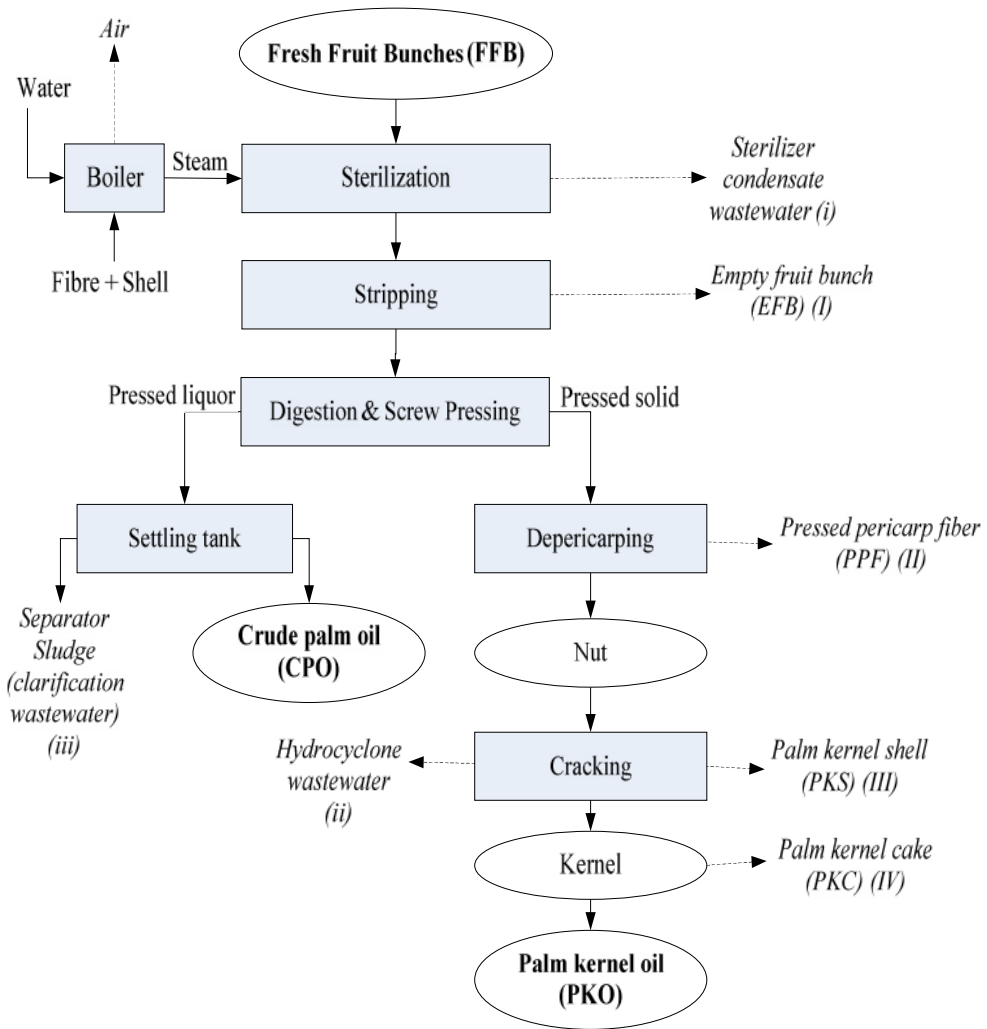
CHAPTER 2

LITERATURE REVIEW

2.1 The predominance of oil palm residues as a substrate for cellulases production

The rapid growth of Malaysian palm oil industry has been phenomenal over the last four decades and it has become a very vital agriculture-based industry which played an important role in the economic development of Malaysia (Hamisan *et al.*, 2009). Despite the obvious benefits, this industry has also significantly contributed to some environmental problems. Oil palm manufacturing processes has not only generated wastewater, commonly known as palm oil mill effluent (POME) and air pollution, but also produced a large quantities of solid wastes as shown in Figure 2.1 (Fauziah, 2010; Wu *et al.*, 2010; Prasertsan and Prasertsan, 1996) and Figure 2.2 (Weeraratne *et al.*, 2007).

In fact, the amount of oil palm residues and its processing by-products are found to be seven times the availability of natural timber with world annual production of oil palm residues amounts to 184.6 million tons (Basiron and Chan, 2004). This figure is expected to increase in the future due to the high global demand for palm oil. The percentage of residues produced from oil palm has increased tremendously since 1980 until recently, contributed by the expansion of crop plantation due to the high demand for palm oil (Shuit *et al.*, 2009).



* (i) + (ii) + (iii) → Palm oil mill effluent (POME)

* (I) + (II) + (III) + (IV) → solid waste

Figure 2.1: General wastes production scheme in palm oil mill (Fauziah, 2010; Wu *et al.*, 2010; Prasertsan and Prasertsan, 1996).

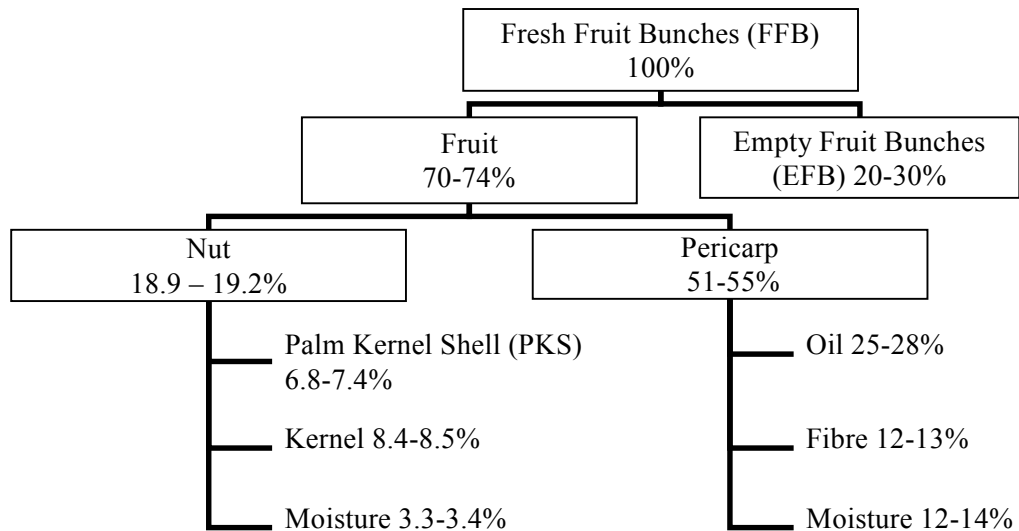


Figure 2.2: Composition of FFB (Prasertsan and Prasertsan, 1996)

Problems occurred as the handling of disposal of the oil palm residues is very expensive due to the high costs transportations and limited availability of landfills. As a result, it is unscrupulously disposed which causes environmental problems. Therefore, the development and implication of cost effective process for treatment and utilization of oil palm residues is essential to convert the wastes into another value-added products, and at the same time can minimize the environmental problems. Furthermore, conversion of oil palm residues into another value-added products or energy is a good means of obtaining carbon credit facility for sustainable management besides meeting the increasing demand for energy in recent year (Sridhar and AdeOluwa, 2009).

Realizing the importance of minimizing carbon emission, industries and scientists are looking for more options in reusing the biomass. Generally in industries, the biomass was used as mulching materials to provide organic nutrients to the oil palm trees in plantations or being used as solid fuels to self-supply steam and electricity required (Bakar *et al.*, 2007; Basiron and Simeh, 2005; Prasertsan and Prasertsan, 1996). Due to their rich mineral contents, oil palm residues had also been

used as fertilizer and soil conditioner to improve the quality of the planting soil (Wan Asma Ibrahim *et al.*, 2007; Wan Rashidah *et al.*, 2000). For instance, EFB and PPF have been used as a successful raw material for mushroom cultivation (Obodai *et al.*, 2003; Prasertsan and Prasertsan, 1996; Klitsaineepaibon and Bungkong, 1990). Meanwhile, the high fibre content and residual fat in palm kernel cake has also led to a higher average daily gain to non-ruminant livestock (Boateng *et al.*, 2008). Other commercializes products such as palm board production (Koh, 2007; Rozman *et al.*, 1997), pulp and paper production (Rodríguez *et al.*, 2008; Mohd. Nor and Rahim, 2007; Okiy, 1987), carotene extraction (Ahmad *et al.*, 2010; Ahmad *et al.*, 2008), activated carbon (Hameed *et al.*, 2008; Wan Asma Ibrahim *et al.*, 2007) and etc. through oil palm biomass reusing have also been reported. Nowadays, studies are focus on the conversion from oil palm residues into a renewable energy due to their lignocellulosic characteristic. The high cellulose and hemicelluloses content owned by the oil palm biomass has made them a renewable and low-cost raw material for the production of fermentable sugars which can be further transformed into another value-added renewable energy such as bio-hydrogen, bio-ethanol and bio-methane (Lam and Lee, 2011; Karimi *et al.*, 2006).

As shown in Table 2.1, oil palm residues mainly consist of cellulose which can be hydrolyzed and fermented into cellulosic ethanol. In addition, the wastes are natural by-products, and non-toxic, because no chemicals are used in the palm oil milling process (Prasertsan and Prasertsan, 1996), and this reduced the inhibition of enzyme hydrolysis and yeast fermentation. However, similar to other lignocellulosic biomass, oil palm residues also contain lignin. Thus, delignification needs to be done in order to reveal more accessible cellulose for glucose recovery from those wastes.

Table 2.1: Oil palm residues as lignocellulosic biomass and their respective composition (wt%) (Shibata *et al.*, 2008; Tong and Hamzah, 1989)

part	cellulose	hemicellulose	lignin	extractives	ash
OPT	30.6	33.2	28.5	3.6	4.1
OPF	39.5	29.8	23.3	1.7	5.7
PKC	35.7	30.3	15.7	11.7	6.7
EFB	37.9	35.0	24.0	2.7	1.5
PPF	39.9	28.9	20.3	-	3.6

* *OPT* = oil palm trunk
OPF = oil palm frond
PKC = palm kernel cake
EFB = empty fruit bunch
PPF = pressed pericarp fibres

2.2 Pressed pericarp fiber (PPF)

2.2.1 Sustainability of PPF

Pressed Pericarp Fiber (PPF) is obtained from palm oil plantation/industry. They are by-products or wastes produced in large quantities by the palm oil mill after screw-pressing of palm fruits during the production of crude palm oil (CPO) (Han et al., 2006). Based on the milling operations, the palm oil extraction is divided into dry and wet process. The majority of palm oil mills in Malaysia have adopted the dry process (as shown in Figure 2.1) since the wet process is unsuitable for use in large-scale productions (Wu et al., 2009).

It has been reported by Tong and Hamzah (1989) that roughly 8.56 metric tons of PPF has been generated as waste materials from oil palm processing each year. In Malaysia, palm oil plantation and industry are governed by the establishment of Roundtable on Sustainable Palm Oil (RSPO) (Tan et al., 2009). It was formally established in 2004 to promote the growth and use of palm oil through co-operation within the supply chain and open dialogue between its stakeholders. RSPO defines sustainable palm oil production as a legal, economically viable, environmentally appropriate and socially beneficial management and operations. This is realized through a policy known as RSPO Principles and Criteria which are

applicable to the management of oil palm plantations and palm oil mills (Tan *et al.*, 2009). Malaysia has always been an active member of RSPO with both the government agencies and private companies taking important roles in the organization. The principles and criteria stated in RSPO are also being strictly followed to ensure the sustainable development in oil palm plantation and production (Shuit *et al.*, 2009; Tan *et al.*, 2009).

Apart from the criteria from RSPO, operations of palm oil companies in Malaysia, from harvesting to palm oil related products production (including PPF), are carried out with best management practice (BMP). These practices are environmental friendly approaches such as zero burning, conservation of wildlife habitat, Integrated Pest Management (IPM) as well as waste minimization and utilization. All these practices have been performed in Malaysia to a large extent which will contribute to environmental conservation and finally contribute towards the sustainable development of oil palm plantation and production in Malaysia (Shuit *et al.*, 2009; Tan *et al.*, 2009).

2.2.2 Properties and composition

Oil palm fiber is a natural fiber extracted from palm oil vascular bundles in the empty fruit bunch (EFB) with < 15% moisture content, < 3% oil content and < 10% impurities (Butler, 2007). According to Ramli *et al.*, (2002), fibers are clean, non-toxic, non-carcinogenic, and non-hazardous biodegradable material and also free from pesticides and soft parenchyma cells. It was reported that the chemical composition of untreated PPF was estimated to be 39.9 wt% cellulose, 28.9 wt% hemicelluloses, 20.3 wt% lignin and 3.6 wt % ash (Tong and Hamzah, 1989). Owing to these characteristic, PPF can be categorized as a lignocellulosic material. The

highest cellulose content of PPF compared to other oil palm residues makes PPF a potential feedstock for cellulases production. Nevertheless, similar with other lignocellulosic biomass, high lignin percentage of PPF also cannot be negligible and need to be removed via pretreatment before going through any enzymatic saccharification and fermentation.

In addition, PPF is expected to contain high level of phospholipids (PL) since palm oil is mechanically extracted without solvents, compared to seed oils, which are extracted via solvent extraction (Choo *et al.*, 2004). Few studies have been carried out previously on the extraction of PL by using solvent such as hexane as well as ethanol (Choo *et al.*, 2004) and this technology was further improved through ultrasound-assisted technology (Chua *et al.*, 2009). On the other hand, the recovered fibers from PPF have also been found as a rich in carotenoid, vitamin E and sterols (Choo *et al.*, 1996). Therefore, researches have been carried out by many researchers to recover and extract carotenes and coenzyme Q10 (Han *et al.*, 2006; Franca and Meireles, 1997; Choo *et al.*, 1996)

Besides that, PPF also contains nutrients such as calcium (Ca), phosphorus (P) ether extract (EE), nitrogen (N), carbon (C), sulphur (S), kalium (K) and others (Tabi *et al.*, 2008; Obese *et al.*, 2001) as summarized in Table 2.2. However, the physical and chemical properties of PPF vary according to the origin of PPF. As reported by a few researchers, the PPF ash was found to contain 1.7-6.6% P, 17-25% K and 7% Ca (Prasertsan and Prasertsan, 1996; Krause, 1994).

Table 2.2: Nutrients composition of PPF in literatures (Tabi *et al.*, 2008; Obese *et al.*, 2001; Prasertsan and Prasertsan, 1996; Krause, 1994).

Nutrients composition	% (w/w)	Reference
Moisture	3.7 – 9	(Obese <i>et al.</i> , 2001)
N	1.2 – 1.3	(Tabi <i>et al.</i> , 2008; Obese <i>et al.</i> , 2001)
Ca	7	(Prasertsan and Prasertsan, 1996; Krause, 1994)
K	17 - 25	(Prasertsan and Prasertsan, 1996; Krause, 1994)
P	1.7 – 6.6	(Prasertsan and Prasertsan, 1996; Krause, 1994)
EE	26.9 – 35.5	(Obese <i>et al.</i> , 2001)
H	6	(Tabi <i>et al.</i> , 2008)
C	31.3 – 54.9	(Tabi <i>et al.</i> , 2008)
S	0.3	(Tabi <i>et al.</i> , 2008)
Ash	5.3 – 8.4	(Tabi <i>et al.</i> , 2008; Obese <i>et al.</i> , 2001)

2.2.3 Application

Due to the environmentally-friendly properties owned by PPF (Ramli *et al.*, 2002), it can be used in several industrial application as shown below:

2.2.3 (a) Solid fuel

Most of the palm oil mill manufacturer used fiber and shell as fuel. The oil retained in the fiber's cell wall after palm oil mill process has made PPF a good combustible material to self-generate steam and electricity to be used in palm oil milling process (Bakar *et al.*, 2007; Basiron and Simeh, 2005; Prasertsan and Prasertsan, 1996). This has helped to reduce the use of fossil fuel. Surplus energy is channeled to the national electricity grid. The bi-drum water tube boiler is the most popular field for palm oil mill burning by palm fiber, shell and EFB (VHM, 2001).

As shown in Table 2.3, the energy produced by fiber and shell as fuel is 750 kWh, which is equivalent to the energy requirement for the palm oil mill production system. Furthermore, the generation of fiber and shell from the system were more than the amount of fuel needed. Meaning that, the fiber and shell can be used as internal fuel for palm oil mill manufacturing.

Table 2.3: Energy production and utilization in Jugra palm Oil Mill (Weeraratne *et al.*, 2007)

	Fuel Generated from System	Fuel Needed	Energy Production	Energy Requirement
Fiber	5.37 tons/h	4.52 tons/h	750 kWh	750 kWh
Shell	3.14 tons/h	1.12 tons/h		

2.2.3 (b) Sources of minerals for plants

PPF was also found to be rich in organic minerals, such as phosphorus (1.7-6.6%), potassium (17-25%) and calcium (7%) which can be used as sources of minerals for plants. In some plantations, all the PPF produced were used as mulching materials to provide organic nutrients to the oil palm trees in plantations (Wan Asma Ibrahim *et al.*, 2007; Wan Rashidah *et al.*, 2000). By leaving the fibers to decompose naturally, a number of objectives such as, improvement of biochemical properties and soil structure, reduction of soil erosion, conservation of soil moisture and increased microorganism activities were achieved (Kadir and Ibrahim, 2007). Another alternative of PPF utilization is composting, which can also be used as soil conditioner and soil replacement in any planting activity including in home gardens. For instance, EFB and PPF have been used as a successful raw material for mushroom cultivation (Obodai *et al.*, 2003; Prasertsan and Prasertsan, 1996; Klitsaineepaibon and Bungkong, 1990).

2.2.3 (c) Food and nutrition

PPF is separated from mesocarp and kernel when FFB was pressed under high pressure to extract the crude oil. The separated fibrous residue contains high amount of oil cells as well as significant amounts of water-soluble and water-

insoluble fibres (Diemer *et al.*, 2005). Both the oil and fibres components from PPF are useful in food and nutrition industry.

Carotenoids are undoubtedly among the widespread and important pigments in living organisms. It can be used as pro-vitamin A and antioxidants. Researches were started since 1997 to extract the oil from residual fiber from palm oil production and found that more than 5% of original oil was retained in the fiber, extracting about 5000 ppm of carotenoids using supercritical CO₂ (Franca and Meireles, 1997). These efforts were further convinced by Choo *et al.*, (1996)'s work by extracting 4000-6000 ppm of carotenoids, 1400-3500 ppm of vitamin E, and sterols 4500-8500 ppm. Subsequent efforts were continued by Han *et al.*, (2006) by using supercritical fluid chromatography to separate coenzyme Q₁₀ from PPF with concentration up to 1000-1500 ppm.

The de-oiled PPF contains high amount of the most desirable sources of dietary fibre (soluble fibre), which can aid in preventing cardiovascular disease, colon cancer and reducing cholesterol (Anderson *et al.*, 2011; Diemer *et al.*, 2005). It has been an alternative raw material for high fibre white bread production instead of whole-wheat / whole grain flour, giving a fibre content of 6.1 g per 100 g serving (Kamarunzaman, 2008). These efforts have opened another pathway for PPF maximum utilization by retrieving some value-added products from both the oil and fibre content of PPF.

2.2.3 (d) Activated carbon

Demand of activated carbon (AC), especially activated wood charcoal is expected to rise sharply in the coming years, largely due to the expansion of pharmaceutical and vegetable oil industries. Activated carbon is a very important

chemical widely employed by certain gases and vapors in purification of catalytic chemical reactions and de-colorization of vegetable oils and sugar solutions.

Since 1998, lots of researches were done on converting palm kernel shell (PKS) into high-quality activated carbon for gas-phase adsorption applications (Hoy and Puad, 2007; Mohd. Din *et al.*, 2005; Wan Daud *et al.*, 2003; Lua and Guo, 1998) due to the characteristic of PKS with high volatile and low ash (2.96%) (Mohd. Din *et al.*, 2005; Wan Rashidah *et al.*, 2000). Till recently, attention has been drawn on oil palm fibers activated carbon for dye adsorption (Hameed *et al.*, 2008; Tan *et al.*, 2007) and metal removal (Isa *et al.*, 2008; Low *et al.*, 1993) by the treated fibers. Satisfactory result with dye adsorption capacity as high as 400 mg/g was achieved by Hameed *et al.*, (2008), showing that the usage of PPF as activated carbon is feasible.

2.2.3 (e) Application as bioresources

As a lignocellulosic material, PPF contains significant amount of cellulose, hemicellulose and lignin as summarized in Table 2.1, Section 2.1. With the increasing amount of world population, the demands for energy also rise exponentially. The depletion of natural fossil fuel resources has urged the scientist to develop a renewable energy to reduce the fuel dependency and/or to replace the fossil fuel (non-renewable energy resources) as the main energy resources. Lignocellulosic materials have been the most popular feedstock for renewable energy production as they are abundantly available on earth.

Since 1997, researches on utilization of PPF as bioresources for biofuel (bioethanol) production have been carried out. The degree of biofuel production is measured by the amount of glucose (which can be further fermented into bioethanol) recovered through enzymatic saccharification and/or fermentation of cellulose

(Subkaree, 2007; Yeoh and Lim, 2000). Subkabree, (2007) was able to obtain 39.5% of digestibility (18.22 g/L of glucose recovery) after hydrolysis with cellulase from *Trichoderma reesei* and β -glucosidase from *Aspergillus niger*, yielding 10.38 g/L ethanol. Another application in the PPF cellulose was carried out by Mashitah et al., (1997) to produce cellulase from POME and PPF cultivation by mixed and axenic culture of *T. reesei* and *A. niger*, obtaining the maximum production of 3.81 IU/mL CMCase, 1.928 IU/mL FPase and 0.310 IU/mL β -glucosidase, respectively. The work was further improved by Teoh and Mashitah, (2010), achieving 50.11 IU/mL of CMCase, 29.01 IU/mL of FPase and 2.49 IU/mL of β -glucosidase, by using pre-sterilized PPF with POME cultivation by *Pycnoporus sanguineus*. Besides cellulose, hemicellulose content in PPF is also gaining more attention as it can be converted into xylose which can be converted to glucose as well. The optimization condition for furfural (mainly xylose) extraction from hemicelluloses from delignified PPF was examined by Rainsa-ngawong and Prasertsan (2011) by using response surface methodology and up to 12.58 g/L of xylose production was achieved.

The literatures have revealed PPF's viability as a bioresources for renewable energy production. Unlike other agricultural crops, PPF, a non-edible source, is a waste that generated bountifully from palm oil production. Therefore, the utilization of PPF as a feedstock for renewable energy would not trigger any food-fuel feud. However, same as other lignocellulosic materials, pretreatment of PPF is a vital primary step before any bioresources conversion due to the lignin content that served as a protective shield which lowers the enzyme activity or microbial activity toward the cellulose (Kuhad and Singh, 2007). Thus, it is important to study the best pretreatment or delignification method for PPF in order to increase the susceptibility of cellulose for enzymatic or microbial action.

2.3 Lignocellulosic materials

A successful commercial enzymatic hydrolysis process must be cost effective. Among various biomass materials, lignocellulosic biomass have been looked on as a promising feedstock due to their abundance, cheapness, and huge potential availability (Kang *et al.*, 2004). Meanwhile, the aerobic cellulolytic bacteria and fungi with high cell yields have also arouse the technological interest in producing microbial cell protein from waste lignocelluloses (Lynd *et al.*, 2002). There are a lot of researches that have been carried out to synthesize cellulase with varying degree of success from various pre-treated lignocellulosic biomass such as corn fibre (Liming and Xueliang, 2004; Vlaev *et al.*, 1997), wheat and rice straw (Kang *et al.*, 2004; Romero *et al.*, 1999), spruce and willow (Juhasz *et al.*, 2005), as well as oil palm residues fibres (Teoh and Mat Don, 2011; Teoh and Mashitah, 2010; Umikalsom *et al.*, 1998; Mashitah *et al.*, 1997; Umikalsom *et al.*, 1997).

2.3.1 Nature of lignocellulosic material

Lignocellulose is the primary building block of plant cell walls which supports and provides structure for plants. It is composed of three major components: cellulose, hemicellulose and lignin. Different lignocellulosic materials possessed different portion of the constituents as mentioned previously (Table 2.4). Basically, cellulose microfibrils are sheltered in the lignocellulose matrix.