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BIOLOGICAL PRETREATMENT OF OIL PALM EMPTY FRUIT BUNCH USING CRUDE LACCASE FROM Pycnoporus sanguineus FOR SUGAR PRODUCTION

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By

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BIOLOGICAL PRETREATMENT OF OIL PALM EMPTY FRUIT BUNCH USING CRUDE LACCASE FROM *PYCNOPORUS SANGUINEUS* FOR SUGAR PRODUCTION

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Chair : Professor Ts. Suraini binti Abd Aziz, PhD
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To date, Malaysia rank in the second place as a global palm oil producer. Despite the rapid growth of oil palm plantation in Malaysia, a non-systematic biomass management contributes to biomass accumulation in huge amount. Oil palm empty fruit bunch (OPEFB) can be categorized as one of the toughest lignocellulosic biomass to be degraded naturally due to its complexity in structure. Conventional industrial practice used chemical and physical pretreatments to treat the OPEFB as it performs faster in hydrolyzing the biomass than biological pretreatment does. However, as the world is moving towards green technology concept, chemical pretreatment is no longer suitable to be practiced because it produces harmful by-products which requires proper management prior to its disposal. One of the methods currently sparking interest is biological pretreatment using laccase. In this study, OPEFB was pretreated biologically using crude laccase and subjected to enzymatic hydrolysis using cellulase to produce sugars. Application of laccase has become a current trend in biological pretreatment of lignocellulosic biomass where laccase aids in degrading and modifying the lignin barrier. This condition subsequently loosens up the biomass structure and helps to improve the cellulase accessibility towards cellulose.

Naturally, wild type fungi produce ligninolytic enzymes in low concentration and inducers have shown promising result in enhancing ligninolytic enzymes production. *Pycnoporus sanguineus* UPM4 was utilized in this study and was found to be a dominant laccase producer. Therefore, enhancement using selected laccase inducers which are veratryl alcohol, ferulic acid, Kraft lignin, copper sulfate and 2,5-xylidine were carried out at different concentration, respectively. Veratryl alcohol, ferulic acid and Kraft lignin were found to be able to enhance the laccase production, meanwhile copper sulfate and 2,5-xylidine inhibited the laccase production. Veratryl alcohol with a concentration of 16 mM

has shown to be to best inducer with resulting laccase production of 6.35 U/mL (2 folds increment).

Response surface methodology (RSM) was employed to optimize the biological pretreatment of OPEFB using enhanced crude laccase synthesized by *P. sanguineus* UPM4. Investigation and screening of significant variables was performed using one factor at time (OFAT) and two-level factorial design. From the analysis of variance (ANOVA), temperature, initial pH and laccase loading have resulted in major effect on the pretreatment process, whereas substrate concentration and incubation time were found to be insignificant. These three variables were further analyzed using Central Composite Design (CCD). The optimum pretreatment condition attained from the model were temperature at 50°C, initial pH of 4.5 and 65 U/g of laccase loading. Hence, biological pretreatment performed at this optimum condition resulted in 13.08% of lignin removal and yielded 20.70 g/L of sugars from pretreated OPEFB.

Additionally, characterization of pretreated OPEFB has revealed a remarkable change occurred on the substrate which was evident through scanning electron microscope micrograph, surface functional groups and surface oxide groups. Scanning electron microscope micrograph displayed formation of craters on substrate surface due to removal of silica bodies on the pretreated OPEFB. Next, an alteration of surface functional groups on the pretreated OPEFB was demonstrated by FTIR spectrum and it was further explained by surface oxide groups analysis showing an increment of carboxyl groups and decrement of lactone and phenolic groups in the pretreated OPEFB.

A study on the feasibility of simultaneous pretreatment and saccharification of OPEFB was carried out. It was observed that cocktail of crude laccase at 45 U/g and Acremonium cellulase at 25 FPU/g has resulted in 8.81% of lignin removal and 8.16 g/L of sugars. As a conclusion, veratryl alcohol has increased the laccase production with 2 folds increment. Optimization of biological pretreatment of OPEFB using crude laccase has increased the lignin removal by 1.2 folds and sugars production by 1.8 folds. Besides that, simultaneous pretreatment and saccharification of OPEFB using cocktail of crude laccase and Acremonium cellulase were a good combination as compared to *Trichoderma reesei* crude cellulase and Celluclast 1.5L, respectively.

Overall, in comparison to previous research, this study demonstrated two novel approaches of biological pretreatment of OPEFB. Optimum operating conditions in separate pretreatment and saccharification of OPEFB has resulted in 56.63% of hydrolysis yield. Meanwhile, the simultaneous pretreatment and saccharification of OPEFB at laboratory scale proved that lignocellulolytic enzymes cocktail was feasible and able to attain hydrolysis yield at 32.62%, in conjunction with reduction of time consumption, number of vessels as well as elimination of substrate washing step.

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

PRA-RAWATAN BIOLOGI TANDAN KOSONG KELAPA SAWIT MENGGUNAKAN LAKASE MENTAH DARI *PYCNOPORUS SANGUINEUS* UNTUK PENGHASILAN GULA

Oleh

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Malaysia kini berada di kedudukan kedua sebagai pengeluar minyak kelapa sawit terbesar di peringkat global. Dengan perkembangan pesat penanaman pokok kelapa sawit di Malaysia, pengurusan biomas yang tidak sistematik telah menyumbang kepada pengumpulan biomas dalam jumlah yang banyak. Tandan kosong kelapa sawit (TKKS) boleh dikategorikan sebagai salah satu biomas lignoselulosa yang paling sukar untuk diurai secara semulajadi kerana strukturnya yang rumit. Amalan industri konvensional menggunakan pra-rawatan kimia dan fizikal untuk merawat TKKS kerana ia menghidrolisis biomas lebih pantas daripada pra-rawatan biologi. Walau bagaimanapun, apabila dunia beralih ke arah amalan konsep teknologi hijau, pra-rawatan kimia tidak lagi sesuai untuk dipraktikkan kerana ia menghasilkan produk sampingan berbahaya yang memerlukan pengurusan yang baik sebelum pelupusannya. Salah satu kaedah yang menjadi tarikan pada masa kini ialah pra-rawatan biologi menggunakan lakase. Dalam kajian ini, TKKS dirawat secara biologi menggunakan lakase mentah dan hidrolisis berenzim menggunakan selulase untuk menghasilkan gula. Penggunaan lakase telah menjadi tren semasa dalam pra-rawatan biologi biomas lignoselulosa di mana lakase membantu mengurai dan mengubah lignin. Keadaan ini akan merenggangkan struktur biomas dan membantu untuk meningkatkan akses selulase terhadap selulosa.

Secara semulajadi, kulat liar menghasilkan enzim ligninolitik dalam kepekatan yang rendah dan penggalak telah menunjukkan hasil yang signifikan dalam meningkatkan penghasilan enzim ligninolitik. *Pycnoporus sanguineus* UPM4 telah digunakan dalam kajian ini dan ia didapati sebagai penghasil lakase yang dominan. Oleh itu, peningkatan penghasilan lakase menggunakan penggalak lakase yang terpilih iaitu veratril alkohol, asid ferulik, lignin Kraft, kuprum sulfat dan 2,5-xilidin telah dijalankan pada kepekatan yang berbeza. Veratril alcohol, asid ferulik dan lignin Kraft didapati dapat meningkatkan penghasilan lakase,

manakala kuprum sulfat dan 2,5-xilidin menghalang penghasilan lakase. Veratril alkohol dengan kepekatan 16 mM telah menunjukkan sebagai penggalak terbaik dengan menghasilkan lakase sebanyak 6.35 U/mL (peningkatan 2 kali ganda).

Kaedah permukaan tindakbalas (KPT) telah digunakan untuk pengoptimuman pra-rawatan biologi TKKS menggunakan lakase mentah yang disintesis oleh *P. sanguineus* UPM4. Penyaringan pemboleh ubah dilakukan menggunakan kaedah satu faktor satu masa (SFSM) dan rekaan dua aras faktorial. Dari analisis varians (ANOVA), suhu, pH awal dan jumlah lakase telah memberi kesan yang besar terhadap proses pra-rawatan, manakala kepekatan substrat dan masa pengeraman didapati tidak signifikan. Ketiga-tiga pemboleh ubah ini seterusnya dianalisis menggunakan rekaan komposit pusat (RKP). Keadaan pra-rawatan optimum yang diperolehi daripada model adalah pada suhu 50°C, pH awal 4.5 dan 65 U/g jumlah lakase. Oleh itu, pra-rawatan biologi yang dilakukan pada keadaan optimum ini telah menghasilkan 13.08% penyingkiran lignin dan menghasilkan 20.70 g/L gula daripada TKKS yang telah diprarawat.

Di samping itu, pencirian TTKS yang telah dirawat telah mendedahkan perubahan yang ketara berlaku pada substrat dan dibuktikan oleh mikrograf mikroskop elektron imbasan, kelompok berfungsi permukaan dan kelompok oksida permukaan. Mikrograf mikroskop elektron imbasan menunjukkan pembentukan kawah pada permukaan substrat kerana penyingkiran badan silika pada TKKS yang telah dirawat. Seterusnya, perubahan pada kelompok berfungsi permukaan pada TKKS yang telah dirawat ditunjukkan oleh spektrum FTIR dan ia dijelaskan secara lanjut oleh analisis kelompok oksida permukaan yang menunjukkan peningkatan kumpulan karboksil dan penurunan kumpulan lakton dan fenolik dalam TKKS yang telah dirawat.

Kajian mengenai kebolehan pra-rawatan dan pensakaridaan serentak TKKS telah dijalankan. Hasilnya, koktail lakase mentah pada 45 U/g dan Acremonium selulase pada 25 FPU/g telah menghasilkan 8.81% penyingkiran lignin dan 8.16 g/L gula. Kesimpulannya, veratril alkohol telah meningkatkan pengeluaran lakase dengan peningkatan 2 kali ganda. Pengoptimuman pra-rawatan biologi TKKS menggunakan lakase mentah telah meningkatkan penyingkiran lignin sebanyak 1.2 kali ganda dan penghasilan gula sebanyak 1.8 kali ganda. Selain itu, pra-rawatan dan pensakaridaan serentak TKKS menggunakan lakase mentah dan Acremonium selulase. adalah gabungan yang baik berbanding *Trichoderma reesei* selulase dan Celluclast 1.5L, masing-masing.

Keseluruhannya, berbanding dengan kajian terdahulu, kajian ini menunjukkan dua kaedah baru untuk pra-rawatan biologi TKKS. Keadaan operasi yang optimum dalam pra-rawatan dan pensakaridaan berasingan TKKS telah menghasilkan 56.63% hasil hidrolisis. Sementara itu, pra-rawatan dan pensakaridaan serentak TKKS pada skala makmal membuktikan bahawa koktail enzim lignoselulolitik dapat mencapai hasil hidrolisis pada 32.62%, di samping dengan pengurangan penggunaan masa, jumlah bekas serta penyingkiran langkah mencuci substrat.

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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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%	Percent
°C	Degree Celsius
µm	Micrometer
ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
$CaCl_2.2H_2O$	Calcium chloride dihydrate
$CaCO_3$	Calcium carbonate
CCD	Central Composite Design
cm	Centimeter
$CuSO_4.5H_2O$	Copper sulfate pentahydrate
DNS	3,5-Dinitrosalicylic acid
ε	Epsilon
$FeSO_4.7H_2O$	Iron sulfate heptahydrate
FPU	Filter Paper Unit
g	Gram
g/L	Gram per liter
HBT	1-Hydroxybenzotriazole
H_2SO_4	Sulfuric acid
keV	Kilo electron Volt
kg	Kilogram
KH_2PO_4	Potassium dihydrogen phosphate
kHz	Kilohertz
M	Molarity
$MgSO_4.7H_2O$	Magnesium sulfate heptahydrate
mL	Mililiter

mm	Milimeter
mM	Milimolar
Na_2HPO_4	Sodium hydrogen phosphate
NH_4NO_3	Ammonium nitrate
nm	Nanometer
OPEFB	Oil palm empty fruit bunch
RPM	Rotation per minutes
SEM	Scanning electron microscope
$ZnSO_4.7H_2O$	Zinc sulfate heptahydrate

CHAPTER 1

INTRODUCTION

1.1 Background study

Palm oil industry is listed as one of the 12 National Key Economic Areas (NKEAs) as announced in the Tenth Malaysia Plan (PEMANDU, 2012). NKEA is the new strategy introduced by government as an important driver of economic activities that directly contributes towards the Malaysian Economic Growth measurable by the National Gross Income (GNI) indicator. Oil palm cultivation in Malaysia has started since 1960s in relation with the government's effort to broaden the crops variety under agricultural diversification program. The expansion of oil palm cultivation results in the increase of biomass being generated from the palm oil industry.

Oil palm empty fruit bunch (OPEFB) is the most abundant oil palm biomass produced in palm oil mil where one metric ton of fresh fruit bunch (FFB) generates 23% (230 kg) OPEFB compared to 14% (140 kg) oil palm mesocarp fibers and 7% (70 kg) palm kernel shells. The OPEFB is usually returned to plantation as mulching agent for nutrient recycling (Omar *et al.*, 2011). However, the drawbacks of this practice are temporary immobilization of a huge pile of nutrients, interference in fruit harvesting process, snakes and rats infestation, increased risk of fire and provides breeding site for rhinoceros beetles (*Oryctes rhinoceros*), a problematic oil palm pest that cause serious damage to young oil palm trees (Stichnothe and Schuchardt, 2010; Sunitha and Varghese, 1999). Hence, this situation needs an urgent and systematic action to curb the problem.

Some of the solution suggested for the problem at hand is to utilize OPEFB as biofertilizer (Razali *et al.*, 2012), raw materials for pulp and paper making (Megashah *et al.*, 2018) and fermentation feedstock for second generation biofuel production (Ibrahim *et al.*, 2012). However, utilization of OPEFB as a fermentation feedstock is challenging due to recalcitrant property of lignin. Lignin is one of the components in plant biomass besides cellulose and hemicellulose. If biomass is directly used as feedstock for fermentation, lignin acts as a physical barrier to prevent enzyme access to the carbohydrates fraction of biomass (Sindhu *et al.*, 2016). Moreover, a demand for higher enzyme loading is required due to the non-productive binding between cellulase and lignin (Yang *et al.*, 2011).

Therefore, pretreatment process is necessary to reduce the recalcitrance of the lignocellulosic biomass through lignin degradation and modification. Biological pretreatment using ligninolytic enzymes has been receiving much attention in recent years for their valuable enzyme system that show effective degradation on the lignocellulosic biomass (Zanirun *et al.*, 2015). In nature, various types of fungi often seen to grow and colonize on dead woody material which is a part of natural decaying mechanism. The ligninolytic enzymes secreted by the fungi are responsible for the decaying process (Isroi *et al.*, 2011). Ligninolytic enzymes which are commonly studied are laccase, manganese peroxidase, lignin peroxidase and versatile peroxidase (Yadav and Yadav, 2015).

Presently, the lignocellulosic biomass is physically and chemically treated prior to obtain the sugars from saccharification process. Conventional pretreatment method requires large consumption of chemicals and energy. In consequence, these processes procure exorbitantly high cost (Alvira *et al.*, 2010). To alleviate this matter, biological pretreatment using ligninolytic enzymes is proposed as a promising alternative pretreatment method. Limited research has been done on the biological pretreatment using enzyme where the potential of the ligninolytic enzymes is less explored. There are several limitations that need to be tackled and improved accordingly in order to develop a full cycle of lignocellulosic biomass conversion into sugars using biological pretreatment approach. It is only logical that further research was carried out on the feasibility of using ligninolytic enzymes to pretreat biomass prior to fermentation, and the effect of other parameters such as temperature, enzyme loading, initial pH and use of mediators in the pretreatment process.

Besides the use of enzyme to pretreat lignocellulosic biomass, research on simultaneously treating and producing sugars from lignocellulosic biomass is practically scarce. Keeping in the outlook of biotechnological applications of laccase, a new approach of combining laccase and cellulase in one vessel was presented in this thesis. Current research involves simultaneous use of lignocellulolytic enzymes such as laccase and xylanase mainly in improving pulp and paper production where recovery of cellulose is vital (Woolridge, 2014). This research point of view hampers the development of simultaneous pretreatment and saccharification processes. Due to success in simultaneous treatment using enzyme cocktail of laccase and xylanase, it is theorized that similar process using laccase and cellulase is possible. This will greatly improve the efficiency of the process and reduce the number of steps needed to produce sugars from lignocellulosic biomass. Hence, simultaneous pretreatment and saccharification is suggested to be one of the alternative methods in lignocellulosic biomass conversion into sugars.

1.2 Problem statements

Low laccase activity affects the performance of biological pretreatment and in term of laccase production, each fungus act differently towards different types of inducers. Therefore, a study on the enhancement of laccase production through the addition of inducers was carried out to improve the laccase production. Next, challenge in utilizing OPEFB to obtain sugars is due to the presence of lignin that prevents enzyme access to the carbohydrates fraction of biomass.

Low lignin removal in biological pretreatment subsequently contributes to low sugars production. Besides that, there are limited studies on parameters influencing biological pretreatment of lignocellulosic biomass. Thus, biological pretreatment needs to be optimized to understand better of the process by investigating the parameters such as temperature, initial pH, laccase loading, substrate concentration and incubation time which subsequently leads to high sugars production.

Furthermore, conventional separate pretreatment and saccharification requires series of sequential steps starting from pretreatment, followed by substrate washing and then saccharification of pretreated substrate. Little knowledge was available on the development of lignocellulolytic enzymes cocktail ratio as well as its compatibility. In conjunction to that, simultaneous pretreatment and saccharification was carried out to investigate its feasibility for potential use in future implementation.

1.3 Significance of study

Application of laccase in biological pretreatment was being explored further in conversion of lignocellulosic biomass into sugars. An enhancement of laccase production which plays crucial role in biological pretreatment has been carried out in order to obtain high laccase production prior to be used in biological pretreatment of OPEFB. A study on optimization of biological pretreatment has demonstrated an increment of lignin removal and sugars production, in which it concomitantly offered an alternative pretreatment method option instead of conventional pretreatment technology that used high energy and chemical. Moreover, this study has unveiled the potential of simultaneous pretreatment and saccharification process because it can be conducted within the same vessel, which makes the process easier, significantly reduces the treatment time and energy consumption as well as it eliminates step for pretreatment hydrolysate management and substrate washing. Overall, exploitation of the massive amount of lignocellulosic biomass generated from crops and agriculture activity into higher value downstream uses was in coherent with the National Biomass Strategy 2020. Therefore, this study subsequently contributes in achieving the aim of the proposed policy to create wealth from lignocellulosic biomass.

1.4 Scope of study and objectives

This study focuses on optimization of biological pretreatment of OPEFB using crude laccase produced by *Pycnoporus sanguineus* UPM4 in order to obtain the sugars from OPEFB. On top of that, a feasibility study on simultaneous pretreatment and saccharification of OPEFB at laboratory scale was performed by utilizing crude laccase and cellulase simultaneously instead of conventional separate pretreatment and saccharification.

The objectives of this study are:

1. To enhance the production of laccase by *Pycnoporus sanguineus* UPM4 using selected inducers.
2. To optimize the parameters for biological pretreatment of oil palm empty fruit bunch using one factor at time (OFAT) and Response Surface Methodology (RSM) for sugars production.
3. To prove the feasibility of simultaneous biological pretreatment and saccharification of oil palm empty fruit bunch for sugars production.

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