UNIVERSITI PUTRA MALAYSIA

LIGNIN PRETREATMENT OF OIL PALM EMPTY FRUIT BUNCH USING LIGNINOLYTIC ENZYME-MEDIATOR AND CELLULOSE HYDROLYSIS FOR FERMENTABLE SUGAR PRODUCTION

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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March 2016

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Lignocellulosic biomass is the source of cellulosic materials which leads to the fermentable sugars productions. The position of Malaysia as among the top producers and major exporter of palm oil generates abundant of palm oil biomass particularly oil palm empty fruit bunch (OPEFB). Conversion to value added products of such organic acid, compost, bioenergy and enzymes besides sugars which is the key step for most of the processes may overcome the issues in future wastes management.

Locally isolated fungus namely *Pycnoporus sanguineus* was found to be the best ligninolytic enzyme producer among the 20 fungi screened on dyed agar plate. Decolorization of *Remazol Brilliant Blue* dye added to agar media by the fungi showed the ability to secrete ligninolytic enzyme and further profiling resulted in the production of laccase as the major enzyme followed by manganese peroxidase and lignin peroxidase with the least activities.

Physical and chemical structural and compositional particularly lignin acts as a barrier to the enzymatic hydrolysis of cellulose. Appropriate pretreatment to remove lignin is necessary to ensure the access of cellulases enzyme to the cellulosic material embedded in the lignocellulosic matrix. In this study, the application of crude ligninolytic enzyme extracts alone from locally isolated fungi namely *Pycnoporus sanguineus* to the oil palm empty fruit bunch as a biological pre-treatment was able to produce 19 g/L of fermentable sugars during enzymatic hydrolysis using commercial cellulase and was increased up to approximately 30 g/L with the addition of combination mediator of HBT-Mn (II) and ABTS-Mn (II). Based on Klason lignin determination, the highest lignin removal was achieved at the concentration of 1.5% HBT, 4 mM ABTS and 2 mM manganese ion as much as 8.02%, 8.68% and 3.7%, respectively as compared to raw OPEFB. Klason lignin was also removed by as much as 8.8% at 50 ℃ and 8.16% at 10% of substrate loading. Supported results on the FTIR and GC-MS analysis showed the changes in the structure and chemical bonds of the

biologically treated OPEFB thus conclude that there are some modification occurred during the pre-treatment.

Cellulase is the key enzyme for the cellulose hydrolysis producing fermentable sugars. The effect of cultivation condition of two locally isolated ascomycetes strains namely *Trichoderma asperellum* UPM1 and *Aspergillus fumigatus* UPM2 were compared in submerged and solid state fermentation. Physical evaluation on water absorption index, solubility index and chemical properties of lignin, hemicellulose and cellulose content as well as the cellulose structure on crystallinity and amorphous region of treated oil palm empty fruit bunch (OPEFB) (resulted in partial removal of lignin), sago pith residues (SPR) and oil palm decanter cake (OPDC) towards cellulases production were determined. Submerged fermentation shown significant cellulases production for both strains in all types of substrates. Crystallinity of cellulose and its chemical composition mainly holocellulose components was found to significantly affected the total cellulase synthesis in submerged fermentation as the higher crystallinity index and holocellulose composition will increase cellulase production. Treated OPEFB was apparently induced the total cellulases from *Trichoderma asperellum* UPM1 and *Aspergillus fumigatus* UPM2 with 0.66 U/mg FPase, 53.79 U/mg CMCase, 0.92 U/mg β-glucosidase and 0.67 U/mg FPase, 47.56 U/mg and 0.14 U/mg β-glucosidase, respectively. Physical properties of water absorption and solubility for OPEFB and SPR also had shown significant correlation on the cellulases production.

The competency of crude cellulase cocktail from both isolates of *Trichoderma asperellum* UPM1 and *Aspergillus fumigatus* UPM2 were mixed at 3:2 ratio and applied on the enzymatically treated OPEFB from local isolate were compared with the commercial cellulase and the result obtained was 30% cellulose hydrolysis percentage for ligninolytic-mediator pretreatment and 44% using commercial cellulase. Regardless of the lower individual cellulase from local isolates compared to commercial cellulase. It is therefore suggested that biological approaches alone using ligninolytic enzyme-mediator as pretreatment and cellulase enzymes produced locally had a promising potential for fermentable sugar productions for OPEFB.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

PRARAWATAN LIGNIN TANDAN KOSONG KELAPA SAWIT MENGGUNAKAN KOMBINASI ENZIM LIGNINOLITIK-PERANTARA DAN HIDROLISIS SELULOSA UNTUK PENGHASILAN GULA FERMENTASI

Oleh

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Sisa buangan lignoselulosa adalah sumber bahan selulosa dimana ia membawa kepada penghasilan gula fermentasi yang sangat bernilai. Kedudukan Malaysia sebagai pengeluar dan pengeksport terbesar minyak kelapa sawit di dunia juga membawa kepada lambakan sisa kelapa sawit terutamanya tandan kosong kelapa sawit. Sisa tandan kosong hampas kelapa sawit ini akan menghasilkan produk yang boleh ditambah nilai seperti asid organik, baja, bio-tenaga dan enzim dan sumbangan yang paling besar adalah penghasilan gula fermentasi yang menjadi tunggak utama kepada proses-proses penghasilan produk sampingan yang mempunyai nilai yang tingi dalam mendepani isu pengurusan sisa terbuang di masa hadapan.

Kulat yang telah dipencilkan yang diberi nama *Pycnoporus sanguineus* merupakan antara kulat yang menghasilkan jumlah enzim ligninolitik terbanyak diantara 20 kulat yang disaring melalui agar yang diwarnakan. Pewarna *Remazol Brilliant Blue* yang digunakan akan dinyah warnakan oleh kulat yang berpotensi menghasilkan enzim lignolitik. Profail yang dijalankan menunjukkan enzim laccase adalah enzim yang terbanyak dihasilkan di ikuti oleh manganese peroksida manakala lignin peroksida merupakan enzim paling sedikit dihasilkan.

Struktur fizikal, kimia dan juga komposisi lignin yang terdapat di dalam bahan lignosellulosa merupakan antara faktor yang menghalang hidrolisis sellulosa. Oleh itu, pra-rawatan yang sesuai adalah perlu untuk menyingkirkan lignin dan seterusnya membenarkan enzim sellulase bergerak menuju ke sellulosa polimer. Di dalam kajian ini, larutan enzim ligninolitik yang di ekstrak dari kulat yang dipencilkan iaitu *Pycnoporus sanguineus* diaplikasikan ke atas tandan kosong kelapa sawit sebagai pra-rawatan biologi pilihan. Pra-rawatan ini mampu menghasilkan 19 g/L gula fermentasi semasa proses hidrolisis menggunakan sellulase komersil dan nilainya meningkat apabila larutan enzim lignolitik tersebut di tambah dengan perantara iaitu HBT-Mn(II) dan ABTS-Mn(II) kepada 30 g/L. Merujuk kepada Klason lignin yang ditentukan,

lignin paling banyak disingkirkan pada kepekatan 1.5% HBT, 4 mM ABTS dan 2 mM manganese ion masing-masing sebanyak 8.02%, 8.68% and 3.7%. Klason lignin juga berjaya disingkirkan sebanyak 8.8% pada 50℃ and 8.16% pada 10% of kemasukan substrat. Keputusan yang selari juga didapati melalui analisis data FTIR dan GC-MS dimana terdapat perubahan yang berlaku pada sesetengah ikatan kimia tertentu dan struktur tandan kosong kelapa sawit. Seterusnya dapat disimpulkan terdapat pengubahsuaian yang berlaku sewaktu proses pra-rawatan.

Selulase pula merupakan enzim yang penting dalam penghasilan gula fermentasi. Kesan kulat yang dipupuk dalam dua keadaan berbeza iaitu kaedah terendam dan kaedah permukaan tindakbalas untuk 2 jenis kulat iaitu *Trichoderma asperellum* UPM1 dan *Aspergillus fumigatus* UPM2 untuk melihat keberkesanan kaedah. Penilaian fizikal ke atas indek serapan dan kelarutan, ciri-ciri kimia bagi kandungan lignin, sellulosa dan hemisellulosa juga struktur selulosa (kristaliniti dan amorfus) ke atas substrat yang berbeza iaitu tandan kosong kelapa sawit, hampas sagu dan kek dekanter kelapa sawit ditentukan untuk melihat penghasilan selulase. Kaedah fermentasi terendam menunjukkan prestasi yang baik untuk kedua kulat. Kesan yang siknifikan terhadap penghasilan selulase dikaitkan dengan tahap kristaliniti dan komposisi kimia substrat. Tandan kosong kelapa sawit mampu menghasilkan selulase masing-masing untuk *Trichoderma asperellum* UPM1 dan *Aspergillus fumigatus* UPM2 secara total 0.66 U/mg FPase, 53.79 U/mg CMCase, 0.92 U/mg β-glucosidase and 0.67 U/mg FPase, 47.56 U/mg and 0.14 U/mg β-glucosidase. Daya serapan dan kelarutan subtrat juga berkait rapat dengan penghasilan selulose. Kebolehupayaan koktel selulase untuk *T. asperellum* UPM1 dan *A. fumigatus* UPM2 pada nisbah 3:2 dan diaplikasikan pada tandan kosong kelapa sawit yang di pra-rawat di bandingkan dengan komersil selulase. Keputusan menunjukkan hydrolisis selulosa sebanyak 30% berjaya dicapai berbanding 44% menggunakan selulosa komersil. Dengan mengambil kira faktor aktiviti selulase dari ekstrak kultur kulat yang lebih rendah berbanding komersil selulase, pendekatan biologi ini mempunyai potensi yang boleh ditingkatkan untuk menghasilkan gula fermentasi yang lebih banyak dimasa hadapan.

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I certify that a Thesis Examination Committee has met on 15 March 2016 to conduct the final examination of Zuraidah binti Zanirun on her thesis entitled "Lignin Pretreatment of Oil Palm Empty Fruit Bunch using Ligninolytic Enzyme Mediator and Cellulose Hydrolysis for Fermentable Sugar Production" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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This is to confirm that:

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LIST OF ABBREVIATIONS

ABTS	2,2`-azinobis-(3-ethyl-)benzthiazoline-6-sulphonate
DW	Distilled water
FTIR	Fourier-transform infrared spectroscopy
MnP	Manganese peroxidase Lac - Laccase
LiP	Lignin peroxidase
VA	Veratryl alcohol - 3,4-dimethoxybenzyl alcohol
OPEFB	Oil palm empty fruit bunch
BSA	Bovine serum albumin
CBH	Cellobiohydrolase
DNS	Dinitrosalicylic acid
FPase	Filter paper cellulase
H_2SO_4	Sulfuric acid
SSF	Solid state fermentation

CHAPTER 1

INTRODUCTION

Malaysia is blessed with a variety of agriculture resources. Being the most important agricultural country with the exports of oil palm, rubber and cocoa, Malaysia generates a substantial amount of biomass specifically in the oil palm industries with estimated amount of 33 million tons of crop residues in the form of oil palm empty fruit bunch (OPEFB), palm kernel shell (PKS) and palm fruit fibre (PFF). Out of these biomass, OPEFB was considered as primary feedstock with 19.5 million tons in 2008 (Omar et al., 2011) generated daily. As part of wealth creation strategy, government support to the use and its utilization and the given incentive has made it possible to be implemented. With this, it will promotes the emerging of new technology in Malaysia creating approximately 70,000 jobs in the future and enhancing the development of rural areas (Malaysia Agensi Inovasi, 2011). Besides, utilizing the biomass wastes could decrease the detrimental impact on the environment by reduction of carbon emission which led to global warming.

Due to their abundant, locally available, non-food sources and inexpensive, the exploitation of the biomass particularly OPEFB could generate further income by the production of value added products. It has been researched by many local researcher that the biomass can be further used as a carbon source for fermentable sugars as the main product for latter application such as biofuel and organic acid production. However, there is limitation since OPEFB biomass is complicated in structure. The basic fundamental of the utilization of biomass arise from the knowledge deals with the rigid structure of lignin and involvement of cellulose and hemicellulose containing disaccharide and monosaccharide. Theoretically, lignin gave a strong structure to plants whilst the component of cellulose and hemicellulose were scaffold in between. Thus, the utilization of cellulose and hemicellulose was blocked by the present of lignin. Therefore, pretreatment is a prerequisite to allow the access of specific enzyme to the targeted component of polysaccharide of sugars. The goals of pretreatment have changed from the lignin removal (as much is possible) in the beginning, to a modification of lignin for easier downstream processing. For this purpose, the responsible enzyme was classified into two categories which is ligninolytic enzyme (lignin peroxidase, laccase and manganese peroxidase) to remove and modify lignin and cellulolytic enzyme (FPase, CMCase and β-glucosidase) to convert cellulose and hemicellulose into fermentable sugars. All of the enzymes were secreted in nature by the ascomycetes and basidiomycete's species of fungi.

A lot of researches had incorporated the use of fungi in the process to achieve a specific target of partial lignin removal or modification. At one stage, the incubation time has to be extended to more than 30 days to attain a satisfactorily lignin removal and it is therefore an obstacle that limits its biotechnological application. Due to time consuming factor, the needs to find an alternative approach surge drastically which led

to the free fungal culture application with lesser hassle of dealing with fungal mycelia, shorter incubation time and reduce the consumed fraction of polysaccharide.
Locally isolated fungi of *Trichoderma asperellum* UPM 1 and *Aspergillus fumigatus* UPM2 was firstly isolated by Abu Bakar et al. (2010). It has been proven to be the potential fungi for cellulase enzyme and fermentable sugars production using OPEFB (Nurul Kartini et al., 2012; Ibrahim et al., 2013). Therefore, further exploration on the improvement of cellulase enzyme and the effect of physical and chemical characteristic of different biomass on cellulase enzyme productions was carried out using both fungi as different biomass properties triggered cellulase composition differently.

Through this study, the target is to fulfil the gaps in bringing an environmentally friendly approach by using ligninolytic enzyme mediator system as a pretreatment for OPEFB lignin removal with the addition of mediators compound to improve the efficiency and followed by the actions of crude cellulolytic enzyme for the production of sugars which both produced by local isolates as an approach to reduce the enzyme cost and to replace the current practices of using list of chemical catalyst such as alkaline and acid as a favourite choices. Mediators are the low molecular weight compounds which covers all the necessary compound besides the enzyme (Call & Mucke, 1997) and its role is very significant to enhance lignin modification and removal (Cho et al., 2008; Elegir et al., 2005; Rico et al., 2014). Therefore, this study focused on the feasibility of using ligninolytic enzyme as a pretreatment to modify and remove OPEFB lignin and subsequent enzymatic hydrolysis for fermentable sugars production in which all the enzyme was produced from locally isolated fungi. In fact, crude enzyme was used throughout the study.

The overall objective of the study was to obtain the lignocellulolytic enzymes with the capability to degrade lignin and hydrolyse the carbohydrate consists of cellulose and hemicellulose for the production of fermentable sugars. The specific research objectives were as follow;

1. To select the most potential isolates producing ligninolytic enzyme for lignin removal
2. To enhance cellulose hydrolysis of OPEFB using ligninolytic enzyme mediator system as pretreatment
3. To enhance cellulases enzyme production by *Trichoderma asperellum* UPM1 and *Aspergillus fumigatus* UPM2 using different biomass as carbon sources
4. To evaluate the feasibility of ligninolytic enzyme-mediator as pretreatment on the cellulose hydrolysis of pretreated OPEFB using crude lignocellulolytic enzyme cocktail for fermentable sugars production

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