UNIVERSITI PUTRA MALAYSIA

PRODUCTION OF CELLULOLYTIC ENZYMES VIA SOLID STATE FERMENTATION OF SPENT MUSHROOM SUBSTRATE BY Trichoderma asperellum UPM 1

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By

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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

PRODUCTION OF CELLULOLYTIC ENZYMES VIA SOLID STATE FERMENTATION OF SPENT MUSHROOM SUBSTRATE BY *Trichoderma asperellum* UPM 1

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Spent mushroom substrate (SMS) is a by-product generated after mushroom harvesting from the commercial mushroom cultivation industry. SMS had lost a significant fraction of their mass (approximately 35-50% depending on their nature) due to metabolic reactions, mushroom production and water evaporation. SMS generally composed of three main components i.e. fungal mycelia, extracellular enzymes secreted during growing of mushroom and unutilized lignocellulosic components. Delignified SMS with small particle size (<1 mm) has been observed to have high cellulose component and lignocellulolytic enzyme content. SMS comprised of high cellulose (45.5%) and partially degraded lignin by the action of *Pleurotus pulmonarius* during mushroom cultivation. SMS in readily available smaller particle size offers a great advantage as a fermentation feedstock. Hence, owing to this untapped potential, this study has investigated the feasibility of SMS as an alternative substrate for lignocellulolytic enzyme recovery. This was done by profiling ligninolytic enzymes activities produced during the growth of *P. pulmonarius* and *P. floridanus*. Crude laccase (3 U/g and 2.6 U/g) and MnP (1.4 U/g and 2.1 U/g) were detected in SMS from *P. pulmonarius* and *P. floridanus*, respectively. After enzyme recovery, optimization of cellulase production from SMS as a feedstock was conducted using Central Composite Design (CCD). Cellulase was produced on SMS from *P. pulmonarius* via solid state fermentation using *Trichoderma asperellum* UPM 1. Significant parameters of temperature, moisture content, and pH employed at three different levels were highlighted and discussed. The optimum condition for maximum production of CMCase (171.21 U/g), FPase (9.85 U/g) and β-glucosidase (6.83 U/g) were as follows; temperature 27.5ºC, moisture content 81% and pH of fermentation 4.5. The optimized condition of cellulase production resulted in increment of 1.4-fold in CMCase and 1.5-fold in β-glucosidase with unoptimized condition produced 125.77 U/g CMCase and 4.67 U/g β-glucosidase..

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PENGELUARAN ENZIM SELULASE MELALUI FERMENTASI KEADAAN PEPEJAL MENGGUNAKAN SISA SUBSTRAT CENDAWAN OLEH *Trichoderma asperellum* UPM 1

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Sisa substrat cendawan (SMS) adalah produk sampingan yang terhasil selepas penuaian cendawan daripada industri penanaman cendawan komersil. SMS kehilangan pecahan yang signifikan daripada jisim asal (anggaran 35-50% bergantung kepada sifat semulajadi) disebabkan reaksi metabolit, penghasilan cendawan dan penyejatan air. Secara umum, ianya terdiri daripada tiga komponen utama iaitu i.e. miselia kulat, rembesan enzim luar sel semasa pertumbuhan cendawan dan komponen lignoselulosa yang tidak digunakan. SMS tanpa lignin bersaiz partikel kecil (<1 mm) menunjukkan enzim lignoselulolitik dan komponen selulosa yang tinggi. SMS terdiri daripada selulosa yang tinggi (45.5%) dan lignin separa terdegradasi semasa proses kultivasi cendawan oleh *Pleutorus pulmonarius*. Partikel saiz SMS sedia ada yang kecil menawarkan kelebihan yang besar sebagai bahan mentah untuk fermentasi. Atas potensi yang belum diterokai ini, kajian ini telah dijalankan untuk mengkaji penggunaan SMS sebagai substrat alternatif untuk pemulihan enzim lignoselulolitik. lanya telah dilaksanakan dengan memprofil aktiviti enzim ligninolitik semasa pertumbuhan *P. pulmonarius* dan *P. floridanus*. Enzim laccase (3 U/g dan 2.6 U/g) dan MnP mentah (1.4 U/g dan 2.1 U/g) telah dikesan masing – masing di dalam SMS dari *P. pulmonarius* dan *P. floridanus*. Selepas pemulihan enzim, pengoptimum untuk pengeluaran selulase menggunakan SMS sebagai bahan mentah telah dijalankan menggunakan reka bentuk gabungan berpusat (CCD). Selulase telah dihasilkan menggunakan SMS dari *P. pulmonarius* melalui fermentasi keadaan pepejal menggunakan *Trichoderma asperellum* UPM 1. Parameter signifikan seperti suhu, kandungan kelembapan dan pH pada tiga paras yang berbeza telah dibincangkan. Keadaan optimum untuk pengeluaran maksimum bagi CMCase (171.21 U/g), FPase (9.85 U/g) dan β-glucosidase (6.83 U/g) adalah seperti berikut; suhu 27.5ºC, kandungan kelembapan 81%, dan fermentasi pada pH 4.5. Keadaan optimum selulase telah menyebabkan peningkatan enzim sebanyak 1.4 kali ganda untuk CMCase dan 1.5 kali ganda untuk β-glucosidase berbanding keadaan tidak optimum sebanyak 125.77 U/g CMCase dan 4.67 U/g β-glucosidase.

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I certify that a Thesis Examination Committee has met on 23 August 2016 to conduct the final examination of Iffah Nabilah binti Mohd Ariff on her thesis entitled ("Production of Cellulolytic Enzymes via Solid State Fermentation of Spent Mushroom Substrate by *Trichoderma asperellum* UPM 1") 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 Master of Science.

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Signature : _____
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LIST OF ABBREVIATION

ABTS	2, 2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid
ADF	Acid Detergent Fibre
ADL	Acid Detergent Lignin
ANN	Artificial Neural Network
ANOVA	Analysis of Variance
AOAC	Association of Official Analytical Chemists
$CaCl_2$	Calcium Chloride
CBH	Cellobiohydrolase
CCD	Central Composite Design
CMC	Carboxymethyl cellulose
CMCase	Carboxymethyl cellulose
$COCl_2$	Cobalt Chloride
DNS	Dinitrosalicyclic Acid
EFB	Empty Fruit Bunch
EG	Endoglucanase
$FeSO_4.7H_2O$	Iron(II) sulphate heptahydrate
FPase	Filter Paper Cellulase
H_2O_2	Hydrogen Peroxide
H_2SO_4	Sulphuric Acid
KCl	Potassium Chloride
KH_2PO_4	Potassium Dihydrogen Phosphate
LiP	Lignin Peroxidase
MARDI	Malaysian Agricultral Research and Development Institute
$MgSO_4$	Magnesium Sulphate
MnP	Manganese Peroxidase
MnSO4	Manganese Sulphate
$MnSO_4.7H_2O$	Manganese Sulphate Heptahydrate
MPOB	Malaysian Palm Oil Board
Na_2CO_3	Sodium Carbonate
Na_2SO_4	Sodium Sulphate
$NaNO_3$	Sodium Nitrate
NaOH	Sodium Hydroxide
NDF	Neutral Detergent Fiber
PDA	Potato Dextrose Agar
rpm	revolution per minute
RSM	Response Surface Methodology
SEM	Scanning Electron Microscopy
SmF	Submerged Fermentation
SMS	Spent Mushroom Substrate
SSF	Solid State Fermentation
UPM	Universiti Putra Malaysia
$ZnSO_4.7H_2O$	Zinc sulphate heptahydrate

CHAPTER 1

INTRODUCTION

Recently, mushroom demand in Malaysia as well as in the global market is escalating and lead to the increasing growth of the mushroom industry. This is reflected with the increment of fresh mushroom import from 80.9 million tonnes in 2007 to 90.8 million tonnes for the subsequent year (Haimid *et al.,* 2013). Additionally, the demand is expected to increase about 15% per year, with around 48,000 tonnes in 2020 (Mat-Amin *et al.,* 2014). Mushroom industry is considered as new and small industry in Malaysia. However, Malaysian government has declared mushroom farming as one of eleven business opportunity under National Key Economic Area (NKEA) with contribution of 182 million in Gross National Income (GNI). Following this, the growing area for mushroom farming is expected to reach 340 ha in 2020 from 78 ha in 2010 (Haimid *et al.,* 2013).

Mushroom exhibit various nutritional values for medical purposes including anti-tumor, anti-diabetic and anti-oxidant (Cohen *et al.,* 2002; Mattila & Ko, 2001). Deliciousness and its health benefits have contributed in increasing demand of mushroom in population and consumption per capita (Mat-Amin *et al.,* 2014). For every one kilogram production of fresh mushroom, it generated five kilogram of residual solid known as spent mushroom substrate (SMS) (Finney *et al.*, 2009; Lau *et al.*, 2003; Phan & Sabaratnam, 2012). The generation rate of SMS varies among countries. Approximately 254,000 tonnes of SMS were produced in Ireland annually (Barry *et al.,* 2012) while in UK was about 200, 000 tonnes (Finney *et al.,* 2009). Meanwhile, in Netherlands, more than 800,000 tonnes of SMS was generated each year (Oei & Albert, 2012). In Malaysia, average mushroom annual mushroom production was approximately 100 tonnes of fresh mushroom with 438 tonnes of SMS (Phan & Sabaratnam, 2012).

Mande, (2005) categorized agricultural residues into two groups; crop-based residues (generated in the field) and processing based-residues (generated during wood and industrial processing). SMS are example of crop-based residues, which are waste materials generated in the field or farm during mushroom production. SMS usually lost a significant fraction of their mass (approximately 35 - 50% depending on their nature) due to metabolic reactions, mushroom production and water evaporation and generally composed of three main components; (1) fungal mycelia, (2) extracellular enzymes secreted during the growth of mushroom and (3) unutilized lignocellulosic components (Koutrotsios *et al.,* 2014).

Generally, majority of this by-product was disposed in landfill and reuse as agricultural fertilisers, causing many environmental issues (Finney *et al.,* 2009;

Lim *et al.,* 2013; Phan & Sabaratnam, 2012). In recent years, the mushroom industry has faced challenges in storing and disposing the SMS. Recently, great attention has been paid in recycling this freely available agricultural crop residue in various applications. For instance, numerous attempts have been made to develop technology to extract the extracellular enzymes from spent (Ko *et al.,* 2005; Lim *et al.,* 2013; Muthangya *et al.,* 2014; Singh *et al.,* 2003; Wu *et al.,* 2013). Employing SMS to bioremediate pollutant derived from industrial and mining industries is another approach that has been focused on (Chen *et al.,* 2005; Chiu *et al.,* 2009).

Another strategy of reusing SMS includes; feedstock for reducing sugar for biofuel production (Balan *et al.*, 2008; Lee *et al.*, 2008); biopesticides (Wu *et al.,* 2013); decolorization of textile effluent (Singh *et al.,* 2012); source for ruminant feed (Dhanda *et al.,* 2005); bioplastic feedstock (Houghton *et al.,* 2004); and fire wood substitute (Williams *et al.,* 2001).

Recently, the demand for cellulases is consistently on the rise due to its diverse applications. Recently, many studies have reported the physicochemical properties of SMS and it is showed that SMS contain high organic content (Paredes *et al.,* 2009), macro and micro-nutrient concentrations (Medina *et al.,* 2012), fungal mycelia, extracellular enzymes and residual lignocellulosic substrates (Lim *et al.,* 2013). These components make SMS attractive and due to this fact, this study has been focusing on employing this freely available agricultural crop as an alternative feedstock for lignocellulolytic enzymes recovery. The objectives of this study were:

1. To profile the ligninolytic enzymes activities produced during the growth of commercial oyster mushroom (*Pleurotus floridanus* and *Pleurotus pulmonarius*).

2. To produce cellulolytic enzymes via solid state fermentation of spent mushroom substrate by *T. asperellum* UPM 1.

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