



UNIVERSITI PUTRA MALAYSIA

***RUBBER WOOD SAWDUST FERMENTATION BY INDIGENOUS
LIGNOCELLULOLYTIC FUNGI FOR CELLULASE PRODUCTION***

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By

HUNDAIDAH BINTI RAMLI

**Thesis Submitted to the School of Graduate Studies, Universiti Putra
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Science**

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in
fulfilment of the requirement for the degree of Master of Science

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By

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October 2017

Chair : Prof. Rosfarizan Mohamad, PhD
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Microorganisms such as bacteria, fungal or yeast are often used for cellulase production using selected substrates via fermentation. However, relatively expensive cellulose powder as a main substrate hinders the industrial application of cellulase. Lignocellulose has been used as a substrate in producing cellulase as an alternative way towards more economic cost. The use of lignocellulolytic fungal in degradation of the lignocellulose are widely explored and studied due to its ability to produce both cellulase and ligninase. In this study, the potential of lignocellulolytic fungi in producing cellulase were investigated in solid-state fermentation of rubber wood sawdust. Four (4) indigenous fungal strains (*Trichoderma aureoviride* UPM 09, *Fusarium equiseti* UPM 09, *Fusarium proliferatum* UPM 09 and *Aspergillus* sp.) were screened for the best lignocellulolytic enzymes (cellulase and ligninase) producer. Solid-state fermentation of rubber wood sawdust was conducted in batch cultivation using modified Mandel's medium in 250 ml shake-flask. A conventional method (*one-factor-at-a-one-time*) of optimization was applied to obtain maximum production of cellulase. Three parameters of medium optimization (types of nitrogen sources, peptone concentration and tween-80) and four parameters of cultural condition (initial medium pH, temperature, particle size and inoculum size) for cellulase production were investigated.

From the experiments, only *T. aureoviride* UPM 09 and *F. equiseti* UPM 09 exhibit lignocellulolytic enzymes production. Hence, the two strains were selected for rubber wood sawdust fermentation. Throughout the fermentation, *T. aureoviride* UPM 09 showed the highest cellulase production of CMCase (12.99 U/g), FPase (1.57 U/g) and β -glucosidase (5.48 U/g). The optimum medium formulation and culture conditions for cellulase production were obtained using peptone as a sole nitrogen source at 2 g/L with addition of 1 % (v/v) tween-80 and initial medium pH of 5.0 at 25 °C. Two agar discs of fungal

culture and 1.7 mm particle size of rubber wood sawdust were used as optimum inoculum and particles sizes. The production of cellulase was increased by 5.5-fold for CMCase (85.04 U/g), 1.9-fold for FPase (4.55 U/g) and 1.7-fold for β -glucosidase (14.92 U/g) using the optimized condition. Enzymatic hydrolysis of different pretreated rubber wood sawdust was carried out to evaluate the performance of crude cellulase activity obtained from the fermentation. The result showed that higher reducing sugar was produced (1.280 mg/ml) using chemical pretreated rubber wood sawdust as compared to biological pretreated rubber wood sawdust (0.577 mg/ml) and raw rubber wood sawdust (0.103 mg/ml). In conclusion, *T. aureoviride* UPM 09 is a high potential strain to be used in cellulase production. The cellulase production by *T. aureoviride* UPM 09 was greatly improved after optimization process which can be further studied using statistical approach.



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sebagai memenuhi keperluan untuk ijazah Master Sains

PENAPAIAN SERBUK KAYU GETAH OLEH KULAT LIGNOSELULOLITIK TEMPATAN BAGI PENGHASILAN ENZIM SELULASE

Oleh

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Mikroorganisma seperti bakteria, kulat ataupun yis seringkali digunakan untuk penghasilan selulase menggunakan substrat tertentu dalam penapaian. Walaubagaimanapun, serbuk selulase yang mahal sebagai substrat menjadi penghalang dalam industri penghasilan selulase. Lignoselulosa digunakan sebagai substrat dalam penghasilan selulase sebagai langkah alternatif untuk kos yang lebih ekonomi. Kegunaan kulat lignoselulose dalam penguraian lignoselulosa telah dikaji secara meluas disebabkan kebolehannya menghasilkan kedua-dua enzim selulase dan ligninase. Dalam kajian ini, potensi kulat lignoselulolitik dalam menghasilkan selulas dikaji menerusi penapaian keadaan pepejal serbuk kayu getah. Empat (4) strain asli kulat (*Trichoderma aureoviride* UPM 09, *Fusarium equiseti* UPM 09, *Fusarium proliferatum* UPM 09 dan *Aspergillus* sp.) telah disaring untuk mencari penghasil enzim lignoselulolitik (selulase dan ligninase) terbaik. Dua strain kulat terbaik dipilih untuk penapaian kaedah pepejal serbuk kayu getah bagi penghasilan selulas. Penapaian kaedah pepejal serbuk kayu getah oleh strain-strain itu dijalankan secara selanjat menggunakan media Mandel yang diubah suai di dalam kelalang kon 250 mL. Pengoptimuman cara konvensional (satu faktor satu masa) telah digunakan bagi mendapatkan penghasilan selulase yang optimum. Tiga faktor pengoptimuman media (jenis sumber nitrogen, kepekatan pepton dan tween-80) dan empat faktor keadaan kultur (pH awal media, suhu, saiz partikel substrat dan saiz inokula) untuk penghasilan selulase telah dikaji.

Daripada hasil kajian, hanya kulat *T. aureoviride* UPM 09 dan *F. equiseti* UPM 09 menghasilkan enzim lignoselulase. Maka, dua strain ini telah dipilih untuk penapaian kaedah pepejal serbuk kayu getah. Sepanjang eksperimen dijalankan, *T. aureoviride* UPM 09 telah menunjukkan penghasilan selulase paling tinggi bagi CMC_{Case}, FPase dan β -glucosidase dengan aktiviti sebanyak

12.99 U/g, 1.57 U/g dan 5.48 U/g berikutan. Pengoptimuman media dan keadaan kultur bagi penghasilan selulase telah diperoleh menerusi pepton sebagai sumber nitrogen pada kepekatan 2 g/L dengan penambahan 1 % (v/v) tween-80 dan pH awal media adalah 5.0 pada suhu 25 °C. Dua ketulan agar kulat dan serbuk kayu getah bersaiz partikel 1.7 mm telah digunakan sebagai inokula dan saiz partikel paling optima. Dengan menggunakan keadaan optimum yang diperoleh, penghasilan selulase telah meningkat sebanyak 5.5-fold untuk CMC_{ase} (85.04 U/g), 1.9-fold untuk FP_{ase} (4.55 U/g) dan 1.7-fold untuk β -glucosidase (14.92 U/g). Hidrolisis enzim serbuk kayu getah yang dirawat dengan cara berbeza telah dibuat untuk menilai aktiviti selulase yang diperoleh melalui proses penapaian. Keputusan telah menunjukkan bahawa gula penurunan paling tinggi dihasilkan (1.280 mg/ml) menerusi serbuk kayu getah yang dirawat dengan perawatan awal kimia berbanding yang dirawat secara biologi (0.577 mg/ml) dan yang tidak dirawat (0.103 mg/ml). Kesimpulannya, *T. aureoviride* UPM 09 adalah strain yang berpotensi untuk digunakan bagi penghasilan selulase. Penghasilan selulase oleh *T. aureoviride* UPM 09 juga telah banyak meningkat setelah proses pengoptimuman dibuat yang mana ianya boleh dikaji lebih lanjut menggunakan cara statistik.

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LIST OF ABBREVIATIONS

ABTS	2,2'azino- bis -3-ethylbenz-thiazoline-6-sulfonic
BGL	β -glucosidase
BSA	Bovine serum albumin
CBM	Cellulosic basal medium
CMC	Carboxymethylcellulose
CMCase	Carboxymethylcellulase
df	Dilution factor
DNS	Dinitrosalicylic acid
DW	Dry weight
EG	Endoglucanase
EX	Exoglucanase
FPase	Filter paper cellulase
Lac	Laccase
LBM	Lignin modifying enzyme basal medium
LiP	Lignin peroxidase
NREL	National Renewable Energy Laboratory
PDA	Potato dextrose agar
PNP	p-nitrophenol
pPNG	β -glucopyronoside
rpm	rotation per minute
RSM	Response surface methodology
SmF	Submerged fermentation
SSF	Solid state fermentation

UniCC	Unit Culture Collection
UPM	Universiti Putra Malaysia
VP	Versatile peroxidase



CHAPTER 1

INTRODUCTION

The rubber industry in Malaysia has become one of the most important socio-economic sectors since 1957. Apparently, almost 35% of all rubber wood biomass processed remain as waste, which must be exploited efficiently if the industry is to remain competitive in the future (Ratnasingam *et al.*, 2012). Malaysia produces abundance of rubber wood sawdust that regarded as wastes, usually from saw milling and furniture industry which accounted almost 2.0 m³ wastes during 2010 (Ratnasingam and Jones, 2011). Rubber wood sawdust contains high cellulose content with an average of 43 % thus it can be a potential substrate for cellulase production (Shaaban *et al.*, 2013).

Cellulase, one of the important industrial enzymes is widely used in industries such as textile, pulp and paper, animal feed, detergent, brewery and wine as well as agricultural sector (Singh *et al.*, 2016; Kuhad *et al.*, 2011). Besides that, recent studies show promising results of cellulase application in biofuel industry (Srivastava *et al.*, 2015; Sweeney and Xu, 2012). The cellulase market itself is expected to grow dramatically when these enzymes were used to hydrolyze pretreated cellulosic materials to sugars which later fermented to commodities such as bioethanol and bio-based products on a large scale (Taha *et al.*, 2016; Mohanty and Abdullahi, 2016; Kang *et al.*, 2014).

The high cost of cellulase production and low yields of cellulase become the major constraints in industrial applications (Prasanna *et al.*, 2016; Lee *et al.*, 2010). Many strategies had been proposed and developed to make it more economic including the use of cheaper substrates such as empty fruit bunch from palm oil (Shahriarinnour *et al.*, 2011), wheat bran, sugarcane bagasse (Kilikian *et al.*, 2014), sawdust (Lo *et al.*, 2005); producing cellulases with higher specific activity on solid substrates and producing enzyme preparations with greater stability for specific processes (Zhang *et al.*, 2006). Recent studies have greatly explored the use of lignocellulosic biomass as a substitute substrate for cellulase production replacing the highly cost of commercial cellulosic substrate.

Lignocellulosic biomass can be a very potential substrate for cellulase production because of its abundance, easily obtain and cheaper raw materials. Lignocellulose is the major structural component of all plants consists of lignin, cellulose and hemicellulose (Sanchez, 2009). Lignocelluloses wastes often refer to plant biomass wastes such as agricultural residue and forest wastes (Mohan *et al.*, 2012). Pretreatment is required before further use of lignocellulosic biomass to improve the digestibility of the biomass itself for maximum sugars recovery (Maurya *et al.*, 2015). Many factors like lignin

content, crystallinity of cellulose and particle size limit the degradation of the cellulose present in the lignocellulosic biomass (Mohan *et al.*, 2012).

However, the major problem to the effective use of lignocellulosic biomass is due to the complex structure of lignin, cellulose and hemicellulose tangles together blocking deconstruction from microbes and enzymes (Ghorbani *et al.*, 2015). Common used pretreatment include physical and chemical pretreatment which relatively high in cost compared to biological pretreatment (Lim and Wang, 2013; Lee *et al.*, 2007).

Biological pretreatment of lignocellulosic biomass involved microorganisms that produced ligninase to degrade lignin content, where fungi currently the main ligninase producer (Maurya *et al.*, 2015; Zhou *et al.*, 2015). Recently, the use of lignocellulolytic fungal in lignocellulose degradation are widely explored and studied because of its ability to produce both cellulase and ligninase (Batista-Garcia *et al.*, 2017; Mtui, 2012). In other words, the need of separated pretreatment process can be eliminated if lignocellulolytic fungal were to be used in cellulase production using lignocellulosic biomass as a substrate.

Productions of cellulase were carried out through fermentation and the common technique is submerged fermentation (SmF) by using various microorganisms. However, solid-state fermentation (SSF) has drawn big attention due to its several potential advantages over SmF. SSF can be defined as the growth of microorganisms on moist, water-insoluble solid substrates in the absence or near-absence of free liquid (Soccol *et al.*, 2017; Moo-Young *et al.*, 1983). Gowthaman *et al.* (2001) pointed out several advantages of SSF including low capital cost, low energy expenditure, low waste water output, less expensive downstream processing and potential higher volumetric productivity.

In this study, rubber wood sawdust was used as an alternative cost-effective substrate in producing cellulase using potential local lignocellulolytic fungi. This research was aimed to improve the production of cellulase using solid-state fermentation of rubber wood sawdust by selected lignocellulolytic fungal strain. The performance of crude cellulase in hydrolyzing the rubber wood sawdust was also investigated. Hence, the specific objectives of this study were:

- a. To determine the best lignocellulolytic fungal used for production of cellulase via solid-state fermentation.
- b. To optimize the medium formulation and cultural conditions of solid-state fermentation of rubber wood sawdust for cellulase production by the selected lignocellulolytic fungal in shake-flask culture.
- c. To identify the function of crude cellulase obtained during solid-state fermentation in enzymatic hydrolysis of rubber wood sawdust.

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