CELLULASES AND XYLANASE PRODUCTION BY *Aspergillus fumigatus* SK1 THROUGH SOLID STATE FERMENTATION FOR ETHANOL FERMENTATION

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SPECIALLY DEDICATED TO MY BELOVED DAD AND MUM

“THANKS FOR ALL SUPPORT AND UNDERSTANDING”

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ABSTRACT

Direct utilization of oil palm trunk (OPT) without chemical pretreatment for cellulases and xylanase production under solid state fermentation (SSF) was conducted in batch culture. A total of 12 fungal strains from Biorefinery Laboratory collections and 5 strains isolated from wooden board were able to secrete cellulases and xylanase based on the clear zones formed on selective agar plates. *Aspergillus fumigatus* SK1 showed significant enzymes productivities with the xylanase activity of 648.448 U g^{-1}, CMCase of 48.006, FPase of 6.860, β-glucosidase of 16.328 U g^{-1} and lignin peroxidase of 4.820 U g^{-1}, respectively. Secretion of cellulases and xylanase by *Aspergillus fumigatus* SK1 was further confirmed by zymographic analysis. The crude cellulases-xylanase cocktail was highly stable at temperature lower than 40℃. The optimum temperature for FPase was 60℃ and 70℃ for CMCase, β-glucosidase, and xylanase. Statistical optimization of cellulases and xylanase production was carried out involving General Factorial Design (GFD), 2-Level-Factorial Design (2LFD), and Central Composite Design (CCD). The GFD optimization demonstrated significant improvement of cellulases and xylanase production in medium supplemented with ammonium sulphate. The significant factors for xylanase production were incubation time and temperature, inoculum size, and ammonium sulphate concentration. These factors were optimized through CCD which produced approximately 4.28 fold higher xylanase activity (1792.43 U/g) compared to that before optimization. The enzymes cocktail produced from SSF was successfully applied in saccharification of chemical untreated OPT, producing a hydrolysate containing a maximum of 15.06 g/L reducing sugars after 24 hours incubation at 40℃. Alcoholic fermentation of the hydrolysate by *Candida tropicalis* RETL-Crl and *Saccharomyces cerevisiae* were resulted in release of 3.067 g/L and 3.151 g/L of ethanol, respectively. The higher ethanol productivity (0.263 g/L/h), $Y_{p/s}$ (0.476 g/g) and specific ethanol productivity (0.0947 g/L/h/g of biomass) of *Saccharomyces cerevisiae* showed a great potential to be used in ethanol fermentation process.

ABSTRAK

Pengunaan batang kelapa sawit (OPT) secara terus tanpa prarawatan kimia untuk penghasilan sellulase dan xilanase melalui penapaian keadaan pepejal (SSF) telah dijalankan dalam kultur kelompok. Sebanyak 12 strain kulat daripada koleksi Makmal Biorefinery dan 5 strain kulat dipencilkan daripada papan kayu telah merembeskan sellulase dan xilanase berdasarkan kepada zon yang jelas atas plat-plat agar selektif. *Aspergillus fumigatus* SK1 telah menunjukkan produktiviti enzim signifikan dengan aktiviti xylanase 648.448 U g^{-1}, CMCase 48.006, FPase 6.860, β-glucosidase 16.328 U g^{-1} dan lignin peroksida 4.820 U/g. Rembesan sellulase dan xilanase oleh *Aspergillus fumigatus* SK1 juga telah disahkan oleh analisis secara *zymographic*. Koktel mentah sellulase-xilanase adalah sangat stabil pada suhu yang kurang daripada 40 ℃. Suhu optimum untuk FPase adalah 60 ℃ dan 70 ℃ untuk CMCase, β-glucosidase, dan xilanase. Pengoptimuman statistik penghasilan enzim sellulase and xilanase terlibatkan *General Factorial Design* (GFD), *2-Level-Factorial Design* (2LFD), dan *Central Composite Design* (CCD) telah dijalankan. Pengoptimuman GFD menunjukan peningkatan yang ketara untuk sellulase dan xilanase dalam medium yang telah ditambahkan dengan ammonium sulfat. Faktor-faktor signifikan adalah masa pengeraman dan suhu, saiz inokulum, dan kepekatan ammonium sulfat. Faktor-faktor ini telah dioptimumkan melalui CCD untuk menghasilkan aktiviti xilanase yang lebih kurang 4.28 ganda (1792.43 U/g) lebih tinggi daripada keadaan sebelum pengoptimuman. Koktel enzim yang dihasilkan melalui SSF telah berjaya digunakan untuk sakarifikasi OPT tanpa rawatan untuk menghasilkan hidrolisat yang mengandungi 15.06 g/L gula penurun selepas dieram pada 40 ℃ sebanyak 24 jam. Penggunaan hidrolisat tertentu untuk fermentasi alkohol telah dijalankan oleh *Candida tropicalis* RETL-Crl dan *Saccharomyces cerevisiae* dan menghasilkan sebanyak 3.067 g/L dan 3.1515 g/L etanol. Produktiviti etanol (0.263 g/L/h), $Y_{p/s}$ (0.476 g/g) dan produktiviti etanol tertentu (0.0947 g/L/h/g biojisim) yang tinggi telah diperolehi daripada *Saccharomyces cerevisiae* dan ini menunjukkan potensinya untuk digunakan untuk fermentasi etanol.

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LIST OF ABBREVIATION

2LFD	-	Two-factorial design
ANOVA	-	Analysis of Variance
BSA	-	Bovine Serum Albumin
CCD	-	Central Composite Design
CMC	-	Carboxymethyl cellulose
CMCase	-	Carboxymethyl cellulase
DNS	-	Dinitrosalicyclic acid
FESEM	-	Field Emission Scanning Electron Microscope
FID	-	Flame Ionized Detector
FPase	-	Filter Paper culture enzyme
FTIR	-	Fourier Transform Infrared Spectroscopy
g	-	Gram
GC	-	Gas Chromatography
GFD	-	General Factorial Design
H_2SO_4	-	Sulphuric acid
HCl	-	Hydrochloric acid
H_2O_2	-	Hydrogen Peroxides
HNO_3	-	Nitric acid
HPLC	-	High Performance Liquid Chromatography
kDa	-	Kilo Dalton
L	-	Liter
min	-	Minute
mL	-	Milliliter
mm	-	Millimeter
MW	-	Molecular Weight
NaOH	-	Sodium hydroxide
N/A	-	Not applicable

nm	-	Nanometer
^{o}C	-	Degree Celsius
OPT	-	Oil Palm Trunk
PAGE	-	Polyacrylamide Gel Electrophoresis
PDA	-	Potato Dextrose Agar
pNPG	-	p-nitrophenyl β-D-glucoside
RID	-	Refractive Index Detector
RSM	-	Response Surface Methodology
SDS	-	Sodium Dodecyl Sulfate
U/g	-	Unit of enzyme per gram
v/v	-	Volume per volume
w/v	-	Weight per volume
μL	-	Micro liter
μm	-	Micro meter

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CHAPTER 1

INTRODUCTION

1.1 Background of Problems

Malaysia is one of the largest palm oil producer in the world with 18.2 million tons of palm oil production in year 2011/2012 (Michael, 2012). In the year 2012, an estimation of 4.56 million hectare land were planted with palm oil trees (Michael, 2012). Normally, after 25 to 30 years, the palm oil trees will be cleared off and replanted with new trees due to decreased yield. It is estimated that the replanting process for each hectare of oil palm trees produces about 66 tonnes of palm trunks and 14.4 tonnes of fronds (Lim, 1986). Therefore, according to some research, the replanting process can generate million tonnes of oil palm biomass including trunks, fronds, and empty fruit bunches (EFB) annually.

Within these few decades, environmental issues and pollutions caused by agriculture wastes have gained public concerns, which in turn have boosted more researches on technologies that promote the reuse of these wastes as alternative materials for commodities production, particularly for chemical, energy and food applications. Most of these commodities are in fact more economical since they require less production energy (Nigam and Pandey, 2009). Due to these reasons, these readily available renewable and free resources have the potential to be transformed into value-added products such as biofuels, biochemical, biopesticides, biopulp, biobleach, biopromoters, and biofertilizer (Nigam and Pandey, 2009). Furthermore, the enforcement of zero burning and strict pollutants diminishing policies is forcing the industries to mitigate the disposal of these biomasses.

Oil palm trunk is a lignocellulose containing waste that is rich in cellulose, hemicellulose, and lignin. The cellulose and hemicellulose are known as reservoir of fermenting sugars due to their structures, remarked as polymers fractions of sugars. Degradation of this complex biomass into monomeric sugars requires a complete multiple hydrolytic enzyme cocktail including cellulases, hemicellulases, and ligninase to act synergistically. Yet, this degradation process can be prevented or limited in natural circumstances due to the existence of robust lignin layers, highly recalcitrant crystalline cellulose, and strong bonding in hemicellulose.

To increase the accessibility of the fibres to enzyme action, some previous works suggested chemical pre-treatment using acid, alkaline or solvents. However, these harsh treatments can cause the losses of some valuable sources such as sugars from hemicellulose and cellulose, and the lignin can be degraded into other by-products such as furfural, 5-hydroxymethyl-2 furfural, acetic acid, phenols, heavy metals, levulinic acid, and formic acid (Mussatto and Roberto, 2004) that have inhibition effects on fermentation yields (Mussatto and Teixeira, 2010). Therefore, the ultimate solution to prevent the formation of inhibitors and reduce the dispersion costs of chemical liquid wastes is to minimize the use of pre-treated biomass, and the use of enzyme-catalysed degradation in this case can provide good production yields without generating side products.

Cellulase and xylanase are major enzyme groups responsible for biodegradation of lignocellulosic materials into polyoses. These cellulases, which include endoglucanase, exoglucanase, and β-glucosidase, work synergistically to degrade cellulose. Endoglucanase initiate the catalytic disruption of internal bonds within the cellulose crystalline structure to produce oligosaccharides. Exoglucanase attack non-reducing end of oligosaccharide chains to produce tetrasaccharides or cellobiose (disaccharides), and finally, β-glucosidase complete the hydrolysis process by converting cellobiose fragment into glucose (Miyamoto, 1997). Xylanase is a group of glycosidase enzymes that catalyse the xylanolytic endohydrolysis of 1,4-β-D-xylosidic linkages in xylan, which is the principal constituent of hemicellulose to produce pentose sugars (xylose and arabinose) as well as hexane sugars (galactose and mannose). These in turn become the primary carbon source for cell metabolisms and good substrates for bioethanol and chemicals production (Collins *et al.*, 2005; Bisaria and Ghose, 1981).

Currently, cellulase, xylanase, and pectinase contribute almost 20% of world enzyme market (Polizeli *et al.*, 2005). However, high enzyme production costs and low production yields have hindered its industrial applications (Kang *et al.*, 2004). Cellulase and xylanase can be produced through submerged fermentation (SmF) (Tolan and Foody, 1999) and solid-state fermentation (SSF) (Pandey *et al.*, 1999). In fact, most of the commercially available cellulase and xylanase are produced through SmF using pure substrates since it is easier to control and maintain the fermentation factors. Nevertheless, SSF is gaining more attentions due to its higher volumetric productivities, higher product stability, lower contamination risk, and lower operating costs (Mitchell *et al.*, 2006). SSF has been reported as the successful method to produce huge amount of important enzymes such as cellulase, ligninase, xylanase, and amylase for industrial usage (Pandey *et al.*, 2000). Therefore, to produce such high potential enzymes through degradation of lignocellulosic materials, filamentous fungi are the superior microbial group which has better adaption to SSF since the hyphae can grow on the surface of moist particles as well as penetrate into inter-particles spaces and colonize it (Pandey *et al.*, 2011; Muller dos Santos *et al.*, 2004).

The production of thermostable cellulase and xylanase by fungi through various palm oil residuals such as palm kernel cake (Kheng and Omar, 2005), palm oil mill residual (Prasertsan *et al.*, 1992), and oil palm empty fruit bunch (Bahrin *et al.*, 2011) have been widely reported. Yet, none of these literatures have demostrated the use of untreated oil palm trunk as the sole carbon sources for fungi in SSF. The oil palm trunk used as sole carbon source in this research is highly suitable for industrial scale applications since it is cheap, readily available, easy to store, has low moisture content, and can be stored in aerobically stable storage (Mielenz, 2009). Therefore, by conducting some comprehensive and highly efficient optimization strategies on all physiochemical factors that can significantly affect the fermentation process, highly potential yet economical crude enzymes cocktail with high activities of xylanases and cellulases can be produced; this is more plausible to accelerate the development of more sustainable biofuel production methods.

1.2 Objectives

The objectives of this research are:

1. To screen, isolate, and identify the most effective fungi for cellulase and xylanase production in solid-state fermentation using untreated palm oil trunk as a substrate.
2. To partially characterize the crude cellulase and xylanase enzyme by selected fungi.
3. To screen and optimize factors influencing cellulases and xylanase production using general factorial design (GFD), two-level factorial design (2LFD) and central composite design (CCD).
4. To optimize the production of polyoses during saccharification of untreated oil palm trunk using crude cellulases and xylanase.
5. To conduct kinetic evaluation on bioethanol production process.

1.3 Scope of Research

The scope of this research is to study the biodegradation of untreated oil palm trunk using locally isolated fungi to produce high activities of cellulases and xylanase through solid-state fermentation. All of the selected fungi were screened through quantitative and qualitative analyses to identify the isolate that was capable of secreting extracellular cellulolytic and xylanolytic enzymes. The cellulases and xylanase production of selected fungi were optimized using statistical approaches. The best nitrogenous supplement in the basal medium was determined using general factorial design (GFD). The two-level factorial design (2LFD) was used to select the most significant parameters that influenced the cellulases and xylanase production, and lastly CCD was used to determine their optimal values. The thermostability and acid-alkaline tolerant of crude cellulases and xylanase were characterized while the major components of cellulases (endoglucanase, exoglucanase, and β-glucosidase) and xylanase were observed in SDS-PAGE and zymogram. The presence of G11 xylanase gene in the crude enzyme was further confirmed through molecular and SDS-PAGE analysis. The saccharification ability of crude cellulases-xylanase cocktails to degrade the untreated oil palm trunk for sugars production was evaluated. Through alcoholic fermentation of reducing sugars in OPT hydrolysates by fermented yeast, better understanding was obtained on the types of sugars that had contributed to ethanol production.

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