PRODUCTION OF LACCASE ENZYME USING RICE HUSK AS SUBSTRATE IN FUNGAL SOLID-STATE FERMENTATION

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FACULTY OF ENGINEERING
UNIVERSITY OF MALAYA
KUALA LUMPUR

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ABSTRAK

Untuk menghasilkan enzim laccase dalam kuantiti yang besar dengan kos yang berpatutan, satu proses fermentasi keadaan pepejal kulat yang menggunakan produk sampingan lignoselulosik dari industri perkilangan beras iaitu sekam padi, dan kulat cendawan *Pleurotus sajor-caju* telah diperkenalkan dalam kajian penyelidikan ini. Penyelidikan ini telah menilai semua aspek utama fermentasi keadaan pepejal kulat untuk penghasilan enzim laccase, termasuk pemilihan dan pengoptimuman keadaan prarawatan substrat, pemilihan kulat, dan penilaian serta pengoptimuman keadaan fermentasi.

Kajian sastera menunjukkan bahawa tiada reagen pernah digunakan dalam prarawatan sekam padi. Oleh hal yang demikian, penyelidikan ini telah mengkaji keberkesanan pelbagai reagen seperti asid sulfurik, asid hidroklorik, asid fosforik, asid asetik, asid nitrik, natrium hidroksida, kalsium hidroksida, 1-butil-3-metilimidazolium klorida, 1-etil-3-metilimidazolium asetat dan 1-etil-3-metilimidazolium dietil fosfat dalam prarawatan sekam padi. Prarawatan dengan asid hidroklorik cair didapati paling berkesan, di mana sekam padi yang telah diprarawat mempunyai komposisi kimia dan struktur yang sesuai untuk fermentasi keadaan pepejal kulat. Kemudian, prarawatan dengan asid hidroklorik cair telah dioptimumkan. Keputusan pengoptimuman menunjukkan bahawa prarawatan hanya perlu dijalankan dalam keadaan sederhana, iaitu pada suhu 125^{o}C selama 1.5 jam dengan menggunakan 0.5% (jisim/isipadu) asid sahaja.

Selepas itu, pemilihan kulat yang mempunyai aktiviti enzim ligninolitik yang tinggi telah dijalankan. Aktiviti enzim ligninolitik yang dihasilkan oleh tiga kulat, iaitu

Pycnoporus sanguineus, *Phlebia radiata* dan *Pleurotus sajor-caju* telah dikaji pada suhu bilik dan 35^{o}C dengan menggunakan piring agar skrining yang mengandungi kromogen seperti guaiacol dan Remazol brilliant blue-R (RBB-R). Semua kulat telah menunjukkan aktiviti pengoksidaan guaiacol dan penyahwarna RBB-R. Namun begitu, hanya *P. sajor-caju* menunjukkan aktiviti pada kedua-dua suhu bilik dan 35^{o}C. Oleh itu, *P. sajor-caju* telah dipilih untuk digunakan dalam fermentasi keadaan pepejal kulat yang berikutnya.

Penyelidikan ini juga mengkaji kaedah penyediaan inokulum kulat, di mana teknik kultur selofan telah dicipta. Teknik ini telah disahkan dapat menyediakan inokulum kulat yang aktif dan sesuai untuk digunakan dalam fermentasi keadaan pepejal. Kemudian, sekam padi yang telah diprarawat dan inokulum *P. sajor-caju* yang disediakan dengan teknik kultur selofan telah digunakan dalam fermentasi keadaan pepejal untuk penghasilan enzim laccase. Keputusan yang diperolehi dari fermentasi menunjukkan bahawa 84 jam fermentasi, 0.5 mM kuprum sulfat, 10 g/L glukosa, dan 0.1 g/L Tween 80 merupakan keadaan optimum bagi penghasilan enzim laccase. Di samping itu, penghasilan enzim laccase dalam keadaan optimum dapat diramalkan dengan menggunakan model logistik. Model tersebut dapat mensimulasi fasa peningkatan dalam penghasilan enzim. Pencirian enzim laccase yang dihasilkan dalam fermentasi telah dijalankan dan didapati bahawa enzim tersebut mempunyai jisim molekul sebanyak 60 kDa, dan ia berkeupayaan untuk menguraikan 2,2'-azino-bis(3-etilbenzothiazolin)-6-sulfonik asid (ABTS), 2,6-dimethoxyphenol (DMP) dan guaiacol.

Hasil kajian penyelidikan ini menunjukkan bahawa penghasilan enzim laccase melalui proses fermentasi keadaan pepejal kulat memberi prestasi yang lebih baik berbanding dengan proses fermentasi yang lain. Penggunaan sekam padi dalam proses fermentasi

dapat mengurangkan sisa pepejal yang dihasilkan dari industri perkilangan beras, di samping menghasilkan enzim laccase yang mempunyai banyak aplikasi industri. Keputusan positif yang diperolehi dalam kajian penyelidikan ini menunjukkan bahawa proses fermentasi keadaan pepejal kulat untuk penghasilan enzim laccase berpotensi untuk dilaksanakan di skala industri.

ABSTRACT

To produce laccase enzyme in large volume at affordable prices, a fungal solid-state fermentation (SSF) process that uses lignocellulosic by-product from rice milling industry – rice husk was introduced. This research aims to provide deeper insights into major aspects of fungal solid-state fermentation from the selection and pretreatment of solid substrate, selection of fungi to assessment and optimization of the process parameters. The research assesses all the mentioned aspects of solid-state fermentation for the production of laccase enzyme using the oyster mushroom *Pleurotus sajor-caju*.

No pretreatment reagent has been reported effective for pretreating rice husk, the research thus reports on various reagents, which include sulphuric acid, hydrochloric acid, phosphoric acid, acetic acid, nitric acid, sodium hydroxide, calcium hydroxide, 1-butyl-3-methylimidazolium chloride, 1-ethyl-3-methylimidazolium acetate and 1-ethyl-3-methylimidazolium diethyl phosphate on the pretreatment of rice husk. Dilute hydrochloric acid pretreatment was found to be most effective for pretreating rice husk by showing desirable chemical and structural characteristics that are favourable for fungal solid-state fermentation. The dilute hydrochloric acid pretreatment was further optimized, and the result showing a relatively mild pretreatment condition of 0.5% (w/v) acid loading, 125^{o}C, and 1.5 hours.

The screening and selection of fungi with ligninolytic enzyme activity was conducted on three pre-selected fungi, namely *Pycnoporus sanguineus*, *Phlebia radiata*, and *Pleurotus sajor-caju*, and the selection was done using screening plates containing chromogens like guaiacol and Remazol brilliant blue-R (RBB-R). Despite all the fungi possessed guaiacol oxidation and RBB-R decolourization activities, *P. sajor-caju*

outperformed the other fungi with activities shown at both room temperature and 35^{o}C. Therefore, *P. sajor-caju* was selected for the subsequent fungal solid-state fermentation.

Prior to solid-state fermentation, an improved fungal inoculum preparation method – cellophane film culture (CFC) technique was developed in this study to overcome the limitations of the existing fungal inoculum preparation methods. This method was verified and validated to be able to produce actively growing fungal inoculum and it is feasible to be used in solid-state fermentation. With the pretreated rice husk and *P. sajor-caju* inoculum prepared using cellophane film culture (CFC) technique, the fungal solid-state fermentation was optimized, and the optimal fermentation condition was at 84 hours fermentation, 0.5 mM copper sulphate, 10 g/L glucose, and 0.1 g/L Tween 80. Laccase production at the optimized fermentation condition fits well into the logistic model. The model is thus used to simulate the increasing phase of laccase enzyme production. The characterization of laccase enzyme shows that the enzyme has a molecular mass of 60 kDa, and it possesses the ability to degrade 2,2'-azino-bis (3-ethylbenzothiazoline)-6-sulphonic acid (ABTS), 2,6-dimethoxyphenol (DMP) and guaiacol.

The research findings demonstrated that the described solid-state fermentation process for laccase production shown comparatively well performance to reported studies. Besides minimizing the 'waste' generated from rice crops, rice husk can be transformed into added-value enzyme that has useful applications in the industries. This signifies the potential of up-scaling the solid-state fermentation for the production of laccase.

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β_n	Parameter in a model
ε	Molar extinction coefficient
μ_g	Gross specific growth
μ_m	Maximum specific growth rate
μ_{net}	Net specific growth
v	Enzyme activity
A_{CS}	Cross-sectional area of nitrogen molecule (BET)
B_1	Weight of oven dried empty crucible before extraction
B_3	Weight of oven dried empty crucible after extraction
B_4	Weight of oven dried empty crucible after hydrolysis
B_5	Weight of empty crucible after ashing (H_2SO_4 lignin)
B_a	Weight of empty crucible after ashing (aNDFom)
B_f	Weight of empty crucible after extraction (aNDFom)
c	Reagent concentration
C	Interaction of nitrogen and sample (BET)
I_{002}	Intensity of diffraction from 002 plane
I_{am}	Intensity of background scatter
k_v	Enzyme production rate constant
K_s	Saturation constant or half-velocity constant
l	Thickness of sample (cm)
M	Molecular weight
N	Avogadro's number
R^2	Coefficient of determination

S	Substrate concentration
t	Time
W	Sample weight (BET)
W_1	Weight of oven dried empty crucible
W_2	Weight of rice husk sample test portion
W_3	Weight of oven dried crucible with sample after extraction
W_4	Weight of oven dried crucible with sample after hydrolysis
W_5	Weight of crucible with sample after ashing (H_2SO_4 lignin)
W_a	Weight of crucible with sample after ashing (aNDFom)
W_f	Weight of crucible with sample after extraction (aNDFom)
W_m	Weight of constituting nitrogen monolayer on sample
X	Biomass concentration
X_n	Variable of a factor
Y	Response in a model
$Y_{X/S}$	Microbial biomass yield
[AMIM]	1-allyl-3-methylimidazolium
[BnMIM]	1-benzyl-3-methylimidazolium
[BMIM]	1-*n*-butyl-3-methylimidazolium
[EMIM]	1-ethyl-3-methylimidazolium
aNDFom	Amylase-treated neutral detergent fibre (organic matter)
ABTS	2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)
ADF	Acid-detergent fibre
ANOVA	Analysis of variance
AOAC	The Association of Official Analytical Chemists
ATF	Trifluoroacetate

BBD	Box-Behnken design
BET	Brunauer, Emmett and Teller
Cl	Chloride
CrI	Crystallinity index
CCD	Central composite design
CFC technique	Cellophane film culture technique
DEP	Diethyl phosphate
DMP	2, 6-dimethoxyphenol
DNS	3, 5-dinitrosalicylic acid
DSMZ	The German Collection of Microorganisms and Cell Cultures
EDTA	Ethylenediaminetetraacetic acid
FT-IR	Fourier-transform infrared
HSO_4	Hydrogen sulfate
LiP	Lignin peroxidases
LOD	Loss on drying
LSF	Liquid-state fermentation
MnP	Manganese-dependent peroxidases
MEA	Malt extract agar
MEPA	Malt extract peptone agar
NDF	Neutral-detergent fibre
NMMO	*N*-methylmorpholine-*N*-oxide
NREL	National Renewable Energy Laboratory
OAc	Acetate
P. chrysosporium	*Phanerochaete chrysosporium*

P. radiata	*Phlebia radiata*
P. sajor-caju	*Pleurotus sajor-caju*
P. sanguineus	*Pycnoporus sanguineus*
RBB-R	Remazol brilliant blue-R
RSM	Response surface methodology
SmF	Submerged fermentation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
SSF	Solid-state fermentation
TAPPI	Technical Association of the Pulp and Paper Industry
UV-VIS	Ultra violet-visible
XRD	X-ray diffraction

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CHAPTER 1

INTRODUCTION

1.1 Research Overview

This dissertation reports on the application of rice husk – a lignocellulosic crop residue to produce laccase enzyme via fungal solid-state fermentation (SSF). The research was initiated with a chemical pretreatment step that aimed to partially disrupt the recalcitrant structure of rice husk. Following the pretreatment study is the solid-state fermentation, which comprised of two major sections. The first section was the screening study to select a fungus capable of producing ligninolytic enzymes. The second section focuses on solid-state fermentation of rice husk that includes inoculum preparation, optimization of solid-state fermentation process and characterization of the laccase enzyme produced.

1.2 Research Background

1.2.1 Lignocellulosic Biomass in Bioconversion

The rising cost and depletion of natural resources for industrial bioprocesses has attracted much attention from researchers around the world to search for other affordable and sustainable alternatives. Agricultural by-products which comprised of lignocellulose generated along with the processing of agricultural crops are inexpensive and they are widely available to be converted into added-value products, such as enzymes (Patel et al., 2009; Rajendran et al., 2011), reducing sugars (Sreenath et al., 1999), furfural (Vazquez et al., 2007), and ethanol (Saha et al., 1998; Saha et al., 2005). Nevertheless, direct bioconversion of lignocellulosic biomass gives low yield. This is due to the recalcitrant nature of the biomass that hinders the accessibility of microorganism and/or hydrolytic enzymes into the matrix. Besides, it also inhibits the

breaking down of the lignocellulose (Khalil, 2002). Therefore, pretreatment is essential to partially disrupt the lignocellulosic structure for better utilization of biomass. Pretreatments commonly employed for pretreating lignocellulosic biomass include physical, chemical, and biological processes. In many cases, a combination of two or more of the mentioned processes is involved in the pretreatment of lignocellulosic biomass. Besides, pretreatment that involves lignocellulose dissolution using ionic liquids is also employed in pretreating lignocellulosic biomass in this study.

1.2.2 Fungal Fermentation in Enzyme Production

The potential conversion of lignocellulosic biomass into valuable products in fungal fermentation has been investigated by numerous researchers worldwide (Alam et al., 2005; Kocher et al., 2008; Mishra et al., 2007; Pang et al., 2006a; Pang et al., 2006b; Reddy et al., 2003; Rezende et al., 2002; Szakacs and Tengerdy, 1997; Vares et al., 1995; Winquist et al., 2008). The most prominent products could be produced from fungal fermentation are enzymes such as laccase (Patel et al., 2009; Rajendran et al., 2011), manganese peroxidases (Rajan et al., 2010), cellulase (Alam et al., 2009; Pang et al., 2006b), amylase (Kunamneni et al., 2005), xylanase (Pang et al., 2006a; Rezende et al., 2002), chitinase (Sudhakar and Nagarajan, 2010), protease (Paranthaman et al., 2009), etc. The ability of fungi in bioconversion is attributed to their capability in consuming lignocellulosic biomass as energy source. Being the natural degrader of lignocellulose in the habitat, fungi are also one of the important sources of commercial and industrial enzymes (Ibrahim, 2008; Singh and Srivastava, 2008).

Currently, most of the commercially available enzymes are produced via liquid/submerged fermentation (Ellaiah et al., 2002; Holker et al., 2004; Toca-Herrera et al., 2007). Liquid/submerged fermentation is well established to cater for the production

of various enzymes. However, enzymes produced via liquid fermentation are costly because the process involves the application of high-end technologies and expensive synthetic raw materials. As a result, its extensive use in industries to improve conversion processes has been limited. Therefore, a more cost effective way has to be developed to make the application of enzymes in industrial scale feasible. Fungal solid-state fermentation, which utilizes inexpensive lignocellulosic biomass as raw materials, offers an attractive solution for the costly enzyme production process. Solid-state fermentation can be applied to produce almost all the enzymes that can be produced by means of liquid/submerged fermentation. Furthermore, solid-state fermentation requires less stringent operating condition and it involves less complicated downstream processing. Thus, it appears to be more efficient in both cost and enzyme yield compared with liquid fermentation.

1.3 Research Objectives

Various crop residues and their applications in fungal fermentation for enzymes production have been investigated (Maas et al., 2008; Pang et al., 2006a; Reddy et al., 2003; Saha and Cotta, 2008; Saha et al., 1998; Saha et al., 2005; Vares et al., 1995). However, the use of rice husk as sole substrate in fungal fermentation for ligninolytic enzyme production has not been reported. A solid-state fermentation process that includes pretreatment of rice husk, screen and selection of fungi, and optimization of solid-state fermentation intended for enzyme production was presented in this dissertation. The research objectives and associated problem statements were detailed as follows:-

i. **To pretreat rice husk in achieving desired structural disruption for solid-state fermentation**

To date, no pretreatment reagent has been reported specifically effective for the pretreatment of rice husk. Pretreatment reagent has to be carefully selected as it modifies the structure of the biomass and affects its digestibility in solid-state fermentation. To have a greater insight into the pretreatment of rice husk, different pretreatment methods were applied namely chemical and ionic liquid dissolution pretreatments in this study. The chemical pretreatment reagents assessed were sodium hydroxide, calcium hydroxide, sulphuric acid, hydrochloric acid, phosphoric acid, acetic acid, and nitric acid. The ionic liquid dissolution pretreatment reagents assessed were 1-butyl-3-methylimidazolium chloride, 1-ethyl-3-methylimidazolium acetate, and 1-ethyl-3-methylimidazolium diethyl phosphate. The best performing reagent was selected for the subsequent optimization of rice husk pretreatment.

ii. **To screen and select a fungus with desired ligninolytic enzyme activity**

Little is known on which fungus can utilize rice husk and produce ligninolytic enzymes. Therefore, a proper selection of fungus prior to solid-state fermentation is necessary. In this study, three strains of fungi, namely *Pycnoporus sanguineus*, *Phlebia radiata* and *Pleurotus sajor-caju*, were screened for their ligninolytic enzyme activities using guaiacol and Remazol brilliant blue-R. One potential fungus showing desired ligninolytic activity was selected for the solid-state fermentation.

iii. **To optimize solid-state fermentation for ligninolytic enzyme production**

The main aims of this study were to assess and improve the solid-state fermentation process to maximize enzyme yield. To improve the process, a new and user-friendly fungal inoculum preparation technique was developed. This technique overcomes limitations frequently associated with existing fungal inoculum preparations. For yield improvement, the effect of enhancers on ligninolytic enzyme production was investigated, and the solid-state fermentation was optimized. The increasing phase of the enzyme production at optimized solid-state fermentation condition was then modelled.

1.4 Scopes of Study

The scopes of study include:

i. The examination of conventional and non-conventional pretreatment reagents and select one reagent that is suitable for pretreating rice husk.
ii. The characterization of the chemical structure of rice husk pretreated with various pretreatment reagents.
iii. The optimization of the pretreatment of rice husk using the selected reagent with Box-Behnken experimental design.
iv. The screening of fungi and selection of a fungus with ligninolytic enzyme activity.
v. The development of an inoculum preparation technique for solid-state fermentation followed by verification and validation of the technique.
vi. The examination of the time profile of laccase production and investigation of the effect of various inducers on the production of the enzyme.

vii. The optimization of the fungal solid-state fermentation using central composite experimental design.

viii. The modelling of the laccase enzyme production.

ix. The characterization of the laccase enzyme produced.

1.5 Structure of Dissertation

This dissertation is presented in 7 chapters and the contents of each chapter are described below.

Chapter 1: Introduction

This chapter presents the background, objectives, and problem statements of this research.

Chapter 2: Literature Review

This chapter presents the literature review for the overall study. The relevant background information and findings by other researchers are detailed in this chapter.

Chapter 3: Materials and Methods

This chapter details the materials and methods used in this research.

Chapter 4: Pretreatment of rice husk

This chapter comprises the results and discussion on the pretreatment of rice husk.

Chapter 5: Selection of Fungus with Ligninolytic Enzyme Activity

This chapter discusses the results obtained from the screening of fungus with ligninolytic enzyme activity.

Chapter 6: Solid-state fermentation

This chapter reports the results and discussion on solid-state fermentation of rice husk for ligninolytic enzyme production.

Chapter 7: Conclusions and Recommendations

This chapter presents the overall conclusions of this research, the novelties and contributions of the study, as well as the recommendations for future studies.

CHAPTER 2

LITERATURE REVIEW

This chapter reviews the long existing solid-state fermentation that is a promising approach in the production of enzymes. Compared to submerged/liquid-state fermentation, solid-state fermentation has not been extensively applied in industrial applications due to its unresolved operational limitations. It is undeniable that many bioprocesses can benefit from the endless potentials of solid-state fermentation. The literatures compiled give an overview of the process intended for laccase enzyme production involving pretreatment of rice husk, selection of fungi and optimization of solid-state fermentation. The first section of this chapter describes the principles of solid-state fermentation, its application in enzyme production and the associated benefits compared with submerged/liquid-state fermentation are reviewed. The following section details the major aspects of solid-state fermentation with emphasis on laccase enzyme production. It also includes the selection of microbe and substrate, process optimization, and isolation and purification of product. The subsequent section reviews the model employed for fungal growth and enzyme production kinetic studies.

2.1 Solid-State Fermentation

Solid-state fermentation involves the growth of microorganism on moist solid substrate in the absence or near-absence of free water, whereby there is a continuous gas phase between spaces of the solid material (Mitchell et al., 2006; Pandey et al., 2001). In general, the term ‘solid-state fermentation’ is used interchangeably with ‘solid-substrate fermentation’. The term ‘solid-substrate fermentation’ is used only when a solid material employed serve as carbon or energy source for the microbe in the absence or near-absence of free water (Pandey et al., 2001). On the other hand, ‘solid-state

fermentation' reflects any fermentation processes occurring in the absence or near-absence of free water, and employing a natural substrate or an inert solid support (Pandey et al., 2001).

Solid-state fermentation, which imitates natural microbiological processes like composting and ensiling, can be employed to produce added-value products in a controlled manner in industry (Rodriguez Couto and Sanroman, 2006). A solid-state fermentation bed is consisted of gas, solid and liquid phases and they are homogeneously distributed in the fermenter on a macroscopic scale (Smits, 1998). Figure 2.1 illustrates the solid and liquid portions in the solid-state, submerged, and liquid-state fermentations. Solid-state and liquid-state fermentations are two extreme systems where the former has the highest proportion of solid substrate and the latter has the least proportion of solid substrate. Submerged fermentation systems fall between the aforementioned systems. The presence of these phases in different proportions results in modified kinetic, diffusional, rheological, and thermodynamic (water activity and osmotic pressure) properties in fermentation processes (Gervais and Molin, 2003).

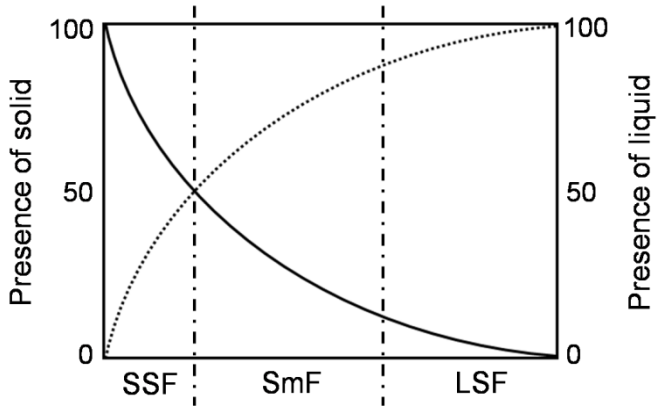


Figure 2.1: Schematic representation of solid (solid line) and liquid portions (dotted lines) in arbitrary units for solid-state (SSF), submerged (SmF), and liquid-state fermentations (LSF) (adapted from Smits (1998)).

Solid-state fermentation has emerged as a promising technology for converting agricultural biomass into valuable products with the aid of microbes (Pandey, 2003). To date, research works in the field of solid-state fermentation focus on its general application in the production of animal feed, food, fuel, enzymes, metabolites and microbial spores (Holker et al., 2004; Pandey, 2003). Some major applications of solid-state fermentation ranging from food, agriculture, fermentation to environmental control sectors are tabulated in Table 2.1. Solid-state fermentation system uses solid substrates like agricultural by-products with combination of different microbes (Holker et al., 2004). In conventional practice, filamentous fungi are employed as they can grow and penetrate into the depth of the solid substrate (Pandey, 2003).

Table 2.1: Major applications of solid-state fermentation

Sector	**Application**	**Reference**
Food industry	Spawn for mushroom production - Edible mushroom	Pandey et al. (2001); Tan and Wahab (1997)
	Fermented food production - Cheese - Soy sauce *koji* - Miso - Tempeh	Pandey et al. (2001)
	Food additives production - Aroma - Flavours - Pigments	Bramorski et al. (1998); Pandey et al. (2001)
Agricultural industry	Bioinsecticide for biocontrol - *Trichoderma* sp.	Esposito and da Silva (1998)
	Bioconversion of agricultural materials - Composting & ensiling - Protein enrichment	Gibbons et al. (1984); Gumbira-Sa'id et al. (1991); Hrubant et al. (1989); Pandey et al. (2001)
Environmental control	Bioremediation of contaminated site - Pentachlorophenol pollution	Ullah and Evans (1999)
	Detoxification of agro-industrial wastes - Coffee pulp and husk	Pandey et al. (2001)

Table 2.1: Continued

Industrial fermentation	Enzyme production - Cellulase & xylanase - Ligninase - Glucoamylase - Phytase - Tannase	Alam et al. (2009); Ellaiah et al. (2002); Pandey et al. (2001); van de Lagemaat and Pyle (2004); Rani and Ghosh (2011)
	Organic acid production - Citric acid - Lactic acid	Pandey et al. (2001)
	Ethanol production	Pandey et al. (2001); Sree et al. (1999); Yu et al. (2008)

Having had a general idea on the underlying principles of solid-state fermentation and its common applications, the following sub-section focuses on the application of solid-state fermentation in enzyme production with emphasis on laccase enzyme.

2.1.1 Laccase Enzyme and its Production

Laccase (benzenediol:oxygen oxidoreductase; EC 1.10.3.2) is a glycosylated polyphenol oxidase enzyme. This copper-containing enzyme catalyzes the oxidation of various phenolic and non-phenolic compounds with simultaneous reduction of molecular oxygen to water (Bourbonnais and Paice, 1990; Gianfreda et al., 1999). In recent years, laccase has been subjected to tremendous investigations due to its versatile applications in different industries (Patel et al., 2009). One important feature of laccase enzyme that contributes to its versatility is its ability to degrade a broad range of substrates – low substrate specificity (Gianfreda et al., 1999; Saito et al., 2003). Compounds that can be mineralized by laccase enzyme are essentially dyes and chromogens, like Remazol brilliant blue-R, azo dye, Crystal Violet, 2,2'-azino-bis (3-ethylbenzothiazoline)-6-sulphonic acid, syringaldazine etc., and they are commonly used in screening of fungi and enzymatic decolourization studies. Unlike some of the

oxidoreductases, laccase does not require the addition or synthesis of co-factor for catalysis reaction (Toca-Herrera et al., 2007). The enzyme requires only oxygen that is present in the environment as its co-substrate.

Laccase enzyme is widely distributed among fungi (Bollag and Leonowicz, 1984), higher plants (Mayer and Staples, 2002), and bacteria (Alexandre and Zhulin, 2000). The primary role of fungal laccase is associated with its lignin degradation ability in humification (Gianfreda et al., 1999; Mayer and Staples, 2002). Besides, it is also reported to constitute the fungal defence mechanism (Baldrian, 2003), take part in the conidial pigment production (Hermann et al., 1983; Mayer and Staples, 2002), involve in spores and fruiting body development (Thurston, 1994), and participate in infestation during phytopathogenesis (Gianfreda et al., 1999). In spite of its putative role in various natural processes, low substrate specificity allows the enzyme to oxidize a wide range of compounds without releasing toxic peroxide intermediates (Claus, 2004; Nyanhongo et al., 2002). The feature associated with oxidation makes laccase enzyme suitable for the application in biotechnological and environmental processes, like delignification in biopulping, textile dye bleaching, bioremediation of soils, decolourization and detoxification of industrial effluent, and enzymatic conversion of added-value compounds from lignin (Kiiskinen et al., 2004; Nyanhongo et al., 2002; Rodriguez Couto and Sanroman, 2005; Toca-Herrera et al., 2007).

However, the use of laccase enzyme in biotechnological and environmental processes is feasible only when the enzyme is produced in large quantity at low cost. A good strategy amidst many sought after is to produce laccase enzyme by solid-state fermentation using agricultural industrial wastes as support substrate (Reddy et al., 2003). Utilization of these agricultural by-products would help to reduce the disposal

problem. In addition, the legislations and environmental issues have also triggered the need to find alternative applications for these lignocellulosic residues (Reddy et al., 2003).

In general, the expression of laccase enzyme in fungal fermentations is influenced by culture conditions, such as carbon source and its concentration, pH of the fermentation environment, the composition of lignocellulosic materials and the nitrogen source. Different parameters affect the culture conditions and the interaction between them has further complicated the optimization of the fermentation. As a consequence, statistical optimization of fermentation medium components appears to be a rational and cost-effective way for efficient laccase enzyme production. The statistical approach also reveals the effect of each component on laccase enzyme production (Rajendran et al., 2011).

Besides the culture conditions, operational considerations from biological and processing perspectives too have influences on solid-state fermentation processes. The major aspects of solid-state fermentation are presented in the next sub-section.

2.2 Major Aspects of Solid-State Fermentation

Although solid-state fermentation uses simpler culture media and requires less stringent sterility than submerged/liquid-state fermentation, the process development for both fermentations is equally laborious. For the development of any solid-state fermentation processes, several important aspects should be considered include: (i) selection of fungi, (ii) selection of substrate, (iii) optimization of process parameters, and (iv) isolation and purification of desired product (Pandey, 2003). These aspects form the backbone of solid-state fermentation processes.

2.2.1 Selection of Fungi

The kingdom of fungi encompasses three main phyla, *i.e.* Ascomycota (sac fungi), Basidiomycota (dub fungi and mushroom), and Zygomycota (bread molds) (Madigan et al., 2003; Soper et al., 1998). The first and second phyla being the major and the most advanced groups. Fungi have been employed in brewing, baking, making cheese and preparation of fermented food. They are also used in the production of antibiotics, commercial enzymes and some other commodity chemicals. Nevertheless, fungi known to involve in the industrial scale applications constitute of only a small fraction of the fungi in the nature.

Fungi colonize diverse natural habitats where some of them are aquatic, while many are terrestrial (Madigan et al., 2003). Most terrestrial fungi live in the soil or on dead plant matter. They are important group of fungi capable of mineralizing organic matter in the nature. Fungi are natural lignocellulose decomposers in the habitat that grow on woody plant or stump and cause wood rotting. Wood rotting involves the disruption of the complex structure of lignocellulosic biomass, and this process is accelerated by the secretion of fungal extracellular lignocellulolytic enzymes. The ability of fungi to grow on almost any lignocellulosic biomass and to produce lignocellulolytic enzymes via fungal fermentation have been well witnessed for the past few decades (Coughlan, 1990).

2.2.1.1 Fungi with Ligninolytic Activity

There are numerous fungi reported to possess the ability of lignocellulose degradation. The three main groups of laccase enzyme-producing fungi are *Ascomycetes*,

Basidiomycetes and *Deuteromycetes*. Majority of the laccase enzyme-producing fungi are comprised of *Basidiomycetes*.

Among the fungi with lignocellulosic activity, the wood rotting fungi can produce extracellular lignin-modifying enzymes consisting of laccase, manganese-dependent peroxidase and lignin peroxidase. Examples of wood rotting fungi are *Phlebia radiata*, *Pleurotus sajor-caju*, *Pycnoporus sanguineus*, and *Phanerochaete chrysosporium*. These fungi belong to the Class *Basidiomycetes* and they are also known as the white-rot fungi. Many members of Class *Basidiomycetes* are saprotroph involving in litter and wood decay that are important in nutrient recycling in natural habitat (Singh and Srivastava, 2008; Webster and Weber, 2007). *Phlebia radiata*, *Pycnoporus sanguineus*, and *Phanerochaete chrysosporium* belong to the Order Polyporates. They are important wood rotting bracket fungi, which cause both brown-rot and white-rot on wood (Singh and Srivastava, 2008; Webster and Weber, 2007). On the other hand, *Pleurotus sajor-caju* belongs to the Order Agaricales (the gilled mushrooms), where most of its members only cause white-rot on wood (Singh and Srivastava, 2008; Webster and Weber, 2007). Some members of Agaricales, like *Pleurotus sajor-caju* and *Pleurotus ostreatus*, are widely cultivated as source of edible protein. Among the fungal strains, *Phanerochaete chrysosporium* has been used as a model organism for lignin degradation studies (Webster and Weber, 2007). The ligninolytic enzyme activities present in the fungi mentioned are summarized in Table 2.2.

Table 2.2: Ligninolytic enzyme activities of the white-rot fungi

Fungus	Enzyme activity				Reference
	Laccase	MnP [a]	LiP [b]	Oxidases	
P. chrysosporium	+	+	+		Okino et al. (2000)
P. sanguineus	+	+	+		Abdul Karim and Mohamad Annuar (2009), Okino et al. (2000), Saparrat et al. (2002)
P. radiata		+	+	+	Niku-Paavola et al. (1988), Vares et al. (1995)
P. sajor-caju	+	+			Patrick et al. (2011), Reddy et al. (2003)

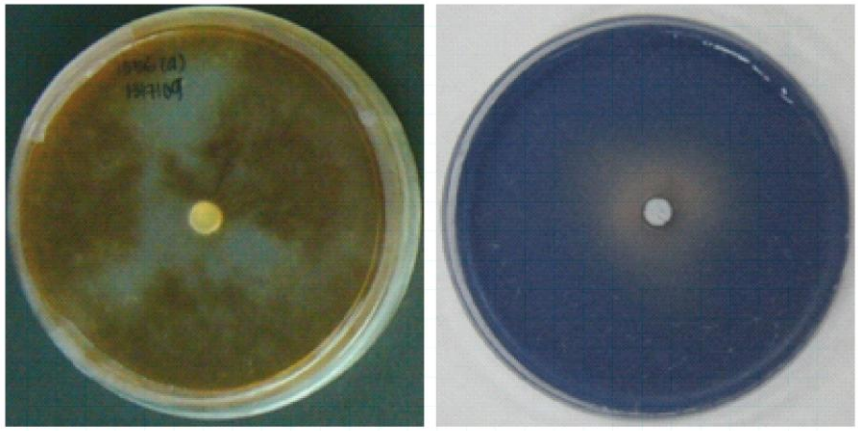
[a] MnP: Manganese-dependent peroxidases
[b] LiP: Lignin peroxidases

2.2.1.2 Screening of Fungi

The wood rotting fungi are known to possess the ability of degrading lignocellulose in biomass. However, a proper screening procedure is required to evaluate their activities in the laboratory. Most of the laboratory screening procedures employed synthetic chromogenic chemicals to screen fungal ligninolytic enzyme activity. Some commonly used synthetic screening chemicals are guaiacol (Coll et al., 1993; Kiiskinen et al., 2004; Viswanath et al., 2008), 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) (Floch et al., 2007; Hao et al., 2007; Saparrat et al., 2000), syringaldazine (Floch et al., 2007) and polymeric dyes such as Remazol brilliant blue-R (RBB-R) (D'Souza et al., 1999).

In the presence of phenol oxidases, the colourless guaiacol is oxidized to form reddish brown-coloured compounds, respectively (Doerge et al., 1997; Okino et al., 2000). Figure 2.2a shows a screening plate of *Phanerochaete chrysosporium* with reddish-brown appearance that is resulted from guaiacol oxidation. Decolourization of RBB-R

is achieved by the combined action of peroxidases and hydrogen peroxide-producing oxidases (Glenn and Gold, 1983; Mtui and Masalu, 2008). RBB-R decolourization appears as a clear zone on the RBB-R screening plate (Figure 2.2b).



(a) Guaiacol oxidation (b) RBB-R decolourization

Figure 2.2: Screening plates of guaiacol oxidation and RBB-R decolourization cultured with *P. chrysosporium*.

Screening of fungi is usually conducted by means of plate culture (Kiiskinen et al., 2004; Mtui and Masalu, 2008; Okino et al., 2000). The diameter of clear zone or coloured halo forming around the growing fungal colonies can usually be seen with naked eyes or after a simple staining procedure. Besides the plate culture technique, Rautela and Cowling (1966) has devised a simple cultural technique using test tube culture for screening of cellulolytic enzyme activity in fungi. This method shows the fungal cellulolytic activity via the magnitude of clear zone formed in the opaque cellulosic medium. In brief, most screening methods interpret fungal ligninolytic enzyme activity as either present or absent in certain fungi (Kiiskinen et al., 2004; Mtui and Masalu, 2008; Okino et al., 2000) with few studies associate enzyme activity to fungal growth (Keyser et al., 1978).

2.2.1.3 Inoculum Preparation

The step that is closely related to the screening of fungi is the preparation of inoculum. It involves the preliminary cultivation of selected microbe on a nutrient-rich medium to produce high yield of biomass (Riviere, 1977) and constitutes as one of the important steps in solid-state fermentation. The choice of inoculum preparation methods is dependent on the selected microbe. Several inoculum preparations being used for solid-state fermentation are spore suspension, mycelial disc, mycelial suspension, and pre-inoculated solid substrate.

Spores are usually produced by surface culture on agar medium or layers of bran or cereals soaked with water (Riviere, 1977). The spores are harvested by washing the surface of agar medium or solid substrate with sterile deionised water, and sometimes with the addition of a suitable amount of Tween 80 (Cavalcante et al., 2008). For optimal spore production, suitable conditions like moisture content of substrate and relative humidity of gas phase are to be determined for specific fungal strain (Riviere, 1977). In addition, spores can also be produced by means of submerged/liquid culture depending on the fungal strain employed (Riviere, 1977).

In some solid-state fermentation processes, mycelial discs are used as inoculum (Patel et al., 2009). Mycelial discs are cut from the actively expanding end of a fungus grown on agar medium. The discs consisted of a layer of fungal biomass and agar medium are applied directly as inoculum. The mycelial disc can be easily prepared, but the nutritious agar attached to the disc is a potential source of contamination in solid-state fermentation.

Inoculum could also be prepared in liquid culture with nutrient medium. This method needs to be conducted in stringent sterile conditions to ensure the quality of the inoculum prepared. Mycelial fungus forms pellets when it is grown in liquid medium. Therefore, homogenization with blender or homogenizer is usually needed to break mycelial clumps before inoculation (Al-Asheh and Duvnjak, 1994; Fukushima and Kirk, 1995). Occasionally, additional washing step is carried out to remove excess nutrient medium before inoculation to reduce the risk of contaminations.

Sterilized solid substrate colonized by fungus can also be used as inoculum for fermentation. The nutrient rich substrate facilitates fungus to increase in its mycelial biomass with a relatively short duration. This strategy is commonly used in the agriculture sectors, for instance the preparation of spawn for mushroom cultivation.

2.2.2 Selection of Substrate

Selection of a suitable solid substrate is another key aspect of solid-state fermentation (Pandey, 2003; Toca-Herrera et al., 2007). Substrates used in solid-state fermentations are generally lignocellulosic and heterogeneous by-products generated from agricultural industries (Raimbault, 1998). The solid substrate, which is insoluble, acts as both physical support and nutrients source for the microbes in solid-state fermentation (Pandey, 2003) to produce metabolites, enzymes, biomass, carbon dioxide, water, and heat (Smits, 1998). In most fungi, solid substrates are their natural media where growth can occur on the surface or within the substrate depending on the porosity (Gervais and Molin, 2003). Therefore, the selection of a suitable solid substrate is crucial as it determines the success of a solid-state fermentation process.

The selection involves screening of a number of agricultural by-products that are fit for microbial growth and product formation (Kunamneni et al., 2005; Patel et al., 2009). The screening evaluation focuses on agricultural by-products mainly due to their potential advantages for filamentous fungi thriving on the solid substrates (Ramachandran et al., 2004). Furthermore, the selection of solid substrate is depended on several factors related primarily to cost and availability (Rodriguez Couto and Sanroman, 2006). Chemical composition of the solid materials plays important role in stimulating the production of desired product (Rodriguez Couto and Sanroman, 2005). For instance, materials with higher lignin content lead to higher expression of fungal ligninolytic activity (Rodriguez Couto and Sanroman, 2005). The availability and accessibility of nutrients in solid substrate depends on substrate porosity and structure, which may be affected by moisture and substrate pretreatment (Cavalcante et al., 2008). Furthermore, substrate structure also has effect in solid-state fermentation as fungi penetrate solids in different ways depending on the porosity and tortuosity of the solid (Cavalcante et al., 2008).

The characteristics of lignocellulosic biomass commonly used as feedstock in solid-state fermentation are presented in the following section with focus given to the rice husk – a potential feedstock in bioconversion.

2.2.2.1 Lignocellulosic Biomass

Lignocellulose that present in all agricultural crop residues is the most abundant biomass on earth. The percent weight composition of some herbaceous and woody lignocellulosic biomass used in bioconversion is tabulated in Table 2.3. Lignocellulosic biomass such as sugarcane bagasse, wheat straw and cottonwood contains more than

50% cellulose and hemicellulose contents, and as high as 23% lignin. The applications of lignocellulosic biomass in various bioprocesses are presented in Table 2.4.

Table 2.3: Percent weight composition of lignocellulosic feedstock

Feedstock	Cellulose (Glucan)	Hemicellulose (Xylan)	Lignin	Reference
Herbaceous				
Alfalfa fiber	33.0	18.0	8.0	Koegel et al. (1997)
Brava straw (Paja Brava)	32.2	22.7	23.1	Sanchez et al. (2004)
Grass clipping	21.6	-	-	Orozco et al. (2007)
Rice husk	35.6	12.0	15.4	Saha and Cotta (2008)
Rice straw	35.1	18.0	15.2	Jiang et al. (2011)
Sugarcane bagasse	41.0	30.1	14.4	Yoon et al. (2012)
Wheat straw	36.3	19.0	25.5	Kootstra et al. (2009)
Woody				
Aspen wood	17.5	3.1	45.9	Torget et al. (1991)
Eastern cottonwood (*Populus deltoids*)	39.0	21.0	26.0	Mok and Antal (1992)
Red bloodwood (*Eucalyptus gummifera*)	38.0	16.0	37.0	Mok and Antal (1992)
Oak	45.2	20.3	21.0	Shafiei et al. (2010)
Spruce	43.8	6.3	28.3	Shafiei et al. (2010)
Sweetgum (*Liquidambar styraciflua*)	23.8	6.6	38.6	Torget et al. (1991)

Note: Minor components are not listed.

Table 2.4: Substrates used in solid-state fermentation and their applications

Substrate/support	Applications	References
Lignocellulosic		
Areca nut husk	Manganese peroxidases production	Rajan et al. (2010)
Banana leaf	Ligninase and cellulase production	Reddy et al. (2003)
Corn stover	Lignin and cellulose degradation for bioremediation	Pometto III and Crawford (1986)
Linseed oil cake	Phytase production	Rani and Ghosh (2011)
Oat husk	Manganese peroxidases and laccase production	Winquist et al. (2008)
Oil palm empty fruit bunch	Cellulase production	Alam et al. (2009), Alam et al. (2005)
Rice straw	Laccase production	Niladevi et al. (2007)

Table 2.4: Continued

Substrate/support	Applications	References
Sugarcane pith bagasse	Fungal growth studies Xylanase production	Saucedo-Castaneda et al. (1992) Rezende et al. (2002)
Wheat bran	Glucoamylase production Fungal growth studies	Ellaiah et al. (2002) Augustine et al. (2006)
Wheat straw	Laccase production Ligninase production	Paranthaman et al. (2009), Patel et al. (2009) Gupte et al. (2007)
Starchy		
Broken rice	Protease production	Paranthaman et al. (2009)
Rice bran	Chitinase production Protease production	Sudhakar and Nagarajan (2010) Chutmanop et al. (2008)
Wheat bran	Amylase production	Kunamneni et al. (2005)
Wheat flour	Fungal growth studies	Koutinas et al. (2003)
Cassava meal	Fungal growth studies	Raimbault and Alazard (1980)
Synthetic support		
Clay granules	Fungal growth studies	Desgranges et al. (1991)
Polycarbonate membrane	Fungal modeling studies	Rahardjo et al. (2004)
Nylon sponge	Lignase production	Dominguez et al. (2001), Rivela et al. (2000)
Others		
Citrus peel	Kinetic studies	Rodriguez-Fernandez et al. (2011)
Apple pomace	Fungal growth studies	Zheng and Shetty (1998b)
Cranberry pomace	Fungal inoculum production	Zheng and Shetty (1998a)

2.2.2.1.1 Rice Husk

Rice husk is a by-product of rice milling activity and it is one of the most abundantly produced lignocellulosic biomass in South-East Asia region (FAOSTAT, 2010). Rice husk is fibrous in nature and rich in cell walls, and has low digestibility, low bulk density and high ash/silica content (Hashim et al., 1996; Juliano, 1985; Juliano and Bechtel, 1985; Saha and Cotta, 2008). This fibrous residue is commonly burnt to generate heat energy for boiler system or household cooking (Ahiduzzaman, 2007; Hashim et al., 1996). Some are utilized in non-energy related areas, for instance as organic compost in agricultural land, filler in animal feed production, poultry bed and

reinforce materials in building, disposed as waste and etc. (Ahiduzzaman, 2007; ESCAP, 2000; Muthadhi and Anitha, 2007). With the accumulation of knowledge and technology development in the utilization of lignocellulosic biomass, rice husk could be used as feedstock for the production of added-value products (Alam et al., 2005; Pang et al., 2006a).

Rice husk has low protein and available carbohydrate contents, and possesses the highest content of crude fibre, crude ash, and silica (Juliano, 1985). The typical composition of rice husk is shown in Table 2.5. Rice husk has relatively low hemicellulose content, approximately 35% of which is consisted of cellulose and lignin comprises about 15-23%. Together both the cellulose and lignin constitute more than 50% of rice husk. Considering its lignocellulose content, rice husk can be converted into biofuel/ethanol (Saha and Cotta, 2008), ligninolytic and cellulolytic enzymes for industrial applications (Darus et al., 2005), etc.

Table 2.5: Typical composition of rice husk

Reference	Content (%, w/w)				
	Cellulose	**Hemicellulose**	**Lignin**	**Ash**	**Moisture**
Saha and Cotta (2008)	35.6	12.0	15.4	18.7	6.2
Vegas et al. (2004)	34.4	16.2	23.0	11.3	9.0

2.2.2.2 Characterization of Lignocellulosic Biomass

In the selection of substrate for solid-state fermentation, the determination of lignocellulosic biomass composition is one of the vital procedures. Characterization elucidates the major constituents of lignocellulosic biomass such as cellulose, hemicellulose, lignin, and ash contents. Information obtained from characterization is essential for predicting microbial substrate digestibility under specified conditions

(Goering and van Soest, 1970). At present, there are a few recognized standard protocols being employed for characterization of lignocellulosic biomass, namely the official methods of analysis of the Association of Official Analytical Chemists (AOAC), the Standard Biomass Analytical Methods of the National Renewable Energy Laboratory (NREL), and the testing procedures of Technical Association of the Pulp and Paper Industry (TAPPI). Each of the mentioned standard protocols uses different analytical approaches, but they give relatively the same outcomes.

A relevant standard protocol is the official methods of AOAC for characterization of lignocellulosic biomass as animal feed. The cellulose, hemicellulose and lignin contents can be determined by a series of extractions with acid or neutral detergents, washings and digestions with acid or enzyme (AOAC, 2005). Extraction with acid detergent solution removes acid-labile carbohydrates and fats leaving primarily cellulose and lignin, the resulting lignocellulose in the extracted biomass is known as acid detergent fibre (ADF) (Goering and van Soest, 1970). For sulphuric acid lignin determination, the acid detergent fibre is subjected to digestion with 72% sulphuric acid (Goering and van Soest, 1970). Extraction with neutral detergent solution and heat-stable α-amylase remove easily digested proteins, lipids and polysaccharides in the lignocellulosic biomass, leaving a fibrous residue consisted of mainly cellulose, hemicellulose and lignin, the resulting lignocellulose is called neutral detergent fibre (NDF) (AOAC, 2005). The difference between acid detergent fibre and sulphuric acid lignin is an estimate of cellulose content. The difference between acid detergent fibre and neutral detergent fibre is an estimate of hemicellulose content.

2.2.2.3 Pretreatment of Lignocellulosic Biomass

Lignocellulose is available in abundant at low cost, but using it directly in bioprocesses incurs high cost. This is because the enzymatic conversion process requires high amount of expensive enzymes (Neureiter et al., 2004; Saha et al., 1998). Moreover, a long incubation time is needed when microbe, such as fungus, is applied in the bioconversion of untreated lignocellulosic biomass due to its recalcitrant nature. Therefore, subjecting lignocellulosic biomass to an efficient pretreatment step would reduce the recalcitrant properties of the lignocellulose (Neureiter et al., 2004).

Apart from the type of substrate, characteristic of the substrate also affects the performance of the solid-state fermentation (Rodriguez-Leon et al., 2008a). Different strategies, namely physical, biological, chemical pretreatments, or a combination of two or more of the pretreatments, have been employed for the pretreatment of lignocellulosic biomass to improve microbial growth in the solid-state fermentation. Besides that, non-conventional methods involve the dissolution of lignocellulosic biomass in a solvent followed by regeneration of the cellulose-rich material also have been developed for similar purpose (Dogan and Hilmioglu, 2009; Dong et al., 2009; Jiang et al., 2011; Li et al., 2009; Zhao et al., 2009).

The selection criteria of a pretreatment process must be effective in treating lignocellulose, economical in large scale, safe and environmental friendly and easy to apply (Chang et al., 1997). Both the application of conventional and non-conventional pretreatment methods in pretreating lignocellulosic biomass is discussed in greater details in the following sections.

2.2.2.3.1 Conventional Pretreatment Methods

Pretreatment is necessary to improve the utilization of lignocellulosic biomass in the bioconversion process. The main purpose of pretreatment is to partially disrupt the recalcitrant structure of lignocellulosic biomass. Conventional pretreatment approaches used in pretreating lignocellulosic biomass are physical, biological (microbial), and chemical methods (Chandra et al., 2007; Ribeiro, 1991).

Pretreatment by physical means involve the breaking-down of the lignocellulosic biomass into smaller fragments or particles by using blender in laboratory scale, and hammer- or ball-mill in larger scale. Physical pretreatment increases the specific surface area of the biomass (Chandra et al., 2007) rendering it more accessible to fungus, while facilitating the mass and heat transfer in solid-state fermentation (Rodriguez-Leon et al., 2008a).

Biological pretreatment is similar to conducting solid-state fermentation on untreated lignocellulosic biomass. Biological pretreatment employs wood degrading fungus, such as soft, brown or white rot fungus, to change the chemical composition of the lignocellulosic biomass (Chandra et al., 2007).

Among the conventional pretreatment methods, chemical pretreatments have been commonly employed. Chemical reagents have been used in pretreating lignocellulosic biomass are sulphuric acid, phosphoric acid, sodium hydroxide, calcium hydroxide, etc. Chemical pretreatment disrupts the lignocellulosic matrix making it more susceptible to be broken down in the subsequent fungal solid-state fermentation or enzymatic hydrolysis (Chang et al., 1998; Zheng et al., 2007). Acid pretreatments generally hydrolyze cellulose/hemicellulose and disrupt the structure of lignocellulose (Orozco et

al., 2007; Saha et al., 2005), while alkaline pretreatments partially remove lignin and solubilise hemicellulose (Gowthaman et al., 2001). Chemical pretreatments can be operated at wide range of temperatures and durations. Most of the chemical pretreatments are carried out at high temperature with a relatively short duration. In short, different pretreatment conditions, *i.e.* level of reagent loading, pretreatment temperature, and duration, are needed for chemical pretreatment of lignocellulosic biomass according to their compositional difference.

Chemical pretreatments of lignocellulosic biomass result in the hydrolysate, and the partially hydrolyzed residue with disrupted structure. The hydrolysate from the pretreatment can be used for subsequent fermentation as it contains reducing sugars. Also, the residue with disrupted lignocellulosic matrix can be used for solid-state fermentation.

2.2.2.3.2 Non-Conventional Pretreatment Methods

In plant, cellulose forms a native composite material with lignin and hemicellulose. Due to the complex supramolecular structure, cellulose is not soluble in water and in most of the organic liquids (Klemm et al., 2005). To dissolve cellulose, specific cellulose solvent capable of disrupting hydrogen bonds and dissolving its supramolecular structure is required (Klemm et al., 2005). Examples of the cellulose dissolving solvents are trifluoroacetic acid (≥99%) (Dong et al., 2009), *N*-methylmorpholine-*N*-oxide (NMMO) (Dogan and Hilmioglu, 2009; Shafiei et al., 2010), and ionic liquids (Jiang et al., 2011; Li et al., 2009; Zhao et al., 2009). The dissolved cellulosic material can be regenerated from the solvent by the addition of an anti-solvent. The latter must be miscible with solvent, but not with dissolved cellulosic material. The commonly used anti-solvents in the regeneration of cellulose are water, alcohol and polar organic liquids

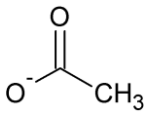
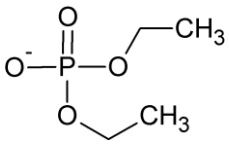
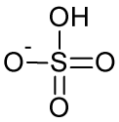
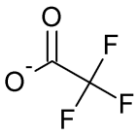
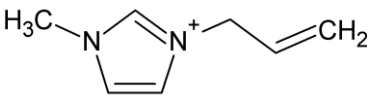
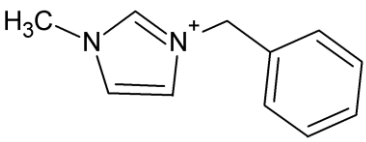
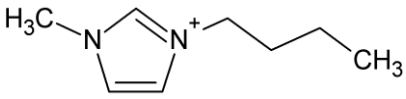
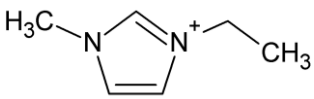
(Swatloski et al., 2002). Most of the dissolution pretreatments are conducted at relatively low temperatures and involved relatively short pretreatment durations from as short as 30 minutes up to a few hours. The dissolution pretreatments are effective in reducing the crystallinity of cellulose (Dong et al., 2009; Kuo and Lee, 2009a; Swatloski et al., 2002; Zhu et al., 2006), and produces regenerated cellulose that are favourable to subsequent conversions.

The use of ionic liquids to dissolve cellulose has been employed in the non-conventional pretreatment (Dadi et al., 2006; Jagadeeswara Rao et al., 2007; Li et al., 2009; Yang and Pan, 2005; Zhao et al., 2009). Various ionic liquids, such as 1-butyl-3-methylimidazolium chloride ([BMIM]Cl) (Dadi et al., 2006), 1-ethyl-3-methylimidazolium acetate ([EMIM]OAc) (Li et al., 2010), and 1-ethyl-3-methylimidazolium diethyl phosphate ([EMIM]DEP) (Li et al., 2009), have been applied in pretreatment step before enzymatic saccharification to enhance reducing sugars yield. The dissolution of cellulose is facilitated by interaction of hydrogen bonding between ionic liquid and cellulose (Klemm et al., 2005), and the dissolved cellulose is regenerated through competitive hydrogen bonding interaction between cellulose and anti-solvent. The structures of some commonly used anion and cation of ionic liquid in dissolution pretreatments are shown in Table 2.6.

Dissolution pretreatment with certain solvents at higher temperatures can hydrolyze the dissolved cellulose. Thus, when regenerated cellulose is the desired product, dissolution pretreatment using trifluoroacetic acid (≥99%) or phosphoric acid (95%) is performed at low temperatures. High pretreatment temperatures can also lead to hydrolysis of cellulose instead of dissolution of cellulose (Fanta et al., 1984; Zhang and Lynd, 2005). Furthermore, NMMO becomes relatively instable at temperatures above 100^{o}C, and

could result in partial decomposition when pretreatment temperature is higher than 130^{o}C (Novoselov et al., 2007; Shafiei et al., 2010).

Table 2.6: Commonly used anions and cations of ionic liquid in dissolution pretreatment of lignocellulosic biomass

Full name	Abbreviation	Structure
Anion		
Acetate	OAc	
Chloride	Cl	
Diethyl phosphate	DEP	
Hydrogen sulfate	HSO_4	
Trifluoroacetate	ATF	
Cation		
1-allyl-3-methylimidazolium	[AMIM]	
1-benzyl-3-methylimidazolium	[BnMIM]	
1-butyl-3-methylimidazolium	[BMIM]	
1-ethyl-3-methylimidazolium	[EMIM]	

2.2.3 Optimization of Process Parameters

Fungal solid-state fermentation is influenced by biological and physico-chemical factors (Rodriguez-Leon et al., 2008b). Biological factors are related to the biology, metabolic process and reproduction of a microorganism, and they determine the behaviour of the particular microorganism in a specific way (Rodriguez-Leon et al., 2008b). Physico-chemical factors are related to the physico-chemical phenomena occurring in the fermentation system, for instance, thermodynamic considerations involving transport phenomena of momentum, energy and mass (Rodriguez-Leon et al., 2008b). All these factors are interrelated, thus it is important to determine which factors are independent or dependent. As the number of parameters increase, optimization of process parameters becomes complicated and time consuming. Therefore, response surface methodology is a useful tool for process optimization where it considers the effect imposed by the parameters while reducing the number of experiments to be conducted.

Response surface methodology is a statistical technique useful for modelling and investigation of process in which the response of interest is influenced by several parameters, and the ultimate objective is to optimize the response (Montgomery, 2009). The use of response surface methodology is not limited to optimization of chemical processes, but also biological processes like solid-state fermentation. Response surface methodology has been employed to optimize the production of enzymes in solid-state fermentation involving multiple parameters (Francis et al., 2003; Vuddaraju et al., 2010). In the optimization, the data are fitted with regression analysis to produce a model.

Prior to the optimization studies, understanding of the effective process parameters is essential for better manipulation of the bioconversion process. The following sub-

sections describe the process parameters relevant to production of enzyme by means of solid-state fermentation.

2.2.3.1 Process Parameters

This section addresses the process parameters of solid-state fermentation with emphasis on the production of enzymes. Besides temporal and spatial variation of the parameters, a mutual interaction exists between the parameters (Smits, 1998). The increase in fermentation temperature leads to changes in the rates of microbial growth and metabolic activity. The temperature changes in solid bed differ locally, and eventually cause microbial growth and metabolic activity to differ at varied spots of the bed. Therefore, the interaction between/among the parameters determines the complexity of a solid-state fermentation process (Smits, 1998).

The process parameters that are commonly investigated in solid-state fermentation are (i) substrate loading; (ii) initial moisture content of solid substrate; (iii) initial pH of moistening agent; (iv) fermentation duration; (v) fermentation temperature; (vi) agitation and aeration; (vii) inoculum loading; and (viii) inclusion of inducer/supplementation.

(i) Substrate Loading

The level of substrate loading influences the porosity and aeration of the substrate in solid-state fermentation conducted in flasks as well as tray solid-state fermentation process (Ellaiah et al., 2002). Substrate weight-to-flask volume ratio of 1:25 and 1:50 are most commonly applied in laboratory-scale solid-state fermentation investigation to provide sufficient air in the headspace of the fermenter to cater for microbial growth and activity.

(ii) Initial Moisture Content of Solid Substrate

Moisture content is closely related to the definition of solid-state fermentation, and with the characteristics of the lignocellulosic materials (Rodriguez-Leon et al., 2008a). Hence, moisture content of solid substrate in solid-state fermentation is a critical parameter for growth, biosynthesis and secretion of metabolites like enzymes (Ellaiah et al., 2002; Ramachandran et al., 2004). Moisture level is governed by the nature of substrate, type of end-product and the requirement of the microbe (Lonsane et al., 1985). Manipulation of this parameter could modify the metabolic production or excretion of a microorganism (Pandey, 2003). This is because the synthesis of enzyme by the fungus is affected by the variation in initial moisture content of solid substrate, and the availability of moisture.

Moisture content of a substrate is affected by its composition and structure. The type of microorganism that can grow in a solid-state fermentation system is determined by water activity (Pandey, 2003). In fungal solid-state fermentation, the moisture content of solid matrix oscillates between 20 to 70% (Perez-Guerra et al., 2003; Rodriguez-Leon et al., 2008a). The wide range of applicable moisture content is advantageous for the development of a specific solid-state fermentation process. Besides, it also has a marked effect on growth kinetics on the fungus used in solid-state fermentation (Raimbault, 1998). Thus, the desirable moisture content level depends on both the fungus and solid substrate used.

(iii) Initial pH of Moistening Agent

The pH of substrate may change during solid-state fermentation due to fungal metabolic activities. During the process, fungus secretes organic acids such as citric, acetic or

lactic acids, which cause decrement in the pH, and thus, the kinetics of pH variation is depended on the fungal strain (Raimbault, 1998). Besides, nature of substrate also has influence on the pH kinetics mainly due to the buffering effect of lignocellulosic materials (Raimbault, 1998). Fungus has an optimal pH range between 3.5 and 6.0 for its growth and activity (Rodriguez-Leon et al., 2008a) as fungal metabolic activities are very sensitive to changes in pH. Similar to initial moisture content, fungal activity is affected if pH level is higher or lower compared to the optimum (Ellaiah et al., 2002). It is suggested that a slightly low pH is preferable for ligninolytic enzymes production via fungal fermentation. However, too low pH is not beneficial for laccase enzyme production, which might be due to their susceptibility to acidic proteases (Mansur et al., 2003).

(iv) Fermentation Duration

The duration of fungal fermentation in enzyme production is closely related to the other parameters, such as inoculum preparation, type and nature of substrate, and conditions that favour the growth and enzyme production of the fungus. Solid-state fermentation of various lignocellulosic biomasses for laccase enzyme production using *Pleurotus ostreatus* exhibited the first sign of growth after 2 – 3 days of incubation and complete colonization of substrates within 8 days of fermentation with maximum laccase activity on day 8 of incubation for the substrates (Patel et al., 2009).

It might seem that longer incubation duration is favourable for solid-state fermentation as there is sufficient time for the fungus to colonize and produce desired enzyme products. However, prolong fermentation duration is not beneficial for enzyme production as incubation beyond the maximum period of 72 hours resulted in decreased

α-amylase enzyme and biomass production, which might be owing to exhausted substrate nutrient and limited space for growth (Ramachandran et al., 2004).

(v) Fermentation Temperature

Temperature is an important parameter influencing fungal metabolic patterns in solid-state fermentation (Ramachandran et al., 2004). It affects the fungal growth, formation/germination of spores, formation of products, denaturation of protein or enzyme, promotion or inhibition on metabolites production, and cell death (Pandey, 2003; Rodriguez-Leon et al., 2008a). A solid-state fermentation study demonstrated little laccase enzyme activity by *Pleurotus ostreatus* at temperatures other than the optimal fermentation temperature (28^{o}C), and also no fungal growth was observed at 50^{o}C due to drying of substrate (Patel et al., 2009). Therefore, fermentation temperature is a significant consideration in the development of biological processes.

In general, fungal growth in aerobic fermentations result in the increase of solid bed temperature, where the rise in temperature is caused by the heat released from exothermic fungal respiration (Perez-Guerra et al., 2003; Rodriguez-Leon et al., 2008a). Due to the limited thermal conductivity and low moisture content of the lignocellulosic biomass, insufficient heat removal also can lead to the formation of thermal gradient in the solid bed (Perez-Guerra et al., 2003; Raimbault, 1998). In addition, heterogeneity nature of the lignocellulosic biomass too contributes to the development of temperature gradients (Rodriguez-Leon et al., 2008a). As a summary, the degree of temperature increase depends on the type of microbe employed, and the porosity, particle size and depth of the substrate bed (Gervais and Molin, 2003).

(vi) Agitation and Aeration

In solid-state fermentation, the reduced liquid phase that is approximately the water holding capacity of the biomass presents a high viscosity to the solid substrate (Gervais and Molin, 2003). Agitation/mixing improves homogeneity and disrupt temperature gradients of solid substrate (Rodriguez-Leon et al., 2008a). Unlike liquid-state/submerged fermentations, it is difficult to mix or agitate solid substrate in solid-state fermentation (Gervais and Molin, 2003). Even though agitation improves homogeneity and helps to control temperature of substrate bed (Perez-Guerra et al., 2003; Rodriguez-Leon et al., 2008a), excessive agitation imposes great shear forces that disrupt the integrity of microbial cell particularly when mycelial fungi are used in solid-state fermentation (Gervais and Molin, 2003). Fungal mycelium was damaged when agitation was constantly applied to mix the solid substrate. Therefore, intermittent agitation is usually employed in solid-state fermentation to avoid causing damage to the fungal mycelium (Rodriguez-Leon et al., 2008a).

Unlike agitation/mixing, aeration can be easily applied in solid-state fermentation compared to liquid-state/submerged fermentation due to free access of atmospheric air to the solid substrate to maintain aerobic conditions, desorption of carbon dioxide, and regulation of substrate temperature and moisture level (Gervais and Molin, 2003; Raimbault, 1998). Gaseous exchange in aeration is important in solid-state fermentation as the gas environment affects the relative production of biomass and enzyme (Raimbault, 1998).

Aeration also serves important function in heat and moisture transfer between the solid and gas phases (Pandey, 2003; Raimbault, 1998). Without aeration, heat removal is very slow due to low thermal conductivity of the substrate (Pandey, 2003). Therefore,

aeration is employed to control temperature of the substrate during solid-state fermentation (Raimbault, 1998). In contrast, high aeration rate reduces moisture content of the solid substrate by evaporation (Raghavarao et al., 2003). In cases whereby high aeration rate reduces moisture content of solid substrate, water-saturated air is supplied (Rodriguez-Leon et al., 2008a).

(vii) Inoculum Loading

The determination of an optimum inoculum loading is one of the crucial steps in solid-state fermentation. A low inoculum loading may be insufficient to initiate the growth of microbe and longer time is required for the microbe to multiply to adequate amount for substrate utilization and production of desired product (Ramachandran et al., 2004). On the other hand, a high inoculum loading may result in competitive inhibition on microbial growth (Ellaiah et al., 2002; Kunamneni et al., 2005), and decrease microbial metabolic activity due to fast depletion of nutrients (Patel et al., 2009). Thus, a suitable loading of inoculum is needed to ensure a rapid proliferation and synthesis of microbial biomass by striking a balance between growth and nutrient availability (Ramachandran et al., 2004).

(viii) Inclusion of Inducer/Supplementation

The production of enzymes by microbes in fermentations can be stimulated by the addition of a variety of inducing substances depending on the targeted enzymes (Osma et al., 2011). Inducing substances such as surfactants can reduce surface tension of the liquid adsorbed on the solid substrate. One commonly used surfactant in fermentations is Tween 80 that increases the bioavailability of less soluble substrates for microbial growth and stimulates the growth of fungal spores (Zheng and Obbard, 2001).

Besides surfactants, some micronutrients play important role in inducing ligninolytic enzymes transcription and production, and also as metal activator for the enzymes. In the case of laccase enzyme, copper is essential to induce the expression and production of the enzyme from fungi (Palmieri et al., 2000; Patrick et al., 2011; Rajendran et al., 2011). This element is also a co-factor for laccase enzyme where each of four copper ions is associated with a single polypeptide chain. The threshold concentration of copper sulphate leading to suppression of fungal laccase expression varies with microbes and types of fermentation.

Furthermore, some aromatic compounds like 2,5-xylidine, lignosulfonates, veratryl alcohol, ferulic acid, vanillic acid, coumaric acid and Kraft lignin (Barbosa et al., 1996; Eggert et al., 1996; Halaburgi et al., 2011; Xiao et al., 2003) have inducing effect on fungal ligninolytic enzymes production. The aromatic inducers such as veratryl alcohol serve as secondary metabolite that plays crucial role in the degradation of lignin (Barbosa et al., 1996). However, some aromatic compounds like guaiacol and syringaldehyde do not have significant effect in inducing fungal laccase enzyme production (Eggert et al., 1996; Halaburgi et al., 2011).

Supplementations of carbon and nitrogen encourage the adaptation of fungus in fermentation and they are useful for the production of ligninolytic enzymes (Patel et al., 2009). The addition of carbon source such as simple sugars promotes fungal growth and allow rapid establishment of the fungus on the solid substrate. Nevertheless, the presence of easily metabolised carbon source accelerates the degradation of lignin from lignocellulosic substrate (Kirk et al., 1976).

2.2.4 Isolation and Purification of Products

In fermentations, the desired product is often released into the aqueous phase. The fermentation product of liquid-state/submerged fermentation is released into the liquid culture medium, whereas fermentation product of solid-state fermentation is released into the thin liquid layer on the solid substrate. Thus, extraction of the desired product is done by separating it from microbial cells and substrate constituents (Riviere, 1977). In solid-state fermentation, the commonly used solvents or extractants for harvesting enzymes are distilled water with or without the surfactant Tween 80 (Ramachandran et al., 2004; Rezende et al., 2002), and buffer solution *i.e.* acetate buffer (pH 4.5) (Cruz-Cordova et al., 1999). During extraction, the mixture of fermented solid substrate and solvent is agitated before they are centrifuged or pressed to get the clear supernatant, which is the crude enzyme filtrate.

Crude laccase enzyme filtrates have low enzyme activity. To obtain preparations with higher enzyme activity and purity, further purification steps are required. A generalized procedure for purification of laccase enzyme includes the concentration of crude enzyme filtrate, dialysis in removing small and unwanted compounds, and separation of targeted protein by chromatography (Coll et al., 1993; Khammuang and Sarnthima, 2009; Mansur et al., 2003).

2.3 Modelling of Solid-State Fermentation

From industrial perspective, monitoring and controlling solid-state fermentation parameters are critical for scaling-up. However, scaling-up of solid-state fermentation processes is complicated and unreliable. This is due to difference in the growth conditions of microbe in large-scale fermenter compared to laboratory-scale fermenter (Toca-Herrera et al., 2007). The inhomogeneity of solid substrate complicates drawing

of representative samples for analysis and subsequently for process control (Smits, 1998). Moreover, the lack of reliable and affordable instrumentations for measuring growth parameters and consumption of lignocellulosic substrates also hampers control strategies in solid-state fermentation (Pandey, 2003; Toca-Herrera et al., 2007). In view of that, simulation using mathematical models has been employed to acquire deeper insight regarding the effects of process parameters in fungal solid-state fermentation. Despite that computer simulation appears to be an attractive way to test new control designs as simulation runs are fast and relatively inexpensive, only simulations with complex models provide reliable results (Toca-Herrera et al., 2007).

Many models can be used for fungal growth in solid-state fermentation and the most applicable fungal growth kinetic model is the logistic model of Okazaki and co-workers (Okazaki et al., 1980). The batch growth curve of logistic model assumes a sigmoidal shape as illustrated by Figure 2.3. This model covers mycelial growth in the stationary and logarithmic phases. It could be used to investigate the relationship between biomass growth and fermentation time for each initial substrate concentration (Al-Asheh and Duvnjak, 1994). The shape of this growth curve can be predicted by combining the Monod equation (equation 2.1) with the growth equation (equation 2.2) and an equation for microbial growth yield based on substrate consumption (Shuler and Kargi, 2002).

$$\mu_g = \frac{\mu_m S}{K_s + S} \qquad (2.1)$$

, where μ_m = maximum specific growth rate (when $S >> K$s), μ_g = gross specific growth (when endogenous metabolism is unimportant), S = substrate concentration, and K_s = saturation constant or half-velocity constant.

$$\mu_{net} \equiv \frac{1}{X}\frac{dX}{dt} \qquad (2.2)$$

, where μ_{net} = net specific growth (this is the difference between gross specific growth rate (μ_g) and the rate of microbial biomass loss due to cell death or endogenous metabolism), X = biomass concentration, and t = time of fermentation.

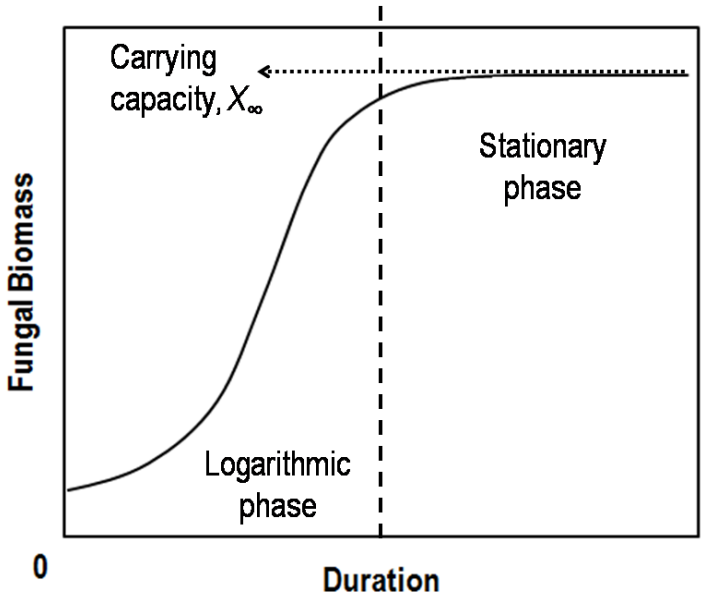


Figure 2.3: Logistic growth curve.

With the assumption of null microbial biomass loss and endogenous metabolism, the net specific growth (μ_{net}) is equal to gross specific growth (μ_g). Therefore, by combining equation 2.1 and equation 2.2 gives equation 2.3.

$$\frac{dX}{dt} = \frac{\mu_m S}{K_s + S} X \qquad (2.3)$$

The relationship between microbial growth and substrate consumption is given by equation 2.4.

$$X - X_0 = Y_{X/S}(S_0 - S) \tag{2.4}$$

, where X_0 and S_0 = initial values for microbial biomass and substrate concentrations, and $Y_{X/S}$ = microbial biomass yield based on the limiting substrate nutrient. Substitution of the term of substrate concentration (S) in equation 2.4 to equation 2.3 yields the rate expression as shown by equation 2.5.

$$\frac{\mathrm{d}X}{\mathrm{d}t} = \frac{\mu_{\mathrm{m}}(Y_{X/S}S_0 + X_0 - X)}{(K_{\mathrm{s}}Y_{X/S} + Y_{X/S}S_0 + X_0 - X)}X \tag{2.5}$$

The rate expression in equation 2.5 describes the sigmoidal-shaped batch growth curve, where the biomass concentration asymptotically approaches the value of $Y_{X/S}S_0 + X_0$. This equation is implicit in its dependence on substrate concentration, and requires a predetermined knowledge of the maximum microbial biomass concentration in the solid-state fermentation system (Shuler and Kargi, 2002). The maximum microbial biomass concentration, which is the carrying capacity of the system, is denoted as X_∞ as shown in Figure 2.3. Since logistic equation characterizes growth in terms of carrying capacity, whereby the specific growth rate is related to the amount of unused carrying capacity, it can be expressed as:

$$\mu_{\mathrm{g}} = \mu_{\mathrm{m}}\left(1 - \frac{X}{X_\infty}\right) \tag{2.6}$$

With the assumption of net specific growth (μ_{net}) equals to gross specific growth (μ_{g}), and the carrying capacity of the system (X_∞) as the maximum biomass concentration

(X_m) that can be attained in the solid-state fermentation system, combining equation 2.2 and equation 2.6 gives equation 2.7.

$$\frac{dX}{dt} = \mu_m X\left(1 - \frac{X}{X_m}\right) \tag{2.7}$$

A lag phase should be considered before biomass increases in solid-state fermentation. During lag phase, dX/dt is equal to zero, and X is equal to X_0 that represents the initial biomass concentration in the system. Integration of equation 2.7 gives:

$$X = \frac{X_m}{1 + \beta e^{-\mu_m t}} \tag{2.8}$$

, where $\beta = X_m/X_0 - 1$.

With this expression, the biomass concentration (X) for a particular substrate concentration at any time of fermentation can be estimated. The values of X_m and μ_m are obtained by fitting experimental data of biomass for each substrate concentration with the logistic law expression.

In solid-state fermentations, enzyme production is divided into phases where enzyme activity increases and decreases as illustrated by Figure 2.4. Logistic law expression can be used for deriving a model to express increasing phase of enzyme activity by taking into account the microbial biomass concentration (Al-Asheh and Duvnjak, 1994). The logistic law assumes enzyme activity is proportional to the biomass concentration during the increasing phase of the enzyme production (Okazaki et al., 1980). By taking the assumption into consideration, the model can be written as:

$$\frac{d\upsilon}{dt} = k_v X \tag{2.9}$$

, where υ = enzyme activity, and k_v = rate constant for enzyme production. Substitution of equation 2.8 into equation 2.9 gives,

$$\frac{d\upsilon}{dt} = \frac{k_v X_m}{1+\beta e^{-\mu_m t}} \tag{2.10}$$

At initial condition, υ is equal to υ_0 that represents the enzyme activity after inoculation. Integration of equation 2.10 leads to equation 2.11, which relates enzyme concentration with fermentation duration and the parameters of logistic law.

$$\upsilon = \upsilon_0 + \frac{k_v X_m}{\mu_m} \ln\left(\frac{\beta + e^{\mu_m t}}{\beta + 1}\right) \tag{2.11}$$

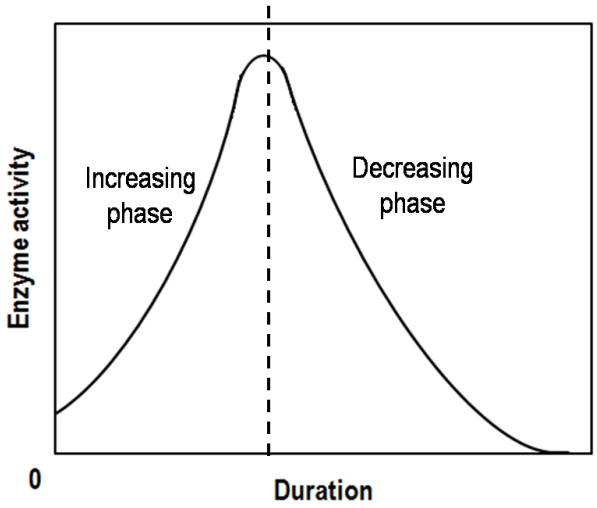


Figure 2.4: Increasing and decreasing phases of enzyme production.

When the enzyme activity has achieved a maximal value, enzyme activity decreases as fermentation duration is prolonged (Figure 2.4). Unlike the increasing phase, the production of enzyme activity does not increase with microbial biomass concentration during the decay phase, but behave independently of the microbial growth. The decrease in enzyme activity is dependent on the concentration of enzyme, and the latter is dependent on the production of hydrolytic enzymes that reduce enzyme activity (Al-Asheh and Duvnjak, 1994). In view of the logistic expression for increasing phase of enzyme production cannot predict the decay phase of enzyme production, another model is required. There are relatively few studies conducted on the decay phase of enzyme production because batch solid-state fermentation processes usually are terminated before the start of this phase (Al-Asheh and Duvnjak, 1994).

CHAPTER 3

MATERIALS AND METHODS

3.1 Characterization of Rice Husk

Rice husk sample was characterized using the Association of Official Analytical Chemists (AOAC) official methods (2005). The moisture, ash, cellulose, hemicellulose, and lignin contents of the rice husk sample were determined and the methodology for each determination was detailed in the following sub-sections.

3.1.1 Preparation of Rice Husk Sample

Rice husk sample was collected from a rice mill in Sekinchan, Selangor, Malaysia. The rice husk was washed and dried at 55^{o}C before being ground into smaller particle size (about 30 mesh size) using a blender (Waring® Commercial, model 32BL80). Ground rice husk was stored in a dry cabinet prior to use.

3.1.2 Determination of Moisture Content

The moisture content of rice husk sample was determined with AOAC Official Method 934.01 (2005). 2 g of rice husk sample was dried in a drying dish at 100^{o}C overnight until reaching a constant weight. The weights of the sample before and after drying were recorded. The moisture content (%, w/w) of rice husk was estimated by equation 3.1 based on loss on drying (LOD) of rice husk sample. The moisture content value was reported as an average of five test portions.

$$\text{Moisture},\%\,(\text{w/w}) = 100 \times \frac{\text{LOD wt (g)}}{\text{Rice husk wt (g)}} \qquad (3.1)$$

3.1.3 Determination of Ash Content

The ash content of rice husk sample was determined with AOAC Official Method 942.05 (2005). 2 g rice husk sample was weighed in a porcelain crucible and placed in a pre-heated furnace at 600^{o}C for 3 hours. The crucible was cooled in an oven at 100^{o}C for 1 hour before it was further cooled to room temperature in a desiccator. The carbon-free ash was weighed immediately and the average ash content (%, w/w) of five test portions was estimated by equation 3.2.

$$\text{Ash, \% (w/w)} = 100 \times \frac{\text{Rice husk wt (g) - Loss on ashing wt (g)}}{\text{Rice husk wt (g)}} \qquad (3.2)$$

3.1.4 Determination of Acid-Detergent Fibre and H_2SO_4 Lignin Contents

Sulphuric acid solutions of 0.5 M and 72.0% (w/v) were prepared from concentrated H_2SO_4 (96%, w/v; analytical grade, Fisher Scientific) using volumetric flasks in cooling bath. The acid-detergent solution was prepared by dissolving 10 g of N-cetyl-N, N, N-trimethyl-ammonium bromide (analytical grade, Merck) in 1 L of 0.5 M H_2SO_4 standardized previously. The solution was stirred using a magnetic stirrer (B. Braun, model REO-S9) at a moderate speed until the detergent was completely dissolved.

Acid-detergent fibre (ADF) and H_2SO_4 lignin contents of rice husk sample were determined with AOAC Official Method 973.18 (2005). This method starts with the extraction of ADF followed by digestion of the sample with 72.0% (w/v) H_2SO_4. The weight of a 50 ml Gooch crucible of porosity grade 3 (W_1) was recorded after drying overnight at 105^{o}C. Approximately 1 g of rice husk sample was weighed (W_2) into a 500 ml round bottom flask. 100 ml acid-detergent solution was added to the rice husk sample. The rice husk-acid-detergent solution mixture was refluxed for 60 minutes from the onset of boiling. After the acid-detergent extraction, the flask was removed from the

heating unit, and the content was filtered into a pre-weighed Gooch crucible to remove the acid-detergent solution. Then, the rice husk residue was rinsed with hot water in the crucible, and the sample was soaked in ~40 ml of hot water for 5 minutes before the hot water was removed by vacuum filtration. The hot water soaking was repeated twice prior to soaking in ~40 ml of acetone (analytical grade, Merck) for 5 minutes in duplicate. Lastly, the sample was dried overnight at 105^{o}C and weighed (W_3). The Gooch crucible was allowed to cool for about 15 minutes in a desiccator before weighing. Blank sample was treated in the same manner without rice husk sample. The average ADF percentage of five test portions was estimated by equation 3.3.

$$\text{ADF},\%\,(\text{w/w}) = 100 \times \frac{(W_3 - W_1) - (B_3 - B_1)}{W_2} \qquad (3.3)$$

, where W_1 = weight of oven dried empty crucible,

W_2 = weight of rice husk sample test portion,

W_3 = weight of oven dried crucible with sample after acid-detergent extraction,

B_1 = weight of oven dried empty crucible before acid-detergent extraction, and

B_3 = weight of oven dried empty crucible after acid-detergent extraction.

The acid-detergent extracted rice husk was used for the subsequent determination of H_2SO_4 lignin content. The rice husk sample in Gooch crucible was covered with cooled 72% (w/v) H_2SO_4 to about three quarter of the crucible. The content was allowed to stand for 3 hours, and it was stirred hourly to break up lumps. After which the H_2SO_4 was removed by vacuum filtration and the sample was washed with hot water until acid free to non-bleeding pH-indicator strips (Merck). The sample was dried overnight at 100^{o}C and weighed (W_4). Following that, the sample was ignited at 500^{o}C in a furnace (Thermconcept, model KL15/11) for 2 hours. The crucible was cooled in an oven

maintained at 100°C for an hour before it was cooled in a desiccator and weighed (W_5). The average H_2SO_4 lignin content of five test portions was estimated by equation 3.4.

$$\mathrm{H_2SO_4\ Lignin,\ \%\,(w/w)} = 100 \times \frac{(W_4 - W_5) - (B_4 - B_5)}{W_2} \qquad (3.4)$$

, where W_2 = weight of rice husk sample test portion,

W_4 = weight of oven dried crucible with sample after H_2SO_4 hydrolysis,

W_5 = weight of crucible with sample after ashing,

B_4 = weight of oven dried empty crucible after H_2SO_4 hydrolysis, and

B_5 = weight of empty crucible after ashing.

3.1.5 Determination of Neutral-Detergent Fibre Content

Neutral-detergent (ND) solution was prepared by dissolving 18.6 g ethylenediaminetetraacetic acid disodium (disodium EDTA, Merck), 4.56 g dibasic sodium phosphate (Na_2HPO_4, Merck), 6.81 g sodium borate decahydrate ($Na_2B_4O_7.10H_2O$, Sigma-Aldrich), 30 g sodium dodecyl sulphate (SDS, Merck), and 10 ml triethylene glycol (Merck) in distilled water and the volume was adjusted to 1 L. The pH of the ND solution was verified and adjusted to between 6.95 and 7.05 with HCl or NaOH, if necessary. The ND solution was stored at room temperature prior to be used in NDF content determination. The Burke iodine solution was prepared using 20 g/L potassium iodide (KI, Merck) and 10 g/L iodine (I_2, Merck) in an opaque bottle.

NDF content of rice husk was determined with AOAC Official Method 2002.04 (2005). The weight of a 50 ml Gooch crucible of porosity grade 3 (W_e) dried at 105°C was recorded. Approximately 0.5 g of rice husk was weighed (S) into a 500 ml round bottom flask. Next, ~50 ml ND solution and 0.5 g sodium sulphite anhydrous (Na_2SO_3, Merck)

were added to the sample, and the reaction mixture was heated to boiling. Upon the onset of boiling, 2 ml of standardized α-amylase working solution was added to the reaction mixture (refer to Appendix A: Standardization of working α-amylase for NDF determination). The reaction mixture was refluxed for 60 minutes at boiling temperature. Subsequently, the flask was removed from the heating unit and the particles were allowed to settle for 30 to 60 seconds. The content of flask was filtered into a pre-weighed Gooch crucible to remove ND solution, and particles attached on the wall were rinsed with hot water into the crucible. The crucible was half-filled with hot water and the second dose of 2 ml α-amylase working solution was added to the solution and stirred. The α-amylase was allowed to react for 45 to 60 seconds before the enzyme solution was removed, and the sample was soaked in ~40 ml of boiling water for 5 minutes. The hot water soaking was repeated twice. The procedure was followed by two times acetone soaking (~40 ml) for 5 minutes. After acetone soaking, the sample was air dried for 10 to 60 minutes to remove excess acetone before it was dried at 105^{o}C overnight and weighed (W_f). The crucible and its content were ignited at 500^{o}C furnace for 5 hours or until the sample is carbon free. The hot crucible was tempered in 105^{o}C for one hour before cooling down to room temperature in a desiccator and weighed (W_a). Blank sample followed the same procedure without the rice husk sample. The average aNDF (organic matter) (aNDFom) of five test portions was estimated by equation 3.5.

$$\text{aNDFom, \% (w/w)} = 100 \times \frac{\left(W_f - W_a - B_f + B_a\right)}{W_2} \qquad (3.5)$$

, where W_2 = weight of rice husk sample test portion,

W_a = weight of crucible with sample after ashing,

W_f = weight of crucible with sample after amylase-treated ND extraction,

B_f = weight of empty crucible after amylase-treated ND extraction, and

B_a = weight of empty crucible after ashing.

3.2 Pretreatment of Rice Husk

Prior to pretreatment of rice husk, screening for both conventional and ionic liquid dissolution pretreatments by assessing various pretreatment reagents were carried out. For conventional pretreatment, the alkalis and acids screened were sodium hydroxide, calcium hydroxide, sulphuric acid, hydrochloric acid, phosphoric acid, acetic acid, and nitric acid. For ionic liquid dissolution pretreatment, the ionic liquids screened were 1-butyl-3-methylimidazolium chloride, 1-ethyl-3-methylimidazolium acetate, and 1-ethyl-3-methylimidazolium diethyl phosphate. The best performing pretreatment reagent was selected and optimized in the subsequent rice husk pretreatment study.

3.2.1 Pretreatment Reagents

The alkaline solutions were prepared from pellets and powder of sodium hydroxide (NaOH, Merck) and calcium hydroxide ($Ca(OH)_2$, Sigma-Aldrich), respectively. The acid solutions were prepared from concentrated acids, namely 96% sulphuric acid (H_2SO_4, Fisher Scientific), 37% hydrochloric acid (HCl, Merck), 85% phosphoric acid (H_3PO_4, Ajax Chemicals), 99.8% glacial acetic acid (CH_3COOH, Riedel-de Haen) and 65% nitric acid (HNO_3, Scharlau Chemie).

The ionic liquids 1-butyl-3-methylimidazolium chloride ([BMIM]Cl, Merck), 1-ethyl-3-methylimidazolium acetate ([EMIM]OAc, Sigma-Aldrich) and 1-ethyl-3-methylimidazolium diethyl phosphate ([EMIM]DEP, Sigma-Aldrich) were used without further purification. The chemical structures of the ionic liquids are shown in Figure 3.1.

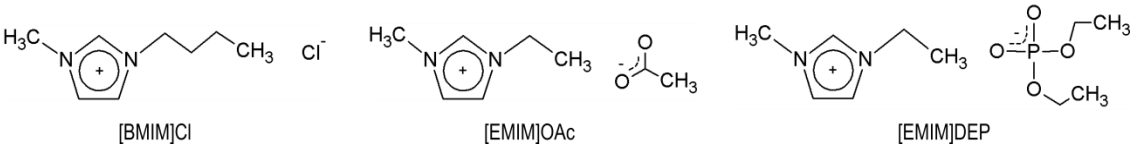


Figure 3.1: Chemical structures of the ionic liquids.

3.2.2 Screening of Pretreatment Reagents

To evaluate the effectiveness of the pretreatment method for rice husk, the application of conventional and dissolution pretreatments in pretreating rice husk were investigated. The assessment for conventional and dissolution pretreatments were conducted separately as both pretreatments are different in nature. The reagents were assessed by examining the effects they exerted on the rice husk composition and structure. The selected reagent also has to be economical feasible and does not cause major harm to the environment when carried out in large scale.

3.2.2.1 Chemical Pretreatment

The screening of sodium hydroxide, calcium hydroxide, sulphuric acid, hydrochloric acid, phosphoric acid, acetic acid and nitric acid was carried out in 25-ml capped test tubes with experimental condition as shown in Table 3.1. The experimental condition was pre-determined from literatures on the pretreatment of lignocellulosic biomass (Chang et al., 1997; Chang et al., 1998; Saha and Cotta, 2008; Torget et al., 1991). The total reducing sugar content of the hydrolysates was determined by means of 3,5-dinitrosalicylic acid (DNS) test. The control of experiment was done by heating rice husk in distilled water. The pH of hydrolysates was adjusted to neutral or slightly alkaline using 1 M NaOH or 1 M HCl before DNS test. The hydrolysates were filtered through 0.45 μm regenerated cellulose filter. All the hydrolysate samples prepared were kept at 4^{o}C prior to analysis. All the screenings were conducted in three replicates.

Table 3.1: Screening condition for conventional pretreatment

Parameter	Condition
Reagent loading	0.5% (w/v)
Rice husk loading	10% (w/v)
Water loading	10 ml/g
Pretreatment temperature	100 $\pm$ 1^{o}C
Pretreatment duration	2 hours

The performance of the reagents was evaluated based on the amount of total reducing sugar detected in the hydrolysate. Total reducing sugar content was employed as an indirect indicator for the extent of rice husk pretreatment. Higher total reducing sugar content in hydrolysate indicates more severe disruption of rice husk structure, and vice versa.

3.2.2.2 Ionic Liquid Dissolution Pretreatment

The performance three ionic liquids, namely 1-butyl-3-methylimidazolium chloride, 1-ethyl-3-methylimidazolium acetate, and 1-ethyl-3-methylimidazolium diethyl phosphate, were evaluated. The ionic liquids selected were based on the dissolution and regeneration of cellulose studies reported previously in the literature (Jagadeeswara Rao et al., 2007; Li et al., 2009; Sun et al., 2009; Swatloski et al., 2002; Zhao et al., 2009; Zhu et al., 2006).

Screening of ionic liquids for dissolution pretreatment was conducted in 25-ml capped test tubes. The condition for dissolution pretreatment is shown in Table 3.2. The reaction mixture of rice husk and ionic liquid was pretreated in an oil bath (Julabo MC (V.2), Germany), and maintained at 100^{o}C for 10 hours. At the end of the pretreatment, the reaction mixture is consisted of ionic liquid-dissolved cellulose and undissolved rice

husk (hereafter called rice husk residue). The dissolution of rice husk was carried out in triplicate.

Table 3.2: Screening condition for ionic liquid dissolution pretreatment

Parameter	Condition
Rice husk-ionic liquid mixture	1.5% (w/v)
Pretreatment temperature	100 $\pm$ 1^{o}C
Pretreatment duration	10 hours

After the dissolution, an equal volume of deionised water (Sartorius, arium® 611UF, Germany) was added to the clear reaction mixture to precipitate regenerated cellulose before the rice husk residue was filtered. The cellulose-rich material (henceforth called regenerated cellulose) precipitated from the mixture was filtered. Both the regenerated cellulose and rice husk residue were washed with deionised water to remove ionic liquid completely, and dried in an oven (Jouan Inc., USA) at 60^{o}C prior to analyses.

3.2.3 Optimization of Pretreatment

The pretreatment reagent selected from screening studies was used as one of the parameter in the optimization of rice husk pretreatment. Three parameters known to have effect on the pretreatment of lignocellulosic biomass, namely reagent loading (X_1), pretreatment duration (X_2), and temperature (X_3), were investigated. To determine the low and high levels of the chosen parameters, preliminary tests were conducted with the following ranges: (i) reagent loading, 0 – 4% (w/v); (ii) pretreatment duration, 1 – 6 hours; (iii) pretreatment temperature, 60 – 140^{o}C. The preliminary tests were performed using one-factor-at-a-time approach, and total reducing sugar content in the hydrolysate was measured as a response for rice husk pretreatment.

The low and high levels of the parameters determined from the preliminary tests were used in the optimization of rice husk pretreatment using Box-Behnken experimental design (BBD). This design is a combination of 2^k factorials with incomplete block design consisted of 17 sets of experiments, including 5 replications of the centre point. Total reducing sugar content of the hydrolysate was determined as response (Y). The response was optimized with response surface methodology (RSM) with the ultimate aim to maximize the release of total reducing sugar from rice husk pretreatment. Regression analysis of data was performed with the aid of Design-Expert version 6.0.6 (Stat-Ease Inc., Minneapolis).

3.2.4 Analytical Methods

The total reducing sugar content in the hydrolysate from the pretreatment of rice husk was determined by 3, 5-dinitrosalicylic acid (DNS) test. The chemical and structural characteristics of the pretreated samples were analyzed using Fourier transform-infrared (FT-IR) spectroscopy, X-ray diffraction (XRD), scanning electron microscopy (SEM), and Brunauer, Emmett and Teller (BET) surface area analysis.

3.2.4.1 3, 5-dinitrosalicylic acid (DNS) Test

The DNS reagent containing 1% (w/v) 3,5-dinitrosalicyclic acid (DNS, Merck), 0.2% (w/v) phenol (Merck), 0.05% sodium metabisulfite (Merck), and 1% (w/v) sodium hydroxide (Merck) was prepared as outlined by Miller (1959). The stock solution was prepared without sodium metabisulfite in an opaque bottle. Appropriate amount of sodium metabisulfite was added to the aliquot prior to use. Rochelle salt solution (40%, w/v) was prepared by dissolving sodium potassium tartrate in distilled water.

The reaction mixture for DNS test containing 3 ml DNS reagent and 0.5 ml hydrolysate was heated in a boiling water bath for 15 minutes. Immediately after boiling, the reaction mixture was cooled to room temperature in a tempering bath, and 1 ml of 40% (w/v) Rochelle salt was added to the reaction mixture to stabilize the colour formed (Miller, 1959). The absorbance of the boiled reaction mixture was read at 575 nm with an ultra violet-visible (UV-VIS) spectrophotometer (Secomam Prim Advanced, France). The concentration of total reducing sugar was determined from a calibration curve constructed with glucose standards prepared from anhydrous D-glucose (Fisher Scientific). Refer to Appendix B: Construction of glucose standard calibration curve.

3.2.4.2 Fourier Transform-Infrared (FT-IR) Spectroscopy

The FT-IR spectra of the sample between 400 and 4000 cm^{-1} at 4 cm^{-1} nominal resolution were recorded at room temperature with a FT-IF/FT-FIR spectrometer (Perkin Elmer, Spectrum 400, USA). The spectra are presented in relative transmittance percentage (%) of wave number (cm^{-1}) and the background was recorded with an empty cell.

3.2.4.3 X-Ray Diffraction (XRD)

The crystallinity of the samples were examined by XRD measurement performed with a D8 Advanced X-Ray Diffractometer (Bruker AXS, USA) using Cu-K_α monochromatized radiation at 40 kV and 40 mA at ambient temperature. The samples were scanned and the intensity was recorded in 2θ range from 10^o to 80^o with a step size of 0.02^o. The crystallinity index (*CrI*) of the samples was calculated with equation 3.6 (Parikh et al., 2007).

$$CrI = \frac{I_{002} - I_{am}}{I_{002}} \times 100 \qquad (3.6)$$

, where I_{002} = intensity of the diffraction from the 002 plane at 2θ = 22.6^{o} (crystalline area), and

I_{am} = intensity of the background scatter measured at 2θ = ~18.7^{o} (amorphous area).

3.2.4.4 Scanning Electron Microscopy (SEM)

The structural changes of the pretreated rice husk was assessed with scanning electron microscope Quanta™ 200 FESEM (FEI, USA) operated at 2 - 5 kV accelerating voltage under low vacuum. Rice husk samples were affixed onto aluminium stubs with double sided adhesive carbon tapes and examined without metal-coating.

3.2.4.5 Brunauer, Emmett and Teller (BET) Surface Area Determination

The surface area and average pore size samples were determined by nitrogen adsorption isotherm at 77 K using a high-performance six-sample surface area and pore size analyzer Autosorb®-6B (Quantachrome, Florida, USA). The nitrogen adsorption-desorption isotherm was operated at relative pressure P/P_0 of 0.3, where P is the system pressure and P_0 is the initial pressure at 1 bar. The BET surface area of samples was determined using equation 3.7.

$$\frac{1}{W\left[\left(P_0/P\right)-1\right]}=\frac{1}{W_m C}+\frac{C-1}{W_m C}\left(\frac{P}{P_0}\right) \qquad (3.7)$$

, where W = sample weight,

W_m = weight of constituting monolayer thickness of nitrogen coverage on sample, and

C = interaction of nitrogen and sample.

The surface are (A) of the sample was calculated using equation 3.8.

$$A = \frac{W_m \times N \times A_{CS}}{M} \tag{3.8}$$

, where $W_m = (S + I)^{-1}$,

S and I = slope and intercept of the plot $\left[1/W\left(\left(P_0/P\right)-1\right)\right]^{-1}$ against P/P_0,

N = the Avogadro's number (6.023×10^{23} molecules/mole),

A_{CS} = cross-sectional area of nitrogen molecule (16.2 $Å^2$), and

M = the molecular weight of nitrogen.

3.2.5 Statistical Analysis

Regression analysis of the optimization data was performed using Design-Expert Version 6.0.6 (Stat-Ease Inc., Minneapolis). A second order model was selected to predict the optimal point and the regression model representation of the three-factor factorial experiment can be expressed by equation 3.9. The type of model equation and statistical significance of regression coefficients was determined by Fischer's test and Student's t-test, respectively.

$$\begin{aligned} Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 \\ + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \end{aligned} \tag{3.9}$$

, where Y = the response,

β_n = parameter whose value is to be determined (n = 1 to 3), and

X_n = variable of a factor (n = 1 to 3).

3.3 Selection of Fungus with Ligninolytic Activity

The selected fungi were screened for their ligninolytic enzyme activity via synthetic chemical reagents, namely guaiacol and Remazol brilliant blue-R. The fungus selected was employed for the subsequent solid-state fermentation of rice husk to produce laccase enzyme.

3.3.1 Fungal Strains

The fungi, *i.e. Phanerochaete chrysosporium, Pycnoporus sanguineus*, *Phlebia radiata*, and *Pleurotus sajor-caju*, were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). Three fungi, namely *Pycnoporus sanguineus* (DSM3023), *Phlebia radiata* (DSM5111), and *Pleurotus sajor-caju* (DSM8265), were screened in this study. These fungi are able to degrade lignocellulose (Esposito et al., 1991; Mane et al., 2007; Niku-Paavola et al., 1988; Okino et al., 2000; Reddy et al., 2003; Thomas et al., 1998; Vares et al., 1995). On the other hand, *Phanerochaete chrysosporium* (DSM1556), which has been extensively reported to possess the ability to degrade xenobiotic substances (De Jong et al., 1992; Fernando et al., 1990; Glenn and Gold, 1983; Okino et al., 2000; Wariishi et al., 1992), was used as a reference strain (positive control) throughout the screening study. The fungi were maintained on malt extract peptone agar (MEPA) at 4^{o}C (Atlas, 2004) and was periodically sub-cultured (Smith and Onions, 1983).

3.3.2 Preparation of Media

Malt extract peptone agar (MEPA) was used for fungal culture maintenance. The MEPA medium (Atlas, 2004) contained malt extract (Becton Dickinson), 30 g/L; Bacto Soytone (Becton Dickinson), 3 g/L; and granulated agar (Becton Dickinson), 15 g/L.

The pH of the MEPA medium was checked and adjusted to pH 5.6 before the addition of granulated agar.

Two types of screening reagents – guaiacol and RBB-R were used in the preparation of ligninolytic enzyme activity screening agar media. The screening reagents were prepared in 2% (w/v) basal malt extract agar (MEA). The 2% (w/v) basal MEA contained malt extract (Becton Dickinson), 20 g/L; and granulated agar (Becton Dickinson), 15 g/L. The guaiacol screening medium contained 0.1 g/L guaiacol (Sigma-Aldrich, catalogue #G5502) in 2% (w/v) basal MEA (Mtui and Masalu, 2008; Okino et al., 2000); whereas, RBB-R screening medium was contained 0.2 g/L RBB-R (Sigma-Aldrich, catalogue #R8001) in 2% (w/v) basal MEA with reference to the method outlined by Mtui and Masalu (2008). The screening media were stored in an inverted position at 4^{o}C before used.

3.3.3 Screening of Fungal Ligninolytic Enzyme Activity

The screening for enzyme activity was conducted at two temperatures, *i.e.* the laboratory room temperature (about 26 – 28^{o}C, hereafter called room temperature) and 35^{o}C. Room temperature approximates the optimal growth temperature of the fungi being screened that is 25^{o}C. The purpose of conducting screening at 35^{o}C was to examine the fungal ability to remain active at a higher temperature. The ability to withstand high temperature is important in solid-state fermentation as the process temperature can rise by 10^{o}C or more in large scale fermentation. Fungus with positive guaiacol oxidation and RBB-R decolourization activities were shown by the formation of reddish brown ring and RBB-R decolourization ring around the fungal colony, respectively.

3.3.3.1 Screening at Room Temperature

The inoculum used in the screenings was 6 mm-diameter agar plug cut from actively expanding end of established fungal culture incubated at their respective optimal growth temperatures (*P. chrysosporium*, 35^{o}C; *P. sanguineus*, 22^{o}C; *P. radiata*, 25^{o}C; and *P. sajor-caju*, 25^{o}C) as indicated by DSMZ. The inoculum was transferred to the screening media by means of standard agar plug technique as detailed by Pommerville (2007) and Fusaro (1972). The screenings were done in five replicates. Negative control was prepared by inoculating the screening media with agar plug cut from a 2% (w/v) MEA. The inoculated plates were incubated for 48 hours in the dark. The fungal colony and halo area resulted from ligninolytic enzyme activity were estimated in cm^2. Figure 3.2 illustrates the grid used for the estimation of fungal colony and halo areas. The ratio of halo ring area to colony area was computed using equation 3.10. The computed ratio indicates the efficiency of fungal ligninolytic enzyme activity, and it was used as a basis for comparison among the fungi.

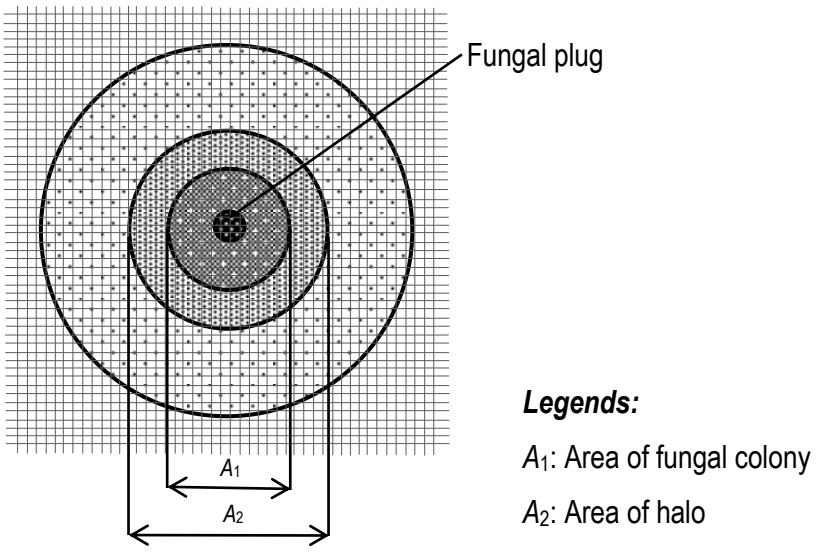


Figure 3.2: Aerial view of an inoculated screening plate. Illustration of the areas spanned by fungal colony and halo resulted from ligninolytic enzyme activity. Each small square spanned an area of 0.04 cm^2.

$$\text{Ratio of Halo / Colony} = \frac{\text{Halo ring area}}{\text{Colony area}} \quad (3.10)$$

3.3.3.2 Screening at Higher Temperature (35^{o}C)

The screenings were repeated with incubation at 35^{o}C to investigate the effect of temperature on the fungal growth and production of ligninolytic enzyme activity. The other screening conditions remain unchanged.

3.4 Solid-State Fermentation

This section includes a brief description of the solid-state fermentation experimental set-up, a newly-developed inoculum preparation method, profiling of fermentation duration and effect of inducers, optimization study of solid-state fermentation, modelling of laccase enzyme production, and characterization of laccase enzyme.

3.4.1 Experimental Set-Up

The solid-state fermentation was carried out in batch using rice husk pretreated under optimized condition and the selected fungus *Pleurotus sajor-caju*. The solid-state fermentation was conducted in a static drum fermenter, which mimic the fermenter for *koji* process. Figure 3.3 shows the experimental set-up of the solid-state fermentation fermenter. The porous seal allows exchange of air in the fermenter. The rotating laboratory mixer is initiated during harvest of enzyme to agitate the solid substrate-buffer solution mixture. The laboratory scale fermenter was set-up in a dark and cool chamber at room temperature humidified with moisten cotton.

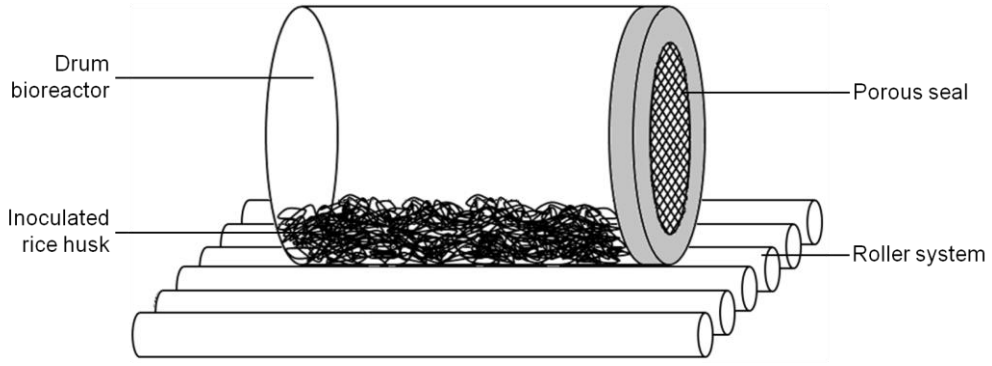


Figure 3.3: Experimental set-up of the laboratory scale fermenter.

3.4.2 Development of Novel Inoculum Preparation – Cellophane Film Culture (CFC) Technique

The inoculum of *Pleurotus sajor-caju* was prepared by transferring 6 mm-diameter agar plug from an established culture to MEPA plates overlaid with autoclaved cellophane films. The inoculated plates were incubated at 25^{o}C in the dark. Figure 3.4 illustrates a cellophane film being overlaid on an agar plate. The fungal biomass separated from the cellophane film culture was used as inoculum in the solid-state fermentation.

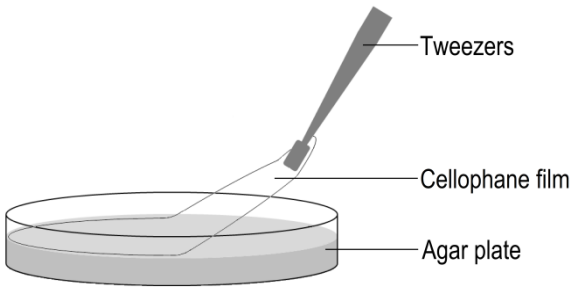


Figure 3.4: Overlaying agar plate with cellophane film in cellophane film culture (CFC) technique.

To quantify fungal biomass for inoculum loading, a regression correlation plot was constructed. The fungal biomass was separated from the cellophane film for the determination of fungal wet weight and the respective dry weight was determined after

drying at 80^{o}C overnight as outlined by Reeslev and Kjoller (1995). A total of 57 replicates were performed, and sampling was carried out from the third day of incubation onwards until complete colonization of the plates. A correlation was established for the fungal wet and dry weight. For regression analysis, the fungal wet and dry weights were fitted to a linear model with linear regression by using SigmaPlot for Windows Version 11.0 (Systat Software, Inc., Germany).

3.4.2.1 Technique Verification and Validation

Verification of the technique was carried out in solid-state fermentation of pretreated rice husk, and the viability of the inoculum was measured by using laccase enzyme activity as an indicator. The technique verification was conducted with a pre-determined solid-state fermentation conditions: initial moisture level of substrate, 55% (w/w); inoculum loading, 0.5% (w/w); fermentation duration, 10 days; and incubation temperature, ~26^{o}C.

For comparison, *P. sajor-caju* was grown on MEPA plates overlaid with nylon membrane, where it has been used to grow mycelial fungus for microbiological studies (Funder and Johannessen, 1957). A total of 20 replicates were carried out in the study.

The validity of the inoculum preparation technique was conducted by using *Pycnoporus sanguineus*, which has also been reported for its laccase production in solid-state fermentation (Abdul Karim and Mohamad Annuar, 2009). A total of 20 replicates were carried out in the validation study.

3.4.3 Time Profile of Laccase Enzyme Production

The laccase enzyme produced by *Pleurotus sajor-caju* over a period of time profile was investigated to determine the period in which the fungus produces the highest laccase enzyme activity. The solid-state fermentation conditions used for the study are shown in Table 3.3. The entire content of one fermenter was sampled every 24 hours for laccase enzyme assay.

Table 3.3: Solid-state fermentation conditions for laccase enzyme production time profile study

Parameter	Condition
Moist solid loading	5 g
Moisture content of solid	65 %
Moistening agent	Distilled water
Inoculum loading	0.5% (w/w)
Fermentation temperature	Room temperature (~25^{o}C)

3.4.4 Effect of Inducers

In this study, three inducers, namely copper sulphate ($CuSO_4$), glucose and Tween 80, reported to possess effects on laccase production were investigated. The inducers were examined at concentrations of 0.2 mM $CuSO_4$ (Sigma-Aldrich), 10 g/L glucose (Fisher Scientific), and 0.015 g/L Tween 80 (Sigma-Aldrich) according to the literatures. The inducer solutions were prepared by dissolving appropriate amount of chemical/reagent in distilled water.

From the time profile study, the fungal laccase enzyme activity achieved peak in 72 hours, after which it decreased and fluctuated at a low level from 192 hours until the end of the investigation. Therefore, the effect of inducers was investigated at two fermentation durations – 72 and 192 hours, respectively. All the parameters remained

the same as in the time profile study, except the moistening agent was replaced with solution containing the respective inducer. The experimental control was conducted in the absent of inducers. The laccase enzyme activity of the crude enzyme filtrate was analyzed.

3.4.5 Optimization of Solid-State Fermentation

The optimization of laccase enzyme production was conducted with four parameters, *i.e.* fermentation duration (X'_1), concentrations of copper sulphate (X'_2), glucose (X'_3) and Tween 80 (X'_4). The optimization was carried out using central composite experimental design (CCD). The experimental design, which consisted of a fractional factorial design, a set of central points and a set of axial points, has 30 runs that were divided into three blocks to reduce the variability transmitted from different batches of pretreated rice husk and inoculum. The ranges of the parameters were determined from the time profile and effect of inducers studies. Laccase enzyme activity and total soluble protein of the crude enzyme filtrate were assayed for the computation of specific laccase enzyme activity. The ultimate aim of the optimization was to maximize the specific laccase enzyme activity (Y') in the crude enzyme filtrate using response surface methodology (RSM). Regression analysis of the optimization data was performed using Design-Expert Version 6.0.6 (Stat-Ease Inc., Minneapolis).

The optimized solid-state fermentation condition was further verified with independent runs in triplicate. The error between predicted and experimental response of laccase enzyme activity was estimated.

3.4.6 Recovery of Enzyme

A 500 ml citrate buffer stock (1 M, pH 4.5) was prepared by dissolving 105 g citric acid monohydrate salt (Merck) in approximately 375 ml of distilled water. The pH of the solution was adjusted to about 4.3 with NaOH, and diluted to a final volume of 500 ml. Citrate buffer (100 mM, pH 4.8) used for harvesting of laccase enzyme was prepared by diluting 1 M citrate buffer stock to an appropriate volume.

To harvest the enzyme, 10 ml of 100 mM citrate buffer was added to the fermented rice husk. The content was agitated on a laboratory rotating mixer for 1 hour before the liquid portion was transferred to a capped-test tube. The test tube was spinned in a refrigerated centrifuged (Eppendorf Centrifuge 5918 R, Germany) at 3,500 rpm for 20 minutes to remove residual rice husk particle and fungal biomass. The cell-free supernatant (hereafter named crude enzyme filtrate) was transferred to another test tube and stored at -20^{o}C prior to analyses.

3.4.7 Analytical Methods

3.4.7.1 Laccase Enzyme Assay (ABTS)

The 2,2'-azino-bis (3-ethylbenzothiazoline)-6-sulphonic acid (ABTS) reagent for laccase enzyme assay was prepared from ABTS chromophore, diammonium salt (Calbiochem, catalogue #194430). The reagent was prepared as 30 mM solution, which was subsequently diluted to 2.5 mM in the final reaction mixture volume. Citrate buffer solution (300 mM) was prepared by diluting 1 M citrate buffer stock to an appropriate volume. The citrate buffer solution was diluted to 100 mM in the final reaction mixture volume.

The laccase enzyme activity was assayed by monitoring the increment in absorbance from the oxidation of ABTS at 420 nm with Secomam Prim Advances UV-VIS spectrophotometer at room temperature using a molar extinction coefficient (ε_{420}) of 36,000 $M^{-1}cm^{-1}$ (Atalla et al., 2010; Gupte et al., 2007; Mtui and Masalu, 2008). The reaction mixture contents of filtrate sample and the blank sample are tabulated in Table 3.4. The absorbance (A) is directly proportional to the concentration of ABTS oxidation product formed (Beer's Law) and the thickness of the sample (Lambert's Law), and their relationship is represented by the Beer-Lambert equation (equation 3.11). The enzyme activity is expressed in U/L, which is defined as the amount of enzyme catalyzing the production of one μmol coloured product per minute per litre.

$$A = \varepsilon \cdot c \cdot l \tag{3.11}$$

, where ε = molar extinction coefficient,

c = concentration of ABTS oxidation product (mol/L), and

l = thickness of the sample (cm).

Table 3.4: Reaction mixture content of (a) filtrate and (b) blank sample of laccase enzyme assay

(a) *Filtrate sample*

Reagent	**Volume (μl)**
ABTS (2.5 mM)	150
Citrate buffer (100 mM, pH 4.8)	600
Distilled water	450
Crude enzyme filtrate	600
(Total volume)	1800

(b) *Blank sample*

Reagent	**Volume (μl)**
ABTS (2.5 mM)	150
Citrate buffer (100 mM, pH 4.8)	1200
Distilled water	450
(Total volume)	1800

3.4.7.2 Total Soluble Protein Content

The total soluble protein content of the enzyme samples was quantified by using Total Protein Kit (product no. TP0300 and L3540, Sigma-Aldrich, USA), which applies Peterson's modification of Micro Lowry method. The chemical reagents, *i.e.* Lowry reagent, and Folin and Ciocalteu's phenol reagent working solutions were prepared according to the instruction given by the manufacturer (Sigma-Aldrich, USA). The prepared reagents were stored at room temperature. Protein standard used in the assay was bovine serum albumin (BSA). The protein standard stock solution (400 μg/ml) was prepared by adding approximately 5 ml of deionised water to 2 mg of BSA, and swirled to completely dissolve the content.

Total soluble protein determination was conducted with reference to the protocol provided by the manufacturer (refer to Appendix C: Protocol for total soluble protein determination). The absorbance of laccase enzyme sample was measured at 765 nm. Absorbance reading of the samples was completed within 30 minutes after colour development. Blank sample was carried out by substituting the sample with 1 ml of deionised water. The protein standards, blank sample, and samples were treated the same during the assay.

3.4.8 Modelling of Laccase Enzyme Production

The increasing phase of enzyme production of the optimized solid-state fermentation was modelled based on the logistic model, and it is assumed that the laccase enzyme activity in the increasing phase is proportional to the fungal biomass concentration (Al-Asheh and Duvnjak, 1994; Okazaki et al., 1980).

The data of specific laccase enzyme activity was collected from a total of 26 runs at random fermentation intervals between 0 and 84 hours under the optimized conditions. The model parameters were estimated from all experimental data simultaneously using the parameter estimation software Matlab® V7.6.0.324 (R2008a) (The MathWorks, Inc., USA). The ordinary differential equations were solved by finding a constrained minimum of the model equations coefficients at an initial estimation. The initial estimate of the coefficients are adopted from Al-Asheh and Duvnjak (1994): (i) maximum specific growth rate (μ_m) = 0.0560 h^{-1}; (ii) maximum biomass concentration (X_m) = 0.078 g/g moist rice husk; (iii) rate constant for enzyme production (k_v) = 0.531 units/g moist rice husk/h. The initial fungal biomass concentration (X_0) = 0.01114 g/g moist rice husk was used in the study. The fitted curve was validated with another set of experimental data that were collected at 12-hour intervals between 0 and 84 hours. Lastly, the root-mean-square (RMS) error between the fitted curve and the experimental data was calculated.

3.4.9 Characterization of Laccase Enzyme

The characterization studies were preceded with partially purification of laccase enzyme followed by the molecular weight determination and substrate specificity investigation of the partial purified laccase enzyme.

3.4.9.1 Partial Purification of Laccase Enzyme

The crude enzyme filtrate was partially purified with Amicon® Ultra-15 centrifugal filter (Millipore, USA) with a 30 kDa cut-off regenerated cellulose membrane. Approximately 15 ml of crude enzyme filtrate was added to the filter device and spinned at 4,000 × g for 15 minutes with a refrigerated centrifuge with swinging bucket

rotor (Eppendorf Centrifuge 5918 R, Germany). Immediately after centrifugation, the concentrated enzyme filtrate was withdrawn from the filter device using a side-to-side sweeping motion to ensure total recovery. The ultrafiltrate was stored in a centrifuge tube. The partially purified enzyme and ultrafiltrate were subjected to subsequent enzyme characterization studies.

3.4.9.2 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Laccase Enzyme

Running buffer for the SDS-PAGE is 1× Tris/glycine/SDS. It was prepared from 10 times dilution of 10× Tris/glycine/SDS buffer stock (Bio-Rad, USA). The diluted running buffer was stored at room temperature.

The apparent molecular mass of the laccase enzyme was estimated by SDS-PAGE with Criterion vertical electrophoresis system (Bio-Rad Criterion™ Cell, USA). The SDS-PAGE was conducted with a 7.5% Criterion Tris-HCl precast gel with 12 wells and thickness 1.0 mm (Bio-Rad, USA) at room temperature. The partial purified laccase sample was treated with 5% β-mercaptoethanol (Bio-Rad, USA), diluted with Laemmli sample buffer (Bio-Rad, USA) at a ratio of 1:1, and the diluted sample was heated at 70^{o}C for 10 minutes. After heating, 45 µl of the treated sample was loaded to the sample well. Precision Plus Protein™ standards containing protein markers with molecular mass of 10 kD, 15 kD, 20 kD, 37 kD, 50 kD, 75 kD, 100 kD, 150 kD, and 250 kD were used as standards. The gel was run at 70 V constant for 145 minutes. The gel was visualised with SimplyBlue™ SafeStain (Comassie G-250 stain, Novex® by Life Technologies, USA). Refer to Appendix D: Protocol for staining SDS-PAGE gel.

3.4.9.3 Substrate Specificity Study

Spectrophotometric measurements of substrate oxidation by the partial purified laccase enzyme of *P. sajor-caju* were carried out at room temperature in a 1.8-ml reaction volume containing the filtrate sample in 100 mM sodium citrate buffer (pH 4.8). Various reagents used in the measurement of specific enzyme activity together with the maximal absorption wavelengths and molar extinction coefficients of the reagents are shown in Table 3.5.

Table 3.5: Maximal absorption wavelengths and molar extinction coefficients of the reagents

Reagent	**Conc.**	**Maximal absorption wavelength (nm)**	**Molar extinction coefficient ($M^{-1}cm^{-1}$)**
2,2'-azino-bis (3-ethylbenzothiazoline) -6-sulphonic acid (ABTS)	30 mM	420	36,000
Guaiacol	50 g/L	470	6,740
Remazol brilliant blue-R (RBB-R)	50 g/L	590	5,820
2,6-dimethoxyphenol (DMP)	30 mM	468	10,000

CHAPTER 4

PRETREATMENT OF RICE HUSK

Pretreatment of rice husk serves as a crucial step in improving substrate digestibility by microbes via partially breaking the recalcitrant structure of rice husk for fungal solid-state fermentation to produce ligninolytic enzyme. This chapter consists of two sections. The first section reports comprehensive screening and selection of pretreatment reagent studies that include conventional and non-conventional reagents for pretreating rice husk. Screening and assessment of pretreatment reagents are necessary for the pretreatment of rice husk. The findings of conventional and ionic liquid dissolution pretreatments of rice husk were reported. The feasibility of employing these pretreatments to rice husk was discussed by considering the modifications that they imparted to rice husk chemical composition and structure. The second section reports the optimization of rice husk pretreatment with response surface methodology by using the selected reagent. This section involves preliminary studies for the determination of suitable ranges for parameters under consideration, followed by optimization using Box-Behnken experimental design (BBD).

4.1 Results and Discussion

4.1.1 Characterization of Rice Husk

Prior to the comprehensive pretreatment studies, the chemical composition of the rice husk sample was characterized. The acid detergent fibre (ADF) and neutral detergent fibre (NDF) contents of the rice husk sample were 72.9 ± 0.7% (w/w) and 77.5 ± 0.2% (w/w), respectively. These values were slightly lower compared to the values reported by Contreras Lara and co-workers (1999) whereby the ADF and NDF contents were 79.0 ± 3.1% (w/w) and 83.1 ± 2.0% (w/w), respectively. The differences in values

might be due to different approaches employed in ADF and NDF determinations. Nevertheless, the rice husk sample was composed mainly of cellulose and lignin (Vegas et al., 2004), both sum up to more than 70% of the biomass composition (Table 4.1). The cellulose content of rice husk sample was comparatively higher compared to the rice husk samples reported by other researchers (Khan and Raza, 2005; Saha and Cotta, 2008; Vegas et al., 2004). However, hemicellulose content of the rice husk sample was comparatively lower. The differences in rice husk lignocellulosic contents might be attributed to geographical locations where the crop was grown and variety of the rice crop. The lignin, ash, and moisture contents of the rice husk sample were fairly consistent compared with rice husk from different localities previously reported (Table 2.5).

Table 4.1: Composition of rice husk sample

Properties	**Content (%, w/w)**	**Method**
Cellulose [a]	53.2 ± 0.4	AOAC 973.18
Hemicellulose [b]	4.6 ± 0.6	AOAC 2002.04
H_2SO_4 lignin	19.7 ±0.3	AOAC 973.18
Moisture	10.6 ± 0.4	AOAC 943.01
Ash	13.9 ± 0.6	AOAC 942.05

[a] Cellulose content was determined from the difference between ADF and H_2SO_4 lignin contents.
[b] Hemicellulose content was determined from the difference between ADF and α-amylase neutral detergent fibre organic matter.

4.1.2 Screening and Selection of Pretreatment Reagent

4.1.2.1 Chemical Pretreatment

In general, the alkalis and acids used in the conventional pretreatments, namely NaOH, $Ca(OH)_2$, H_2SO_4, HCl, H_3PO_4, CH_3COOH, and HNO_3, hydrolyse hemicellulose and fraction of cellulose in rice husk to simple sugars (Weil et al., 1994). The simple sugars were detected as total reducing sugar content in pretreatment hydrolysates, and it

reflects the extent of the conventional pretreatments. The performance of the acids and alkalis in rice husk pretreatment is illustrated by Figure 4.1. Based on the findings of screening, each alkali and acid had pretreated rice husk to a varying extent. Low amount of total reducing sugar was detected in the hydrolysates of NaOH and $Ca(OH)_2$ pretreatments. This is because the alkalis only fractionally hydrolysed hemicellulose and cellulose (Weil et al., 1994), but mainly delignified rice husk (Brannvall, 2004). The delignification action of the alkalis is evidenced by the dark brown appearance of hydrolysate that is imparted by the solubilised lignin. In overall, acids were found to be better pretreatment reagents than alkalis. The highest total reducing sugar content was detected in the hydrolysates of HCl pretreatment (15.0 $\pm$ 0.6 mg/ml), followed by HNO_3 (12.2 $\pm$ 0.1 mg/ml) and H_2SO_4 (7.2 $\pm$ 0.3 mg/ml) pretreatments. Pretreatments with CH_3COOH, H_3PO_4, NaOH, and $Ca(OH)_2$ only produced less than 1 mg total reducing sugar/ml hydrolysate, which is close to that of the control. In the experimental control, boiling of rice husk in water at 100^{o}C hydrolyzed the amorphous hemicellulose fraction of the lignocellulosic biomass to reducing sugars (Mok & Antal, 1992; Sreenath et al., 1999; Teleman, 2009; Weil et al., 1994).

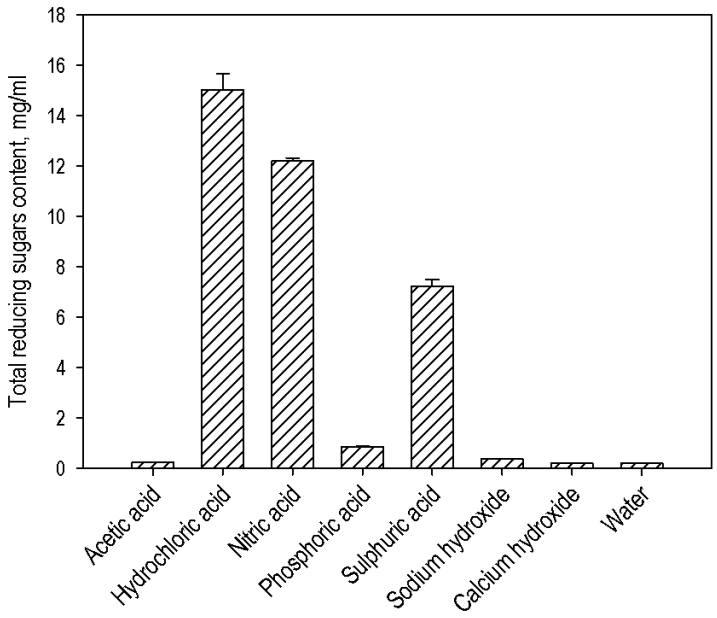


Figure 4.1: Performance of acids and alkalis in rice husk pretreatment.

The chemical composition of rice husk residue after pretreatments is shown in Figure 4.2. The characterization of rice husk samples showed that all acid-pretreated rice husk had reduced hemicellulose content, which explains the main role of acids were to hydrolyze amorphous hemicellulose in the substrate (Orozco et al., 2007). The hydrolysis of hemicellulose in rice husk contributed to the total reducing content in hydrolysate. Furthermore, cellulose was also partially hydrolyzed during the acid pretreatments (Weil et al., 1994), particularly pretreatments using strong acids like HCl, HNO_3 and H_2SO_4. The availability of more reactive protons disrupted the hydrogen bonding of cellulose chain prior to hydrolysis and resulted in higher total reducing sugar yields (Orozco et al., 2007). On the other hand, the lignin contents of the rice husk residue from alkaline pretreatments were slightly reduced compared to acid-pretreated samples signified the delignification action of the alkali.

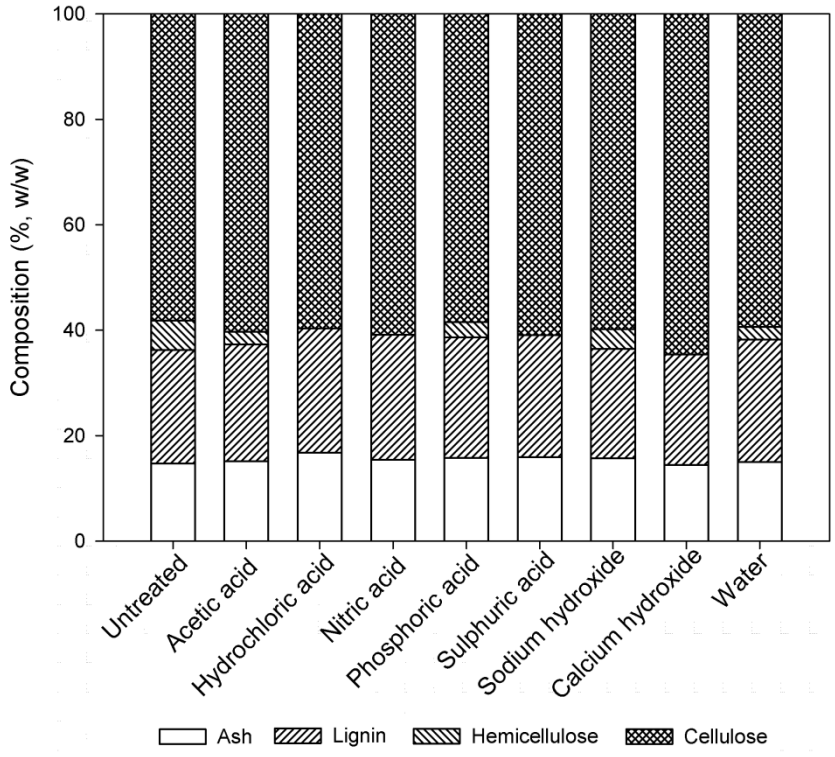


Figure 4.2: Chemical composition of rice husk after acid and alkali pretreatments (based on the same initial rice husk sample weight).

Among the reagents studied, HCl hydrolysed rice husk and released the highest amount of total reducing sugar during pretreatment. This signifies the effectiveness of HCl pretreatment in disrupting rice husk structure, while retaining significant amount of cellulose (~60%) and lignin (~20%) in the pretreated rice husk. To obtain a deeper understanding of the action of HCl in rice husk pretreatment, the HCl-pretreated rice husk were subjected to structural characterization with Fourier transform-infrared (FT-IR) spectroscopy, X-ray diffraction (XRD), scanning electron microscopy (SEM), and Brunauer, Emmett and Teller (BET) surface area determination.

The FT-IR spectroscopy spectra of HCl-pretreated rice husk were illustrated in Figure 4.3. Changes in relative transmittance were observed in five bands related to chemical structure of lignocellulosic biomass, namely 790, 1046, 1423, 1511 and 2924 cm^{-1}. Table 4.2 shows the group frequency of absorption bands of lignocellulosic biomass and their assignments. FT-IR spectroscopy spectra revealed that the pretreated rice husk exhibited prominent enhancement of cellulosic content as shown by the intensified bands at 1046 and 2924 cm^{-1}. The band at 1046 cm^{-1} represents C-O stretching vibration in cellulose/hemicellulose and aryl-OH group in lignin (Guo et al., 2008; Hurtubise and Krassig, 1960; Labbe et al., 2005), whereas the band at 2924 cm^{-1} is attributed to C-H stretching in methylene of cellulose (Liu et al., 2007; Oh et al., 2005). In addition, cellulose portion of the HCl-pretreated rice husk has more disordered structure, which is represented by the intensified band of C-H deformation vibration for pretreatment of rice husk (Labbe et al., 2005). The HCl-pretreated rice husk also possessed lignin that is reflected by the slight enhancement of bands at 1511 and 1423 cm^{-1}. These bands reflect the aromatic skeletal stretching in lignin (Coates, 2000; Hsu et al., 2010), and asymmetric bending of methyl and methoxy groups present in lignin (Guo et al., 2008;

Labbe et al., 2005; Sun et al., 2009), respectively. The findings of FT-IR spectroscopy complemented and confirmed the chemical composition analysis of pretreated rice husk mainly consisted of cellulose and lignin.

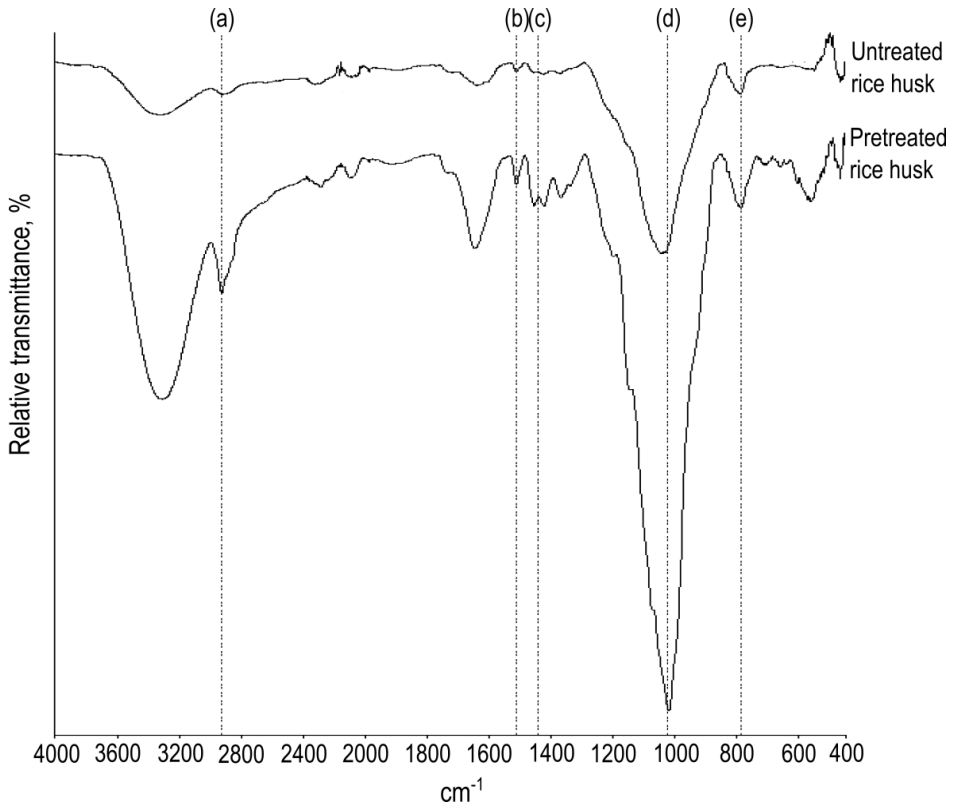


Figure 4.3: FT-IR spectra of untreated and HCl-pretreated rice husk.

FT-IR spectral bands (cm^{-1}): (a) 2924; (b) 1511; (c) 1423; (d) 1046; (e) 790.

The crystallinity of rice husk samples was determined via XRD analysis. The HCl-pretreated rice husk pretreated was found to be slightly more crystalline than the untreated rice husk as indicated by the occurrence of a sharper peak at $2\theta = 22.6^{o}$ (Figure 4.4). The increased in the crystallinity index (*CrI*) from 51.5 of the untreated rice husk to 56.3 of the pretreated rice husk is attributed to the removal of amorphous hemicellulose and a fraction of amorphous cellulose during HCl pretreatment. Similar findings on acid-pretreated substrates with higher *CrI* than the untreated substrates were also reported by Barl et al. (1991). The evidence for hemicellulose/cellulose removal is

confirmed by the SEM micrographs. At 1500× magnification, pretreated rice husk clearly exhibited a smoother surface than the untreated rice husk (Figure 4.5).

Table 4.2: Group frequency of absorption bands for lignocellulosic biomass

Group frequency (cm^{-1})	**Origin**	**Assignment**	**Reference**
800 – 950	C-H	C-H deformation vibration in cellulose	Labbe et al. (2005)
~1035	C-O	C-O stretching vibration in cellulose/hemicellulose and aryl-OH group in lignin	Guo et al. (2008) Hurtubise and Krassig (1960) Labbe et al. (2005)
~1457	C-H	Asymmetric bending of CH_3 and methoxy (-OCH_3) groups present in lignin	Guo et al. (2008) Labbe et al. (2005) Sun et al. (2009)
~1513	C=C-C [a]	Aromatic skeletal stretching in lignin	Coates (2000) Hsu et al. (2010)
~1637	O-H	O-H bending vibration of adsorbed water molecules	Hurtubise and Krassig (1960) Proniewicz et al. (2001)
~2919	C-H	C-H stretching in cellulose-rich material	Liu et al. (2007) Oh et al. (2005)
2995 – 4000	O-H	Free and hydrogen-bonded OH stretching	Hurtubise and Krassig (1960) O'Connor et al. (1958) Oh et al. (2005)

[a] C=C–C is used as an approximation of the aromatic skeleton.

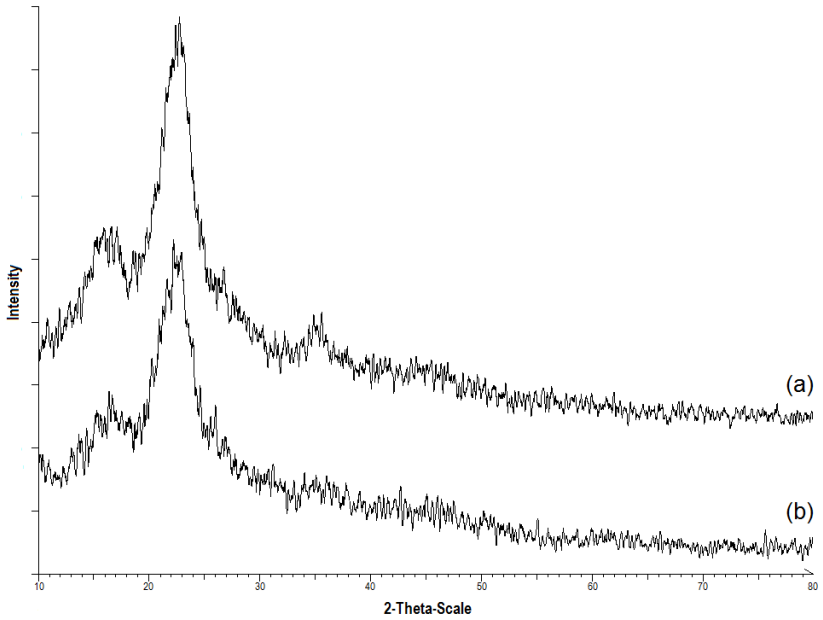


Figure 4.4: X-ray diffractograms of (a) HCl-pretreated and (b) untreated rice husk.

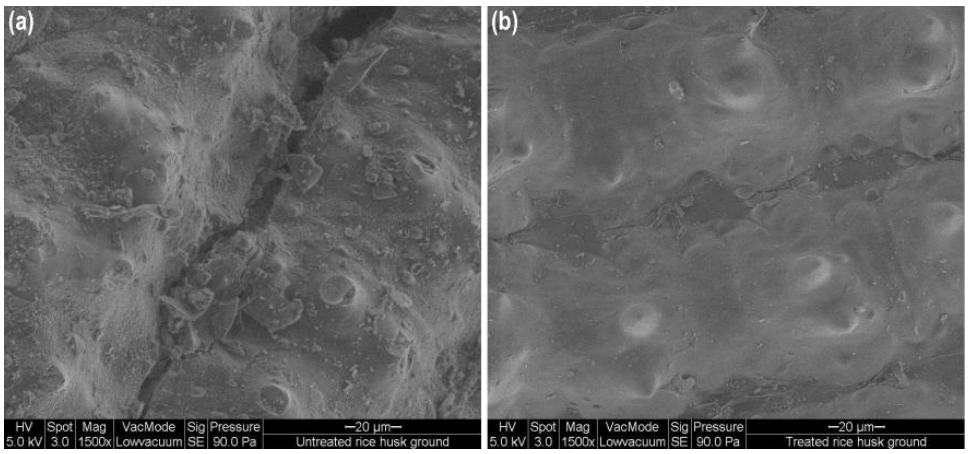


Figure 4.5: SEM micrographs of (a) untreated and (b) pretreated rice husk.

Furthermore, BET surface area analysis was performed to investigate the changes in specific surface area of rice husk after HCl pretreatment. The results showed that specific surface area of pretreated rice husk was significantly reduced from 21.2 to 9.6 m^2/g. The reduction in specific surface area is due to the hydrolysis of amorphous hemicellulose/cellulose portion in rice husk that results in the merging of smaller pores into larger pores. The pore volume and pore size of the rice husk after pretreatment increased from 1.76×10^{-2} to 1.81×10^{-2} cc/g and 33.2 to 75.2 Å, respectively. Similar

observation has been reported by Hsu et al. (2010), hydrolysis of hemicellulose and cellulose by HCl results in increased pore volume in pretreated rice husk. The pretreated solid residue with higher pore volume and bigger pore size is advantageous for fungal growth during fermentation (Hsu et al., 2010; Zhu et al., 2008), albeit its slightly higher *CrI* and lower specific surface area compared to the untreated rice husk. The change in *CrI* and specific surface area of cellulosic substrates has been reported to have no effect on the enzymatic hydrolysis rate (Grethlein, 1985). Nevertheless, the underlying relationship of structural features and biomass digestibility of the pretreated substrate depends on the size of enzyme used in enzymatic hydrolysis (Zhu et al., 2008), and the accessibility of microbes to substrate during fermentation. All these characteristics shown in the pretreated rice husk shows that HCl is a suitable reagent for the pretreatment of rice husk.

4.1.2.2 Ionic Liquid Dissolution Pretreatment

A non-conventional pretreatment of rice husk using ionic liquids was also conducted. The ionic liquid dissolution pretreatment of rice husk was assessed at 100^{o}C for 10 hours. The experimental conditions were chosen as a compromise to allow sufficient dissolution of rice husk for subsequent investigations. Moreover, pretreatment at such temperature and duration minimizes the possibility of cellulose degradation as previous study showed that prolong pretreatment at high temperatures poses risk of degradation of dissolved cellulose (Sun et al., 2009). In dissolution pretreatment, the ionic liquids namely [BMIM]Cl, [EMIM]DEP and [EMIM]OAc were employed for dissolution of rice husk and subsequent regeneration of cellulose. The regenerated cellulose was precipitated from the reaction mixture after being agitated in the anti-solvent indicating that all the ionic liquids under investigation dissolved rice husk.

The ability of ionic liquids in the dissolution of rice husk cellulose and subsequent regeneration is illustrated in Figure 4.6. After 10 hours of dissolution at 100^{o}C, the ionic liquids did not completely dissolve rice husk. The acetate-based ionic liquid [EMIM]OAc and chloride-based ionic liquid [BMIM]Cl produced 0.37 and 0.31 g regenerated cellulose/g rice husk, respectively. Under the same pretreatment conditions, [EMIM]DEP produced less regenerated cellulose that was about 0.16 g regenerated cellulose/g rice husk. The amount of cellulose regenerated depends on the degree of rice husk dissolution, which reflects the dissolution ability of the ionic liquid in dissolving rice husk. Rice husk dissolution is influenced by the interactions between anion of the ionic liquid and hydroxyl group of the cellulose (Dadi et al., 2006; Kosan et al., 2008; Zhao et al., 2009). Anion of the ionic liquid acts as the hydrogen-bond acceptor in dissolution where it interacts specifically with the hydroxyl protons of the cellulosic materials (Ha et al., 2011; Remsing et al., 2006) and facilitates the formation of hydrogen bonds between cellulose and ionic liquid. Among the ionic liquids, the acetate-based [EMIM]OAc has higher hydrogen-bond basicity (MacFarlane et al., 2006), which explains its better dissolubility than [BMIM]Cl and [EMIM]DEP. It is worth noting that other than dissolving cellulose/hemicellulose, the ionic liquids also dissolve lignin. This could be observed from the dark brown appearance of the reaction mixtures caused by dissolved lignin of lignocellulosic biomass after dissolution process (Jagadeeswara Rao et al., 2007; Li et al., 2009; Sun et al., 2009; Zhao et al., 2009).

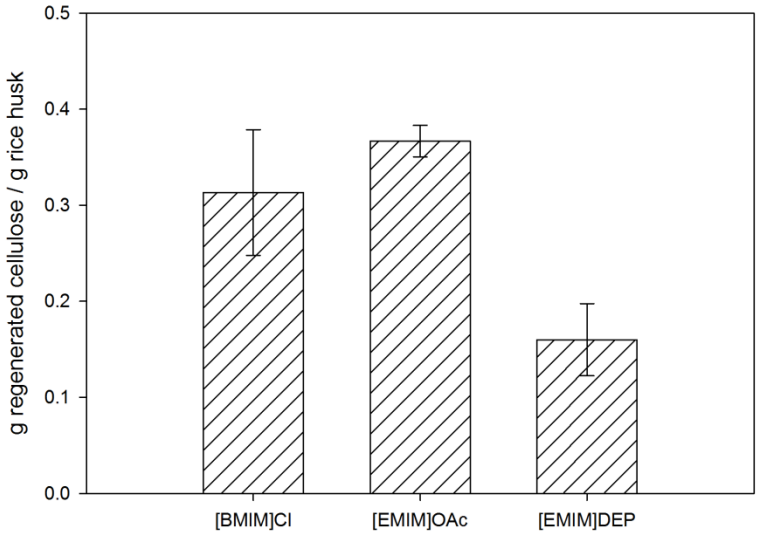


Figure 4.6: Cellulose regenerated from ionic liquid pretreatments.

From visual observation, cellulose regenerated from the ionic liquids had different structures compared to the untreated rice husk. The regenerated cellulose had clumpy appearance, whereas the untreated rice husk had flake-like structure. Furthermore, rice husk residues separated from the reaction mixtures were also swollen compared to the untreated rice husk. This is due to the diffusion of the ionic liquids into rice husk matrix that eventually facilitates the dissolution of rice husk (Brandt et al., 2010). Among the ionic liquids examined, rice husk residue from the [EMIM]OAc pretreatment swelled the most, whereas [BMIM]Cl and [EMIM]DEP did not show extensive swelling.

Unlike conventional pretreatments, the extent of ionic liquid dissolution pretreatment could not be examined by direct measurement of total reducing sugar in the hydrolysate/reaction mixture. The quantitative yield of regenerated cellulose indicates only the efficiency of the ionic liquids in dissolving rice husk. Besides, measuring the yield of regenerated cellulose does not necessarily reflect their performance in the pretreatment of rice husk. In order to gain a better insight into ionic liquid pretreatment of rice husk, the chemical and structural characteristics of the regenerated cellulose and

rice husk residue were investigated using FT-IR spectroscopy, XRD, and SEM. The information on structural characterization of regenerated cellulose as well as rice husk residues is helpful in the selection of a suitable ionic liquid for pretreating rice husk.

The structural changes of regenerated cellulose were analyzed by FT-IR spectroscopy in the region of 600 – 4000 cm^{-1}, which is commonly used to study the fine structural characteristics of cellulose (Hurtubise and Krassig, 1960; Nelson and O'Connor, 1964). The spectra of regenerated cellulose from the ionic liquids and untreated rice husk are presented in Figure 4.7. The regenerated cellulose from all ionic liquids was found to have altered chemical and structural characteristics compared to the untreated rice husk. The absorption bands at 798, 1035, 1457, 1513, 1637, 2919, 3312, and 3750 cm^{-1} in the spectrum of untreated rice husk are associated with raw rice husk lignocellulose. Both cellulose/hemicellulose- and lignin-associated bands are present in the spectrum of untreated rice husk, and this suggests that rice husk is composed of lignin-carbohydrate matrix. Spectra of all the regenerated cellulose show the strongest absorption band at around 1035 cm^{-1}. This band corresponds to the C-O stretching vibration in both cellulose/hemicellulose and lignin, and it explains the lignocellulosic nature of rice husk (Guo et al., 2008; Hurtubise and Krassig, 1960; Labbe et al., 2005).

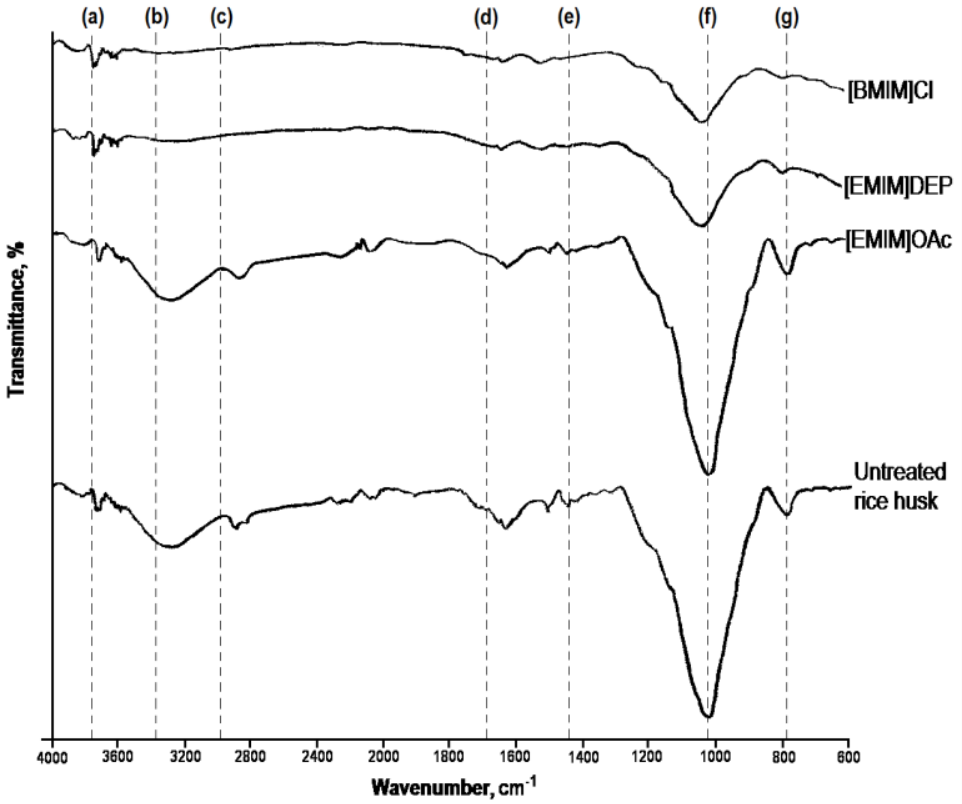


Figure 4.7: FT-IR spectra of the regenerated cellulose and untreated rice husk.

FT-IR spectral bands (cm^{-1}): (a) 3750; (b) 3312; (c) 2919; (d) 1637; (e) 1457; (f) 1035; (g) 798.

The findings demonstrate that [EMIM]OAc possessed the highest degree of rice husk dissolution. The spectrum of cellulose regenerated from [EMIM]OAc has all the absorption bands resembling the spectrum of the untreated rice husk. This clearly suggests that [EMIM]OAc does not selectively dissolve cellulose, but both cellulose and lignin in the rice husk matrix. The ionic liquid has been reported to be capable of dissolving cellulose and lignin (Lee et al., 2009), and various lignocellulosic biomass (Lee et al., 2009; Sun et al., 2009; Zavrel et al., 2009). Furthermore, [EMIM]OAc-treated cellulose showed higher intensity at band 797 cm^{-1} than the untreated rice husk, indicating that the regenerated cellulose was more amorphous. The band around 800 cm^{-1} is sensitive to the amount of amorphous cellulose present in the regenerated

cellulose, in which broadening of this band indicates higher amorphousity. The dissolution and subsequent regeneration of the cellulose/hemicellulose fraction contribute to higher degree of amorphousity of the regenerated cellulose.

In comparison, the spectra of regenerated cellulose from [BMIM]Cl and [EMIM]DEP dissolution were different from the spectrum of the untreated rice husk, where some absorption bands were absent. The regenerated cellulose of [BMIM]Cl and [EMIM]DEP exhibited broader band in the region of 800 – 950 cm^{-1} imply a higher amount of disordered cellulosic structure (Oh et al., 2005; Proniewicz et al., 2001). The disorder of cellulosic structure is very likely caused by the deformation vibration of β-glycosidic linkages and hydrogen bond rearrangement (Labbe et al., 2005; Proniewicz et al., 2001). In addition, [BMIM]Cl- and [EMIM]DEP-regenerated cellulose exhibited reduced absorbance at 1035 cm^{-1}, which might have resulted from the degradation of cellulose/hemicellulose during heating. The shorten cellulose chains lead to the reduction in C-O-C pyranose ring skeletal stretching (Liu et al., 2007). Moreover, the degradation of cellulose also reduced C-H stretching at 2896 cm^{-1} and free/hydrogen-bonded OH stretching at 3312 cm^{-1} of these regenerated cellulose. The disappearance of absorption band at 1457 cm^{-1} suggests the removal of lignin in regenerated cellulose of [BMIM]Cl and [EMIM]DEP.

Apart from regenerated cellulose, rice husk residues of the ionic liquid pretreatments are potential substrates to be converted into valuable products. The spectra of rice husk residues from ionic liquid dissolution pretreatments were also recorded using FT-IR spectroscopy (Figure 4.8). All the absorption bands that appeared in the spectrum of untreated rice husk were present in the spectra of the rice husk residues, indicating that both have similar compositions. An obvious change in intensity was observed in the

band of approximately 1035 cm^{-1}. Compared with their untreated counter-parts, the transmittance of this band increased in all the rice husk residues indicating they contain considerable amount of cellulose/hemicellulose, and possibly lignin, after the ionic liquid pretreatments. Besides, this also implies that the ionic liquid pretreatments dissolve components other than cellulose/hemicellulose contributing to proportionate increase in the relative cellulose/hemicellulose content of rice husk residues. The intensity of absorption band in the region 800 – 950 cm^{-1} remained unchanged, signifying that both the rice husk residues and untreated rice husk do not vary very much in terms of amorphousity.

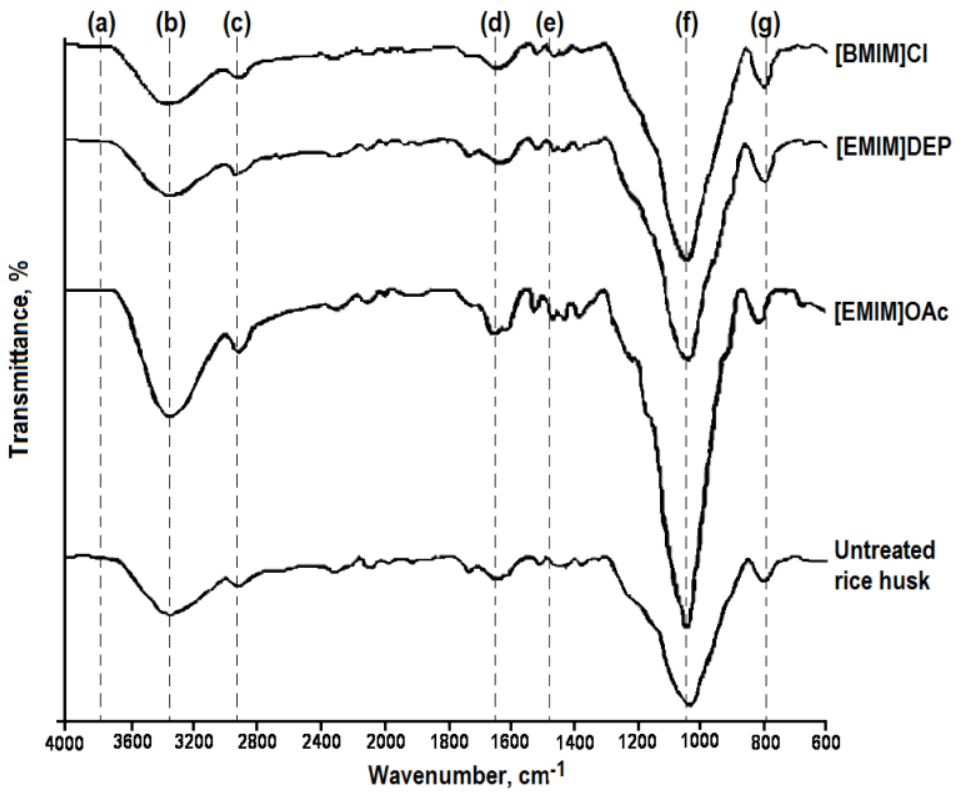


Figure 4.8: FT-IR spectra of rice husk residues and untreated rice husk.

FT-IR spectral bands (cm^{-1}): (a) 3750; (b) 3312; (c) 2919; (d) 1637; (e) 1457; (f) 1035; (g) 798.

On the other hand, a less crystalline regenerated cellulose structures was confirmed by XRD analysis with the occurrence of a sharper peak at $2\theta = 18.7^{o}$ compared with the untreated rice husk (Kumar et al., 2009). The lower crystallinity index indicates a higher amount of amorphous cellulose was present in the regenerated cellulose (Kuo and Lee, 2009b). The results complemented and confirmed the findings of FT-IR analysis reported previously, in which the cellulose regenerated from ionic liquid pretreatments exhibited higher amorphousity. All the regenerated cellulose has an estimated 25% lower crystallinity index compared to untreated rice husk (Table 4.3). Among the regenerated cellulose, cellulose regenerated from [EMIM]DEP pretreatment gave the lowest crystallinity index (32.0), followed by regenerated cellulose of [EMIM]OAc and [BMIM]Cl pretreatments, which were 34.4 and 37.7, respectively.

Table 4.3: Crystallinity indexes of untreated rice husk, regenerated cellulose and rice husk residue

Rice husk sample	Crystallinity index
Untreated rice husk	46.0
Regenerated cellulose	
[BMIM]Cl	37.7
[EMIM]OAc	34.4
[EMIM]DEP	32.0
Rice husk residue	
[BMIM]Cl	56.1
[EMIM]OAc	39.1
[EMIM]DEP	49.5

Interestingly, rice husk residues of [BMIM]Cl and [EMIM]DEP pretreatments showed higher crystallinity index compared with the untreated rice husk. Rice husk residue of [EMIM]OAc pretreatment showed slightly lower crystallinity index than the untreated rice husk (Table 4.3). The dissolution of amorphous cellulose/hemicellulose in rice husk

leaving the more crystalline lignocellulosic matrix in the residue, which accounts for the higher crystallinity index in rice husk residues of [BMIM]Cl and [EMIM]DEP pretreatments. The lower crystallinity of rice husk residue of [EMIM]OAc pretreatment could be due to the swollen structure of rice husk caused by ionic liquid pretreatment.

The structural morphology of cellulose regenerated from the ionic liquids was examined by SEM (Figure 4.9). All the regenerated cellulose showed rough and conglomerate textures (Swatloski et al., 2002) and these ribbon-like fibre aggregates were disorderly arranged in the matrix. The well organized structure commonly present in raw lignocellulosic biomass was absent signifying the structure of the regenerated cellulose was more amorphous (Cuissinat et al., 2008). The findings of SEM were in agreement with the findings of FT-IR analysis where ionic liquid pretreatment reduces crystallinity of the cellulosic biomass.

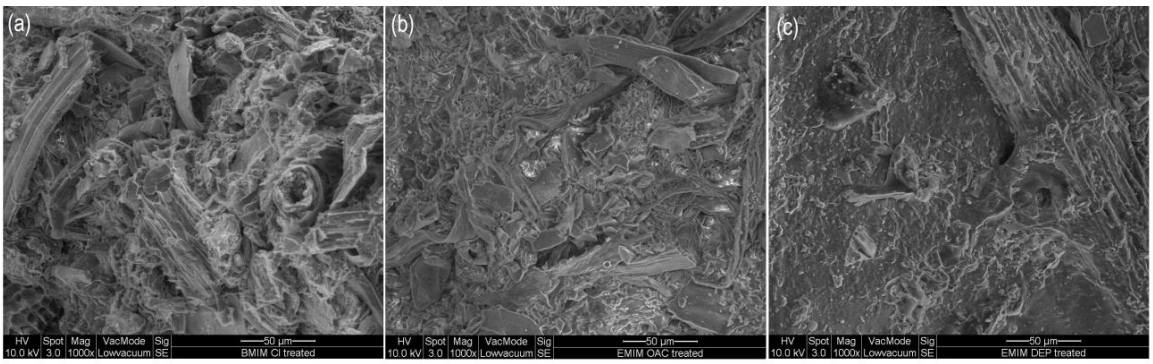


Figure 4.9: SEM images of regenerated cellulose from (a) [BMIM]Cl, (b) [EMIM]OAc, and (c) [EMIM]DEP pretreatments.

Although FT-IR analysis did not demonstrate many changes in chemical compositions of the residues compared to untreated rice husk, the SEM micrographs show that the surface structure of the rice husk residues changed significantly (Figure 4.10**)**. The untreated rice husk had an intact and rather smooth surfaces (Figure 4.10a) while the

surface of rice husk residues appeared to be uneven and had crevices. Rice husk reside from [EMIM]OAc pretreatment was the most severely disrupted (Figure 4.10c). The disruption of residue surface might be caused by the solvating action of the ionic liquids, in which the outer lignocellulosic matrix of the rice husk was swollen before it was dissolved in the ionic liquids. The dissolution of rice husk depends on the degree of swelling of the lignocellulosic biomass. The relationship between structure disruption and rice husk dissolution could be observed from [EMIM]OAc pretreatment. [EMIM]OAc pretreatment yielded the highest amount of regenerated cellulose, and its rice husk residue was rigorously swollen at the end of the pretreatment.

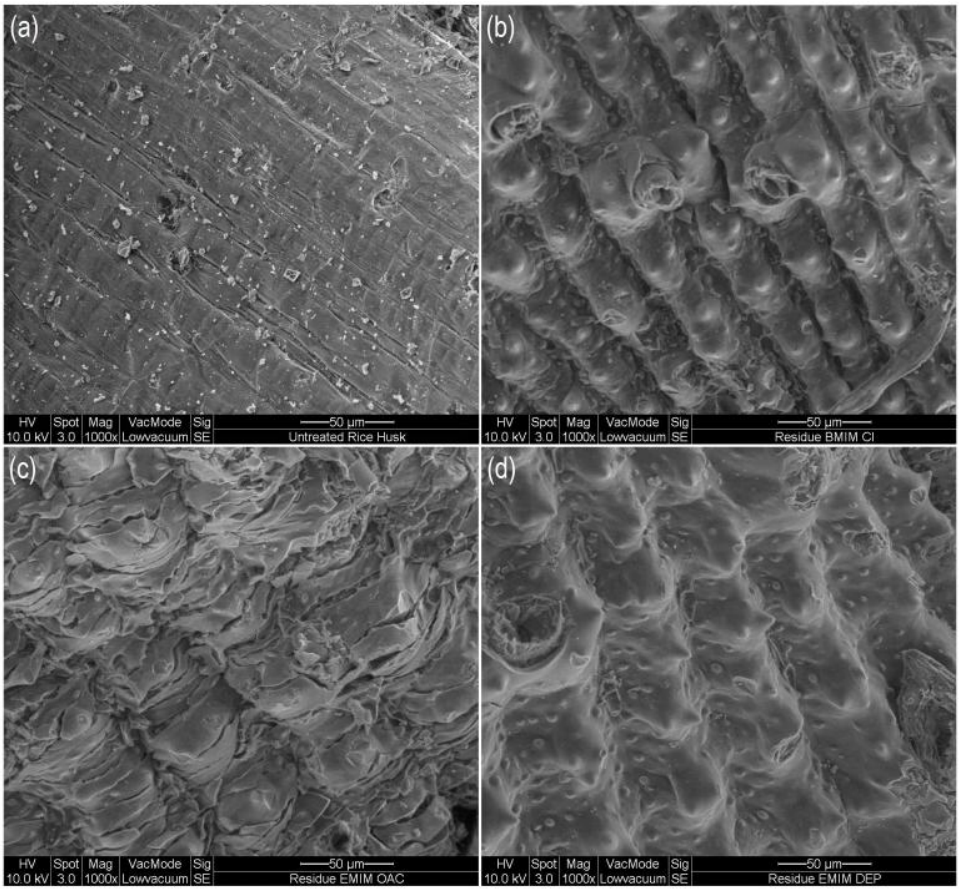


Figure 4.10: SEM images of (a) untreated rice husk and rice husk residues of (b) [BMIM]Cl, (c) [EMIM]OAc, and (d) [EMIM]DEP pretreatments.

The collective findings of structural characterization suggest that the pretreatment products – regenerated cellulose and rice husk residue exhibit favourable properties for solid-state fermentation. In general, the regenerated cellulose is comprised of low crystallinity, amorphous cellulose-rich materials; whereas, the rice husk residues are composed of low crystallinity lignocellulosic biomass with disrupted surface structure. Among the ionic liquids assessed, [EMIM]OAc that dissolved rice husk and regenerated the highest amount of regenerated cellulose is a promising reagent for pretreatment of rice husk. Rice husk pretreated with [EMIM]OAc demonstrated the most severe rice husk surface structure swelling. The disrupted surface structure of rice husk residues was favourable for solid-state fermentation where it facilitates microbial growth by allowing access of microbes to lignocellulosic matrix.

4.1.2.3 Comparison between Chemical and Ionic Liquid Dissolution Pretreatments

The assessments of various reagents in the pretreatment of rice husk using acids, alkalis and ionic liquids suggest that HCl and [EMIM]OAc are suitable reagents. Both the pretreatment reagents modified the rice husk structure, and the pretreated biomass exhibited favourable characteristics for fungal solid-state fermentation. To select a pretreatment reagent for pretreating rice husk, pros and cons associated with the mentioned pretreatment methods in terms of operational and economical feasibility, and handling of reagent were discussed.

The feasibility of a pretreatment process takes into consideration whether the process could be scaled-up for industrial applications as well as the cost of reagent associated with the pretreatment process. In the assessment studies, the HCl pretreatment was conducted at a bigger scale than the [EMIM]OAc pretreatment because of operational and cost constraints. Due to the availability of many reactor designs and relatively low

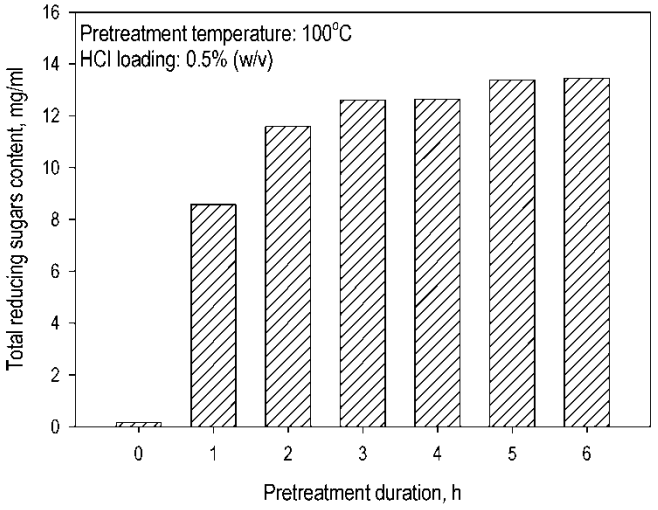
cost of chemical reagent, HCl pretreatment could potentially be scaled up for pilot or industrial application. On the contrary, the dissolution pretreatment with [EMIM]OAc was conducted at a smaller scale due to the limitation of ionic liquid cost that is very much higher compared to acids or alkalis. When the scale of pretreatment increases, the amount of ionic liquid required increases proportionately, so does the overall cost of pretreatment process. Besides, ionic liquid pretreatments need more sophisticated reactors that could handle viscous and semi-solid reaction mixture during the processes.

Considering the economical feasibility, the preparation of pretreatment reagent in-house should reduce the overall cost of the dissolution pretreatment. While HCl pretreatment requires only dilution of concentrated HCl, the synthesis of ionic liquids is more complicated and involves hazardous organic solvents and chemicals, for instance 1-methylimidazole, 1-chlorobutane and diethyl ether (Jiang et al., 2006; Lateef et al., 2009). Ionic liquids too have been named as green solvents for pretreatment of lignocellulosic biomass because they could be recycled and reused in the pretreatment processes (Seoud et al., 2007; Zhu et al., 2006). However, regeneration and recycling of ionic liquids incurs additional cost to the overall dissolution process because the process involves the use of regeneration chemicals as well as purification column for removal of impurities (Li et al., 2009).

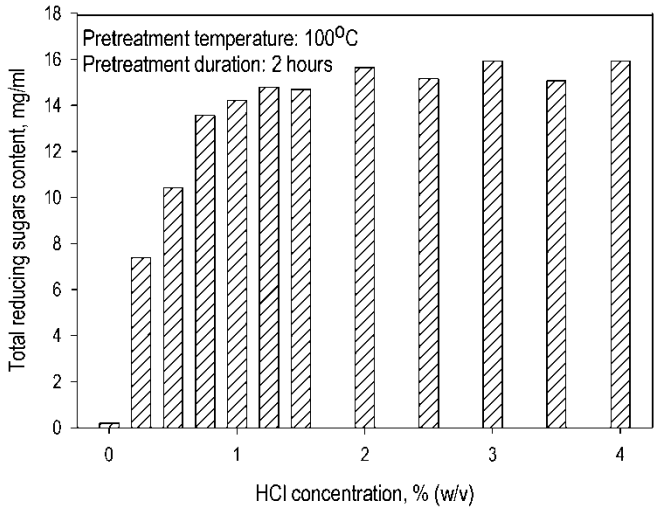
Besides, the selection of reagent for the pretreatment of rice husk depends largely on the final achievable aim, cost of overall process, and handling of the reagent. From the discussed points, HCl was found to be superior pretreatment reagent for rice husk than [EMIM]OAc because HCl pretreatment is more economically feasible compared to the ionic liquid pretreatment.

4.1.3 Optimization of Rice Husk Pretreatment

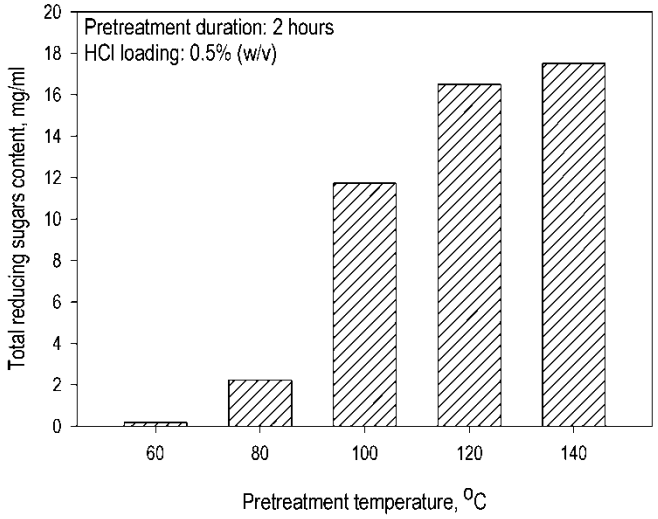
Preliminary studies were conducted to determine the range of pretreatment duration, HCl loading, and pretreatment temperature prior to optimization of rice husk pretreatment. The mentioned parameters are important and affect rice husk pretreatment. Figure 4.11 illustrates the findings of the preliminary studies. Under the pretreatment conditions at 100^{o}C with 0.5% (w/v) HCl loading, the total reducing sugar content increased sharply in the first two hours of pretreatment and reached plateau after heating for more than three hours (Figure 4.11a). This indicates that it is not necessary to prolong the heating duration in the pretreatment of rice husk. From the HCl loading study, total reducing sugar content increased sharply with HCl loading range between 0.25 and 0.75% (w/v) (Figure 4.11b). Little increment in total reducing sugar content was observed with HCl loading higher than 1.0% (w/v). In the study, total reducing sugar content increased proportionally with the pretreatment temperature (Figure 4.11c). Several attempts to pretreat rice husk at temperature higher than 140^{o}C were conducted resulted in charred rice husk sample, and the HCl solution was boiled to dry. Therefore, the maximum pretreatment temperature for the study was 140^{o}C. Moreover, pretreatment at higher temperatures requires higher energy cost and expensive reactor.



(a) Pretreatment duration



(b) HCl loading



(c) Pretreatment Temperature

Figure 4.11: Range determination for (a) pretreatment duration, (b) HCl loading, and (c) pretreatment temperature for optimization of rice husk pretreatment.

Based on the findings from the preliminary studies, the low and high levels of HCl loading (X_1), pretreatment duration (X_2), and temperature (X_3) were determined. The design matrix of Box-Behnken experimental design including the response (Y) is given in Table 4.4, where Y is the total reducing sugar detected in the hydrolysate. From the runs, the highest total reducing sugar (23.9 mg/ml) was obtained with pretreatment conditions at 0.75% (w/v) HCl loading, 120^{o}C heating for 2 hours. The least total reducing sugar (10.9 mg/ml) was detected when pretreatment was conducted at 0.25% (w/v) HCl loading, 100^{o}C heating for 2 hours.

Table 4.4: Design matrix of BBD and the response

Run	Parameter			Response
	X_1 (%, w/v)	X_2 (h)	X_3 (^{o}C)	Y (mg/ml)
1	0.25	1	120	18.3
2	0.25	3	120	21.6
3	1.25	1	120	21.5
4	1.25	3	120	22.3
5	0.25	2	100	10.9
6	0.25	2	140	23.6
7	1.25	2	100	19.0
8	1.25	2	140	19.5
9	0.75	1	100	16.1
10	0.75	1	140	23.5
11	0.75	3	100	19.1
12	0.75	3	140	20.2
13	0.75	2	120	23.9
14	0.75	2	120	23.3
15	0.75	2	120	23.1
16	0.75	2	120	23.1
17	0.75	2	120	22.2

The analysis of variance (ANOVA) of the reduced quadratic model confirmed the significance and goodness of fit of the model (Table 4.5). The reduced model expressed in actual terms is given by equation 4.1, where Y represents the total reducing sugar content as a function of HCl loading (X_1), heating duration (X_2) and heating temperature (X_3).

$$Y = -156.32 + 49.74X_1 + 9.89X_2 + 2.36X_3 - 7.39X_1^2 - 0.008X_2^2 - 0.31X_1X_3 - 0.08X_2X_3 \quad (4.1)$$

Table 4.5: Analysis of variance (ANOVA) of quadratic model for optimization of rice husk pretreatment

Source	Sum of square	Degree of freedom	Mean square	*F*-value	*P*-value
Model	173.04	7	24.72	25.44	<0.0001
X_1	7.88	1	7.88	8.11	0.0192
X_2	1.85	1	1.85	1.91	0.2007
X_3	59.46	1	59.46	61.19	<0.0001
X_1^2	14.43	1	14.43	14.85	0.0039
X_3^2	39.43	1	39.43	40.58	0.0001
X_1X_3	37.33	1	37.33	38.42	0.0002
X_2X_3	9.83	1	9.83	10.11	0.0112
Residual	8.75	9	0.97		
Lack of fit	7.28	3	1.46	3.96	0.1034
Pure error	1.47	4	0.37		
Corrected total	181.79	16			

The reduced model explained 95.2% of the variability in the optimization of rice husk pretreatment (determination of coefficient, R^2 = 0.95; adjusted-R^2 = 0.91). The values of the adjusted-R^2 and predicted-R^2 (0.70) fall in a reasonable range of agreement, and this signifies that the model is fit for prediction of response for new experiments. In the optimization study of pretreatment, HCl loading and pretreatment temperature had higher effect compared to pretreatment duration. Both the parameters – HCl loading and pretreatment temperature interacted in the pretreatment of rice husk (Figure 4.12). The interaction plot illustrates higher temperature is needed to achieve the maximal total reducing sugar content when lower HCl loading is applied, and vice versa. The total reducing sugar content demonstrated a quadratic dependence on the pretreatment temperature, which was also shown by the high effect value of the term X_3^2.

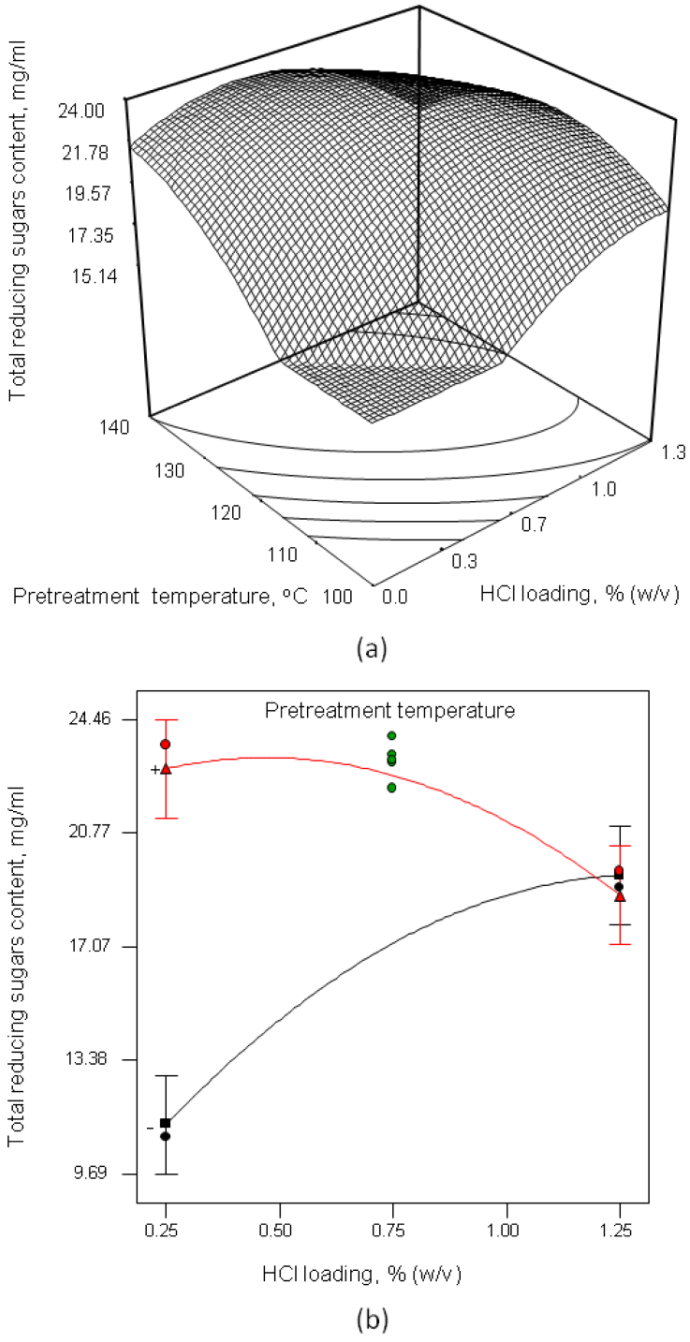


Figure 4.12: (a) Response surface and (b) interaction plots of HCl loading and pretreatment temperature when heating for 2 hours.

Numerical optimization was conducted for maximization of total reducing sugar content with the aid of Design-Expert software. The optimum pretreatment conditions were 0.5% (w/v) HCl loading, 125^{o}C, and 1.5 hours of heating, which has a predicted total reducing sugar content of 22.8 mg/ml of hydrolysate. The pretreatment condition is

relatively mild compared to similar acid pretreatment studies (Kootstra et al., 2009; Saha et al., 2005; Vazquez et al., 2007), where it involves low acid loading and pretreatment temperature. The predicted optimum was verified with triplicate pretreatment runs. The average total reducing sugar released from the runs was 22.3 ± 0.3 mg/ml. A mere 2% discrepancy between the actual experimental and predicted total reducing sugar content suggests good predictability of the reduced model.

4.1.4 Concluding Remarks

There are relatively few pretreatment studies carried out to assess the performance of different types of reagents in pretreating rice husk. In this study, various reagents including acids, alkalis and ionic liquids were investigated in the pretreatment of rice husk. Among the reagents investigated, HCl was found to be more superior for pretreating rice husk. The comprehensive structural studies demonstrated that HCl-pretreated rice husk, which composed mainly of cellulose and lignin, possessed enlarged pore size and pore volume. Despite slightly higher crystallinity index, bigger pore size and volume of the pretreated rice husk is favourable to the attachment and growth of microbe during solid-state fermentation. The optimization of HCl pretreatment gives relatively mild optimal condition (0.5% (w/v) HCl loading, 125^{o}C, and 1.5 hours) involving low HCl loading, low pretreatment temperature and short duration. The reduced model exhibits 2% discrepancy between the predicted and experimental suggests its accurate predictability.

CHAPTER 5

SCREENING OF FUNGI WITH LIGNINOLYTIC ACTIVITY

Following the pretreatment of rice husk, screening of fungi with ligninolytic activity was performed. The aim of which is to select a fungus with desired characteristics intended for various applications, particularly with ligninolytic activity. In general, most of the screenings of fungal enzyme activities are conducted via colourimetric method, in which colour changes of synthetic chemicals in media are associated with specific fungal enzyme activity. In the case of ligninolytic activity, fungi possess ligninolytic activity are capable of degrading synthetic chemicals with structure similar to lignin (Esposito et al., 1991; Glenn and Gold, 1983; Gowthaman et al., 2001; Wunch et al., 1997). Most screening methods interpret ligninolytic activity as either being present or absent in fungi (Kiiskinen et al., 2004; Mtui and Masalu, 2008; Okino et al., 2000), while very few approaches associate ligninolytic activity to fungal growth (Keyser et al., 1978). Hence, qualitative observations of the presence or absence of enzyme activity in screenings provide little information on the fungus. Typically, fungal ligninolytic activity has a close relationship to the growth of the fungus. The halo ring formed on the screening plates indicates fungal ligninolytic activity, while the size of colony is an estimate of fungal growth. Due to the fact that evaluating fungal growth from dry fungal cell weight is tedious, the measurement of colony area serves as an alternative and simpler method to estimate fungal growth in screenings.

A quantitative method to measure fungal ligninolytic activity using media plates containing screening reagents, namely guaiacol and Remazol brilliant blue-R was developed. In addition to quantifying fungal ligninolytic activity, the effect of incubation temperatures on the expression of ligninolytic enzymes was also assessed.

From the screening assessments, a potential fungus with desired ligninolytic activity was selected for subsequent solid-state fermentation.

5.1 Results and Discussion

5.1.1 Screening of Fungal Ligninolytic Enzyme Activity

In this study, a quantitative method to measure ligninolytic activity was developed by considering the ratio of halo ring to fungal colony against incubation period. A higher ratio signifies higher ligninolytic activity, and it indicates the fungus has relatively high ligninolytic activity with respect to its growth. Furthermore, with desirable high ratio values, fungus does not need to have densely grown biomass before it exhibits enzyme activity. Densely grown fungal biomass in enzyme filtrate is problematic as it requires additional processing steps and cost in separating and purifying the target enzyme. However, a proper separation of fungal biomass from enzyme filtrate is necessary as some by-products from fungal metabolism inactivate enzyme activity such as lactic acid (a fungal metabolic by-product) that changes the pH of the enzyme extract and inactivates the enzyme under acidic conditions.

In the screening studies, the desired ligninolytic activity is shown by the formation of reddish brown halo ring and/or clear halo ring around fungal colony resulting from fungal guaiacol oxidation and/or RBB-R decolourization activity, respectively. The areas of fungal colony (A_1) and halo ring (A_2) formed are tabulated in Table 5.1, where the areas of fungal colony and the resulting halo ring are indications of fungal growth and ligninolytic activity, respectively. The findings show that all the fungi demonstrated ligninolytic activity, and they were in agreement with the results of the other researchers (Gowthaman et al., 2001; Mane et al., 2007; Reddy et al., 2003; Thomas et al., 1998).

Table 5.1: Areas of fungal colony (A_1) and halo ring (A_2) for guaiacol oxidation and RBB-R decolourization screenings (incubation period: 48 hours)

(a) ***Guaiacol oxidation***

	Area (cm^2)											
T [a]	***P. chrysosporium***			***P. radiata***			***P. sajor-caju***			***P. sanguineus***		
	A_1	A_2	***R*** [c]	A_1	A_2	***R*** [c]	A_1	A_2	***R*** [c]	A_1	A_2	***R*** [c]
RT [b]	29.3 (±1.3)	0.0 (±0.0)	0	0.4 (±0.0)	4.7 (±0.4)	12.9	0.8 (±0.2)	5.3 (±0.4)	6.8	2.5 (±1.3)	7.3 (±0.2)	3.0
35°C	56.8 [d] (±0.0)	0.0 (±0.0)	0	0.4 (±0.0)	3.1 (±0.5)	8.5	0.4 (±0.0)	5.4 (±0.4)	14.9	6.7 (±0.1)	7.4 (±0.3)	1.1

(b) ***RBB-R decolourization***

	Area (cm^2)											
T [a]	***P. chrysosporium***			***P. radiata***			***P. sajor-caju***			***P. sanguineus***		
	A_1	A_2	***R*** [c]	A_1	A_2	***R*** [c]	A_1	A_2	***R*** [c]	A_1	A_2	***R*** [c]
RT [b]	27.1 (±0.8)	0.0 (±0.0)	0	0.4 (±0.0)	1.5 (±0.1)	3.9	1.3 (±0.1)	1.9 (±0.1)	1.5	1.8 (±0.3)	2.9 (±0.3)	1.6
35°C	56.8 [d] (±0.0)	3.2 (±0.9)	0.1	0.4 (±0.0)	0.0 (±0.0)	0	0.4 (±0.0)	1.7 (±0.3)	4.6	5.6 (±0.6)	3.4 (±0.2)	0.6

[a] *T* = incubation temperature.
[b] *RT* = laboratory room temperature (in the range of 26 to 28°C).
[c] *R* = ratio of halo ring to fungal colony area.
[d] Total colonisable surface area of a 9-cm diameter Petri plate = ~56.8 cm^2.

In guaiacol oxidation screening, the reference strain *P. chrysosporium* demonstrated the highest growth compared to the fungi being screened (Table 5.1a), where its colony area was 29.3 cm^2 after 48 hours of incubation at room temperature. When incubated at 35^oC, *P. chrysosporium* fully colonized the screening plate within 48 hours. Among the fungi being screened, the highest growth was shown by *P. sanguineus*, whereby its colony areas were 2.5 cm^2 and 6.7 cm^2 at room temperature and 35^oC, respectively. The other two fungi *P. sajor-caju* and *P. radiata* did not show extensive growth under the screening conditions. Their colony areas remained at ~0.4 cm^2 at both screening temperatures, except *P. sajor-caju* demonstrated a slightly higher growth (0.8 cm^2) when being incubated at room temperature. The general trend of fungal growth in descending order is *P. sanguineus*, *P. sajor-caju* and *P. radiata*. The guaiacol oxidation screening suggests that *P. sanguineus* exhibited the highest halo area among the fungi when incubated at room temperature (7.3 cm^2) and 35^oC (7.4 cm^2) (Table 5.1a). The next highest guaiacol oxidation halo area was given by *P. sajor-caju* and followed by *P. radiata* at both screening temperatures. As for the fungal guaiacol oxidation activity, *P. sanguineus* has the highest activity, followed by *P. sajor-caju* and *P. radiata*. Unexpectedly, the guaiacol oxidation activity appeared to be independent of fungal growth. Despite *P. radiata* did not show significant changes in growth, it showed observable guaiacol oxidation activity. This signifies the fungus might have utilized the nutrient substrate for non-growth associated processes such as the production of ligninolytic enzymes for the metabolism of guaiacol in screening agar medium as reported by Lester and Birkett (1999). On the other hand, the reference strain *P. chrysosporium* that exhibited the highest growth did not show guaiacol oxidation activity after 48 hours of incubation at both temperatures. A low degree of guaiacol oxidation activity was detected only after being incubated at 35^oC for 72 hours and beyond (Figure 5.1). The delay in the occurrence of guaiacol oxidation activity could be

attributed to the fungal requirement for longer adaptation time to synthesize ligninolytic enzymes (Lester and Birkett, 1999). The length of the adaptation time varies, and depends on the physiology of fungal strain. The observations from *P. chrysosporium* shown that incubation temperatures affect the expression of ligninolytic enzymes, where guaiacol is oxidised only at the fungal optimum growth temperature of 35^{o}C.

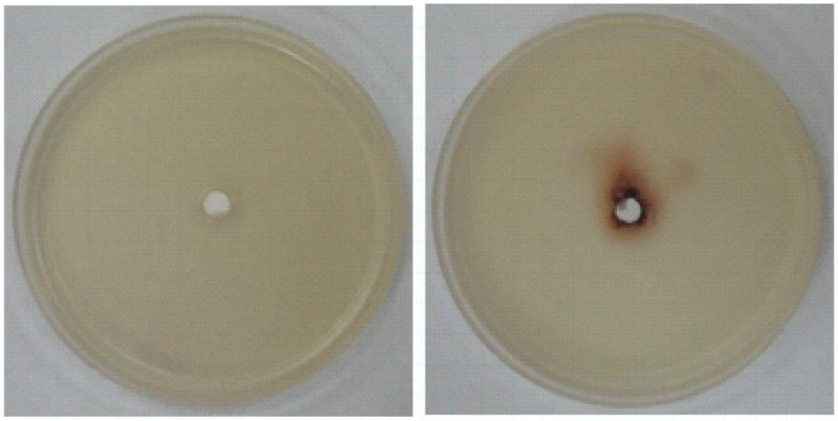


Figure 5.1: Guaiacol oxidation screening plates of *P. chrysosporium* after 72 hours incubation at (a) room temperature and (b) 35^{o}C.

In RBB-R decolourization screening, the growth of fungi on RBB-R screening plate did not vary greatly compared to guaiacol oxidation screening. The findings show that *P. chrysosporium* was the fastest growing fungus after 48 hours of incubation at both screening temperatures (Table 5.1b), with colony area of 27.1 cm^2 and 56.8 cm^2 at room temperature and 35^{o}C, respectively. The growth of fungi being screened in this study was comparable to the guaiacol oxidation screening. Similarly, *P. sanguineus* demonstrated the highest growth of 1.8 cm^2 and 5.6 cm^2 at room temperature and 35^{o}C, respectively. Meanwhile, the other two fungi did not show significant growth under the screening conditions, except *P. sajor-caju* showed slightly higher growth (1.3 cm^2) at room temperature. The general growth trend of the fungi being screened is in the descending order of *P. sanguineus*, *P. sajor-caju* and *P. radiata*. While all the fungi

being screened exhibited RBB-R decolourization activity at both the screening temperatures, *P. radiata* only showed the activity at room temperature (refer Table 5.1b). Among the fungi screened, *P. sanguineus* has the highest areas of decolourization halo of 2.9 cm^2 and 3.4 cm^2 at room temperature and 35^oC, respectively. The sequence was followed by *P. sajor-caju* and *P. radiata*. The reference strain did not show any guaiacol oxidation activity at both room temperature and 35^oC, but RBB-R decolourization at 35^oC. It had relatively high decolourization halo area (3.2 cm^2) that was comparable to *P. sanguineus*. At both screening temperatures, the RBB-R decolourization activity of the fungi was in descending order of *P. sanguineus*, *P. sajor-caju* and *P. radiata*.

In both guaiacol oxidation and RBB-R decolourization screenings, the reference strain demonstrated the highest growth, and it colonized the plates completely after 48 hours at 35^oC. The fungus with the next highest growth was *P. sanguineus* followed by *P. sajor-caju* and *P. radiata*. It was observed that the growth of *P. sanguineus* was approximately two to three folds higher when incubated at 35^oC compared to room temperature in both screenings. In contrast, the oyster mushroom *P. sajor-caju* has two to three folds higher growth when incubated at room temperature than at 35^oC. Meanwhile, the growth of *P. radiata* appeared to be unaffected by the screening incubation temperatures. Despite the unusual behaviour of *P. radiata*, all other fungi demonstrated that incubation temperatures have effect on fungal growth.

In addition to the areas of halo ring and fungal colony, the ratio of halo ring area to fungal colony area as previously mentioned in equation 3.10. Comparatively, all fungi have about two to five folds higher guaiacol oxidation activity than RBB-R decolourization activity. At room temperature, the halo to colony area ratio of guaiacol

oxidation as in descending order of *P. radiata* (12.9), *P. sajor-caju* (6.8), and *P. sanguineus* (3.0). However, at 35^{o}C, *P. sajor-caju* has the highest ratio (14.9) followed by *P. radiata* (8.5) and *P. sanguineus* (1.1). The reference strain *P. chrysosporium* did not exhibit guaiacol oxidation activity in the screening. For RBB-R decolourization screening, the ratio of decolourization halo ring area to colony area at room temperature in descending order was *P. radiata* (3.9), *P. sanguineus* (1.6), *and P. sajor-caju* (1.5). At 35^{o}C, the edible oyster mushroom *P. sajor-caju* possessed the highest ratio (4.6) followed by *P. sanguineus* (0.6) and the reference fungus *P. chrysosporium* (0.1).

5.1.2 Selection of Fungus for Solid-State Fermentation

The fungus selected was applied in solid-state fermentation of rice husk to produce ligninolytic enzymes. The selection was made based on fungus having high ratio values in guaiacol oxidation and RBB-R decolourization screenings. The ratio values provide a basis for ligninolytic activity of the fungi, and they indicate the fungal performance in producing the enzymes. In the selection of fungi, there might be more than one strain of fungi showing desired characteristics. Thus, the ultimate applications of the fungus have to be taken into consideration during the selection of fungus.

Based on the ratio values computed, *P. radiata* might be a potential fungus for the subsequent solid-state fermentation showing the highest ratio values in both screenings at room temperature. However, when this fungus was incubated at 35^{o}C, it exhibited a low ratio value in guaiacol oxidation screening, and the RBB-R decolourization activity was completely absent. On the other hand, the edible oyster mushroom *P. sajor-caju*, which has moderate ratio values at room temperature, showed a better ratio values than *P. radiata* at 35^{o}C. This signifies that *P. sajor-caju* is a good producer of ligninolytic

enzymes at both room temperature and 35^{o}C. The other fungus *P. sanguineus* showed only insignificant ratio values in the screenings at the incubation temperatures.

Fungi do not strive equally well on all the lignocellulosic agricultural by-products as these lignocellulosic substrates vary in their nutrient compositions. The lignocellulosic biomass – rice husk used in this study, which has been identified as rich in cellulose, has been reported to be beneficial for the cultivation of *Pleurotus* species oyster mushroom (Frimpong-Manso et al., 2011; Hanai et al., 2005). The presence of rice husk in the growth substrate improves the biological efficiency (yield potential) of the mushrooms (Frimpong-Manso et al., 2011).

In view of the discussion presented, *P. sajor-caju* was selected for the subsequent solid-state fermentation of rice husk. Its application in solid-state fermentation has several advantages as the fungus could grow and produce ligninolytic enzymes at ambient temperature, and maintaining its growth as well as ligninolytic activity when the temperature of the solid bed increases during the process.

5.1.3 Concluding Remarks

In the screening assessments, all the fungi had demonstrated positive guaiacol oxidation and RBB-R decolourization activities suggesting their ability in producing ligninolytic enzymes. The findings of the screenings show *P. sajor-caju* is the most suitable fungus for solid-state fermentation of rice husk as it exhibits ligninolytic activity at both room temperature and 35^{o}C. The capability of *P. sajor-caju* in preserving its ligninolytic enzymes expression at higher temperatures is crucial in solid-state fermentation to ensure fungal performance as the temperature of the solid bed increases during fermentation.

CHAPTER 6

SOLID-STATE FERMENTATION

Comprehensive pretreatment reagent screening study and the selection of fungus have been addressed in the previous chapters. This chapter reports on the production of laccase enzyme via the application of *Pleurotus sajor-caju* in solid-state fermentation of rice husk. *Pleurotus sajor-caju* that shows desirable ligninolytic activity was selected from the screening of various strains. Novel findings contributing to the development of fungal solid-state fermentation was presented with the development of a novel and user-friendly inoculum preparation technique. The challenges involving the preparation of actively growing and quantifiable fungal inoculum were addressed. Prior to solid-state fermentation studies, the time profile of laccase enzyme production of the fungus and the effect of inducers on the fungal laccase enzyme production system were also conducted. Lastly, the fungal laccase enzyme was characterized and its performance was evaluated.

To present the findings comprehensively, five main sections of namely (i) preparation of inoculum, (ii) time profile study and the effect of inducers on laccase enzyme production, (iii) optimization of solid-state fermentation, (iv) modelling of laccase enzyme production, and (v) characterization of laccase enzyme will be discussed.

6.1 Results and Discussion

6.1.1 Development of Novel Inoculum Preparation – Cellophane Film Culture (CFC) Technique

To date, most of the solid-state fermentations were conducted at laboratory scale with only a few conducted in industrial scale due to the unresolved technological and

operational constraints particularly when mycelial fungus is employed. One of the constraints is the preparation of inoculum. In current practice, inoculum for solid-state fermentation is prepared by growing mycelial fungus in liquid culture or by taking plugs from actively expanding end of an established culture (Gupte et al., 2007; Matsubara et al., 2006). Unlike unicellular bacteria and yeast, inoculum preparation by growing fungus in liquid culture is troublesome due to the changes of its physiological state in liquid. The fungus tends to clump and distribute unevenly in liquid culture; therefore, this complicates the quantification of fungal biomass. The fungal plug method too has limitation in quantifying fungal biomass as inoculum can only be quantified discretely by the number of plugs introduced in solid-state fermentation. Furthermore, nutrient agar attaching to fungal biomass in the plugs could be an inherent source of contaminations.

In view of the abovementioned concerns, cellophane film culture (CFC) technique that can overcome the limitations is developed in this study. This technique is a simple and effective method in which fungal biomass can be separated from the cellophane film-overlaid agar plate, and common contamination problem during inoculum preparation can be alleviated. The thin transparent cellophane film used to overlay the agar plates is made of plant-based cellulose, and it is semi-permeable to moisture. In addition, the film can be autoclaved and withstand heat up to 190^{o}C making it suitable for sterile microbiological works. Most importantly, cellophane film is biodegradable and does not have negative impact on the environment.

6.1.1.1 Regression Analysis of Fungal Dry and Wet Weights

Estimation of fungal dry weight is essential during inoculum preparation as it is frequently used to report inoculum loading in solid-state fermentation studies. The

technique developed offers a quick estimation of dry weight of fungal biomass while maintaining the viability of the inoculum. Besides, the application of cellophane film prevents penetration of hyphae into agar medium, and makes the separation of fungus for biomass determination possible. Figure 6.1 illustrates the correlation plot of dry to wet weight of *P. sajor-caju* cultivated on agar plates overlaid with cellophane film. The linear regression has a coefficient of determination (R^2) of 0.93, which explains ~ 93% variability of the relationship between the fungal dry and wet weights. The regression equation gives a higher accuracy for fungal wet weight below 0.8 g, which equivalent to the fungal weight of 6 to 8 days incubation period. As the weight of fungal biomass increases, the estimation of fungal dry weight becomes less accurate due to the absorption of water on the biomass. However, this does not reduce the reliability of the technique as inoculum is usually harvested on day 7 when the fungus is actively growing.

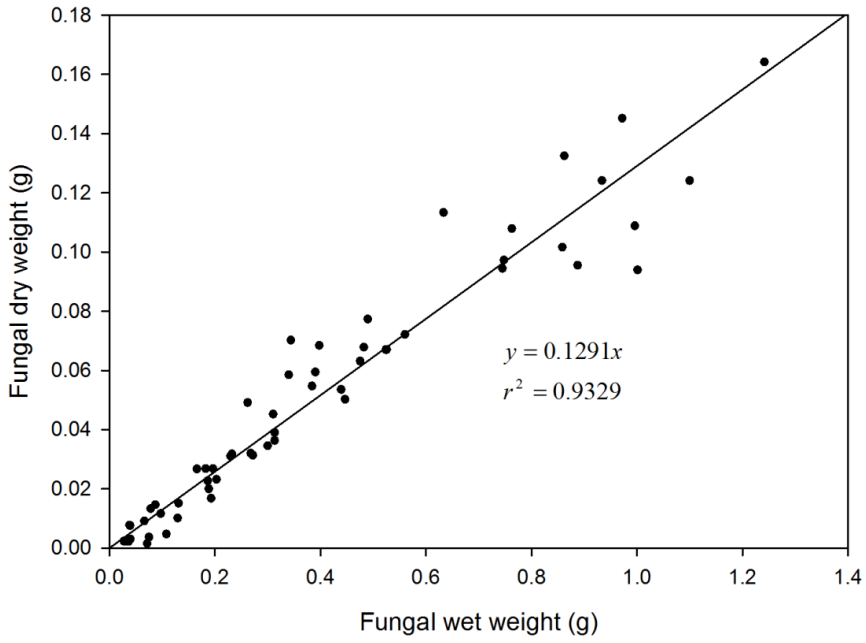


Figure 6.1: Regression plot of dry and wet weights of *P. sajor-caju* (cellophane film).

From the findings of regression analysis, the cellophane film culture technique is feasible for inoculum preparation of *P. sajor-caju*. To consolidate the reliability of this newly developed method, further verification and validation studies were performed and the outcomes will be discussed in subsection 6.1.1.2.

6.1.1.2 Cellophane Film Culture (CFC) Technique Verification and Validation

From the verifying investigation, the inoculum of *P. sajor-caju* prepared via the technique gives the desired laccase activity of 5.03 U/L in solid-state fermentation of rice husk under a sub-optimal condition. The findings demonstrated that the fungal mycelia were not damaged when it was separated from the cellophane film as the fungus was striving on the rice husk.

For comparison purpose, *P. sajor-caju* was also grown on MEPA plates overlaid with nylon membrane. Figure 6.2 depicts the regression plot of dry to wet weights of *P. sajor-caju* cultivated on agar plates overlaid with nylon membrane. The regression plot shows a high R^2 (0.96), which was slightly higher compared to the R^2 exhibited by the fungus grown on cellophane film. In spite of this, the use of nylon membrane in inoculum preparation has some disadvantages. First of all, it incurs higher cost than cellophane film, especially when production of a large volume of inoculum is involved. Besides, separation of fungal biomass from nylon membrane is difficult due to its relatively porous surface structure compared to cellophane film. From visual observations, the biomass of *P. sajor-caju* grown on nylon membrane was half of the fungal biomass grown on cellophane film for a same incubation period. The difference in fungal biomass density observed between the two overlaying materials might be attributed to the fungal physiology on different growing surfaces.

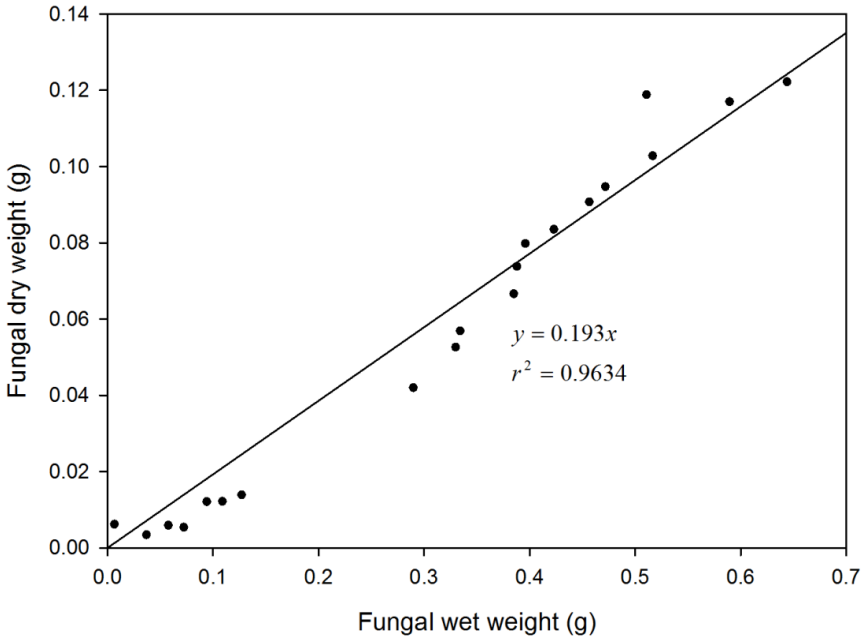


Figure 6.2: Regression plot of dry and wet weights of *P. sajor-caju* (nylon membrane).

To evaluate the applicability of the cellophane film culture technique for other strains of fungi, further validation of the technique was conducted by using another strain of white-rot fungus (*P. sanguineus*). The findings showed that *P. sanguineus* can also grow on cellophane film and its regression plot shown in Figure 6.3 gives a good correlation of fungal dry to wet weights. This signifies that the technique is applicable to other fungal strains. Besides that, the cellophane film culture technique has added benefits compared to the existing inoculum preparation methods, which will be detailed in the following section.

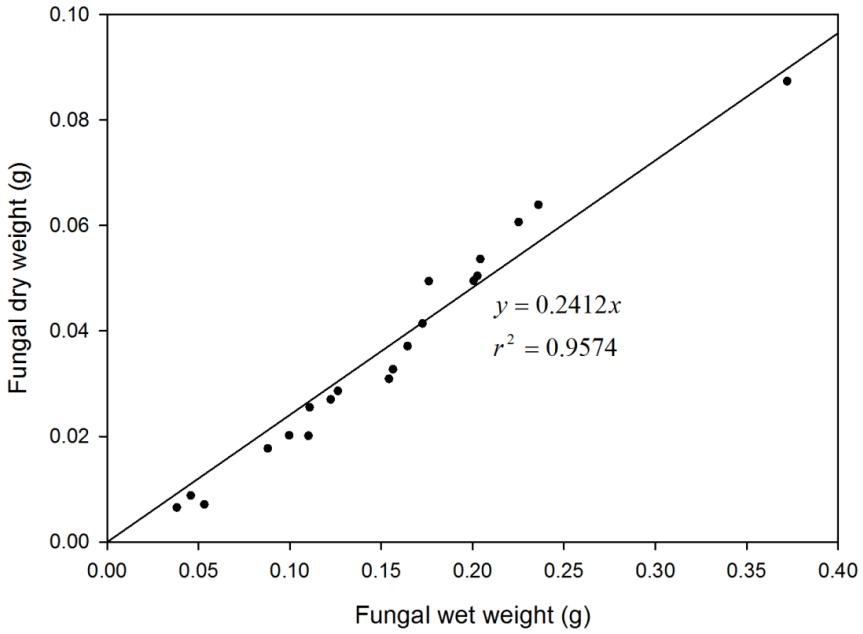


Figure 6.3: Regression plot of dry and wet weights of *P. sanguineus* (cellophane film).

6.1.1.3 Effectiveness of Cellophane Film Culture (CFC) Technique

Added advantages to the cellophane film culture technique are its ability to produce actively-growing inoculum and its association with lower risk of contamination compared to liquid culture approach. The actively-growing fungus from this proposed technique enables rapid colonization of the solid substrate following inoculation as the fungus has been adapted to growing on solid surface during the preparation stage. This could be substantiated by the fungus manifesting homologous morphological appearance when it was grown on the rice husk and agar plate overlaid with cellophane film as shown in Figure 6.4a and 6.4b, respectively.

Figure 6.4: Morphology of *P. sajor-caju* grown on (a) rice husk, (b) agar plate, and in liquid culture observed from (c) aerial and (d) bottom views.

In contrast to growing in the solid state, the fungus has a distinctive morphology when it was grown in liquid state, where the mycelial fungus assumes a globular shape in the absence of solid support (Figure 6.4c and 6.4d). Fungal inoculum prepared in liquid state results in a longer colonization time (48 – 72 hours) after inoculation onto the solid-state fermentation substrate. This could be due to changes in the fungal physiology associated with the difference in morphology when it is grown in different states.

Furthermore, preparation of fungal inoculum in liquid culture requires stringent sterility and vulnerable to higher risk of contamination. Figure 6.5 illustrates an example of contamination observed during inoculum preparation using liquid culture approach. The nutrient medium absorbed in the interstitial space of the fungal globular structure is a

potential source of contamination. This is because it may attract contaminants when conducting solid-state fermentation under non-aseptic condition. Likewise, another method involves the direct inoculation of fungal plugs with nutrient agar, also shows higher risk of contamination.

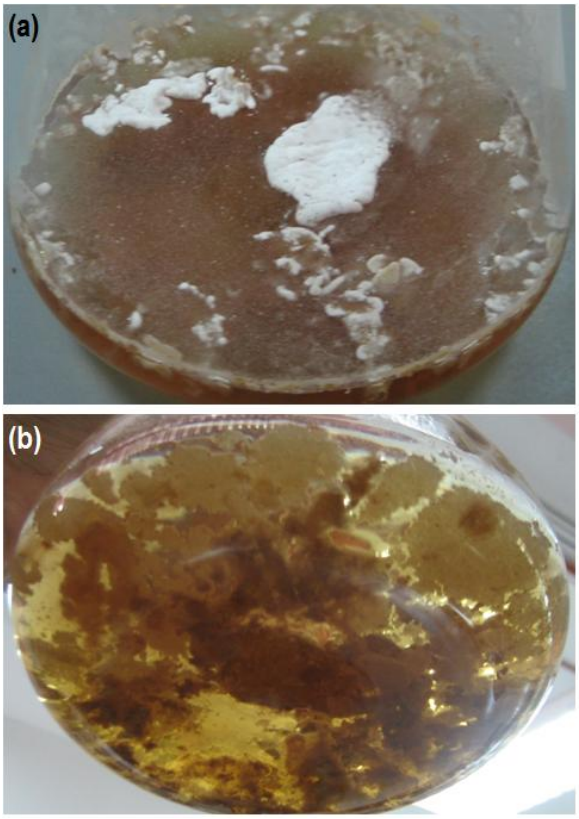


Figure 6.5: Observed contamination of liquid medium during inoculum preparation. The liquid culture contains 3% malt extract broth was inoculated with 3 fungal agar plugs, and incubated on an orbital shaker at room temperature.

From the perspective of scaling-up, preparation of inoculum using liquid culture approach becomes intricate when large quantity of inoculum is involved as it requires costly and sophisticated bioreactor. However, the cellophane film culture technique developed allows preparation of large quantity of inoculum in a simple manner by sub-culturing fungus on more agar plates overlaid with cellophane films. As long as the agar medium and cellophane films are sterilized and handled aseptically during the process,

this technique is capable of producing good quality inoculum. With respect to the sterility of the autoclaved cellophane films on agar plates, it can be maintained up to a period of 12 months or longer under room temperature. In addition to that, the transfer of inoculum from agar plates to solid substrate can be preformed without the need of a laminar flow cabinet.

6.1.2 Time Profile Study and Effect of Inducers

A careful experimental design for solid-state fermentation can save time and resources by avoiding unnecessarily long fermentation duration. Thus, the time profile of laccase enzyme production is useful in deciding a suitable length of duration for the solid-state fermentation. Besides, the inclusion of inducers into the fermentation substrate can also improve the laccase enzyme production. Selected inducers like copper sulphate, glucose and Tween 80 were examined on the enhancement of laccase enzyme activity, which is essential for the subsequent solid-state fermentation process and optimization study.

The time profile investigation was conducted with rice husk as the sole nutrient source without the addition of inducers. The time profile plot of laccase activity of the crude enzyme filtrate from solid-state fermentation of rice husk is shown in Figure 6.6. The laccase enzyme activity increased sharply during the first 48 hours of fermentation and reached its maximum of 45 U/L in 72 hours. After that the laccase enzyme activity gradually declined and remained at a low enzyme activity level of ~10 U/L from 192 until 480 hours. The sharp increase in laccase enzyme activity within 48 hours of inoculation was evidenced from the rapid colonization of the rice husk substrate by the fungus. The fungal inoculum employed in this study has been acclimatized to grow under solid-state condition, and thus, it colonized rice husk without a significant lag phase. As the fungus grows, it produces ligninolytic enzymes such as laccase to

breakdown lignin in rice husk. When sufficient degradation of lignin in the rice husk was achieved at localized spots, laccase enzyme may reduce in production leading to a gradual decrease in its activity.

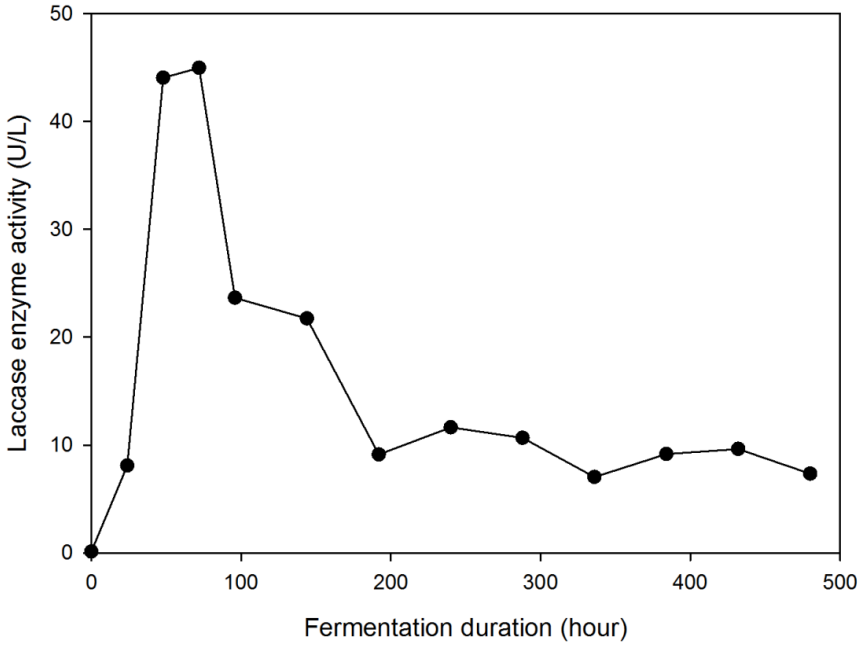


Figure 6.6: Time profile plot of laccase activity of the crude enzyme filtrate in solid-state fermentation of rice husk.

Maximum laccase enzyme activity was observed in less than 100 hours of fermentation, which is earlier than a similar fungal solid-state fermentation of wheat straw that employs *Pleurotus ostreatus* whereby its maximum enzyme activity was shown at 192-hour of fermentation (Patel et al., 2009). The difference could be attributed to the fungal physiology of the different type of fungal inoculum employed. Patel and co-workers (2009) applied mycelial plugs that contained residue agar medium, which is easily utilized by the fungus before it produces ligninolytic enzymes for breaking down the lignocellulosic wheat straw. On the other hand, the inoculum prepared in this study consisted mainly of actively growing fungal biomass, thus it is essential for the fungus to produce ligninolytic enzymes before the biomass can be utilized.

To investigate the effect imposed by the inducers on the fungus, the studies were carried out at two durations, *i.e.* at maximal laccase enzyme activity 72 hours, and at 192 hours when the enzyme activity was at the lowest level. In the absence of inducers, the controls exhibited comparable laccase enzyme activities at both 72 and 192 hours of fermentation that were 24 and 27 U/L, respectively (Figure 6.7). At 72 hours of fermentation, supplementation of copper sulphate and Tween 80 enhanced laccase enzyme activity at least two-fold to 51 and 49 U/L respectively, compared to the control. Apart from playing the role as co-factor of laccase enzyme, copper also serves as a metal activator that induces laccase enzyme expression in the fungus (Palmieri et al., 2000). Therefore, copper could be used as a replacement of the expensive and toxic aromatic compounds like veratryl alcohol and syringaldazine to enhance laccase enzyme production. Tween 80 has been reported to act on the structure of fungal cell membrane by changing its permeability to promote the secretion of ligninolytic enzymes (Asther et al., 1987; Patel et al., 2009) and they are capable of increasing the bioavailability of the less soluble substrates to the fungus (Zheng and Obbard, 2002). On the other hand, supplementation of glucose decreased laccase enzyme activity to 15 U/L after 72 hours of fermentation. It is hypothesized that the availability of glucose delayed laccase enzyme production because the fungus is prone to utilize the glucose rather than to synthesize ligninolytic enzymes for breaking down the rice husk.

When fermentation duration was extended to 192 hours, the inclusion of copper sulphate and Tween 80 reduced enzyme activity approximately two-fold to 8 and 12 U/L, respectively. Despite both copper sulphate and Tween 80 have been reported to benefit laccase enzyme production, the exact cause of the decrease in enzyme activity is not known. The phenomenon might be triggered by the fungal physiology in reaction to

prolong exposure to copper sulphate and Tween 80. In contrast, supplementation of glucose improved laccase enzyme activity by two-fold to 47 U/L at longer fermentation duration of 192 hours. The presence of glucose led to increased growth of the fungus, which eventually resulted in more laccase enzyme production.

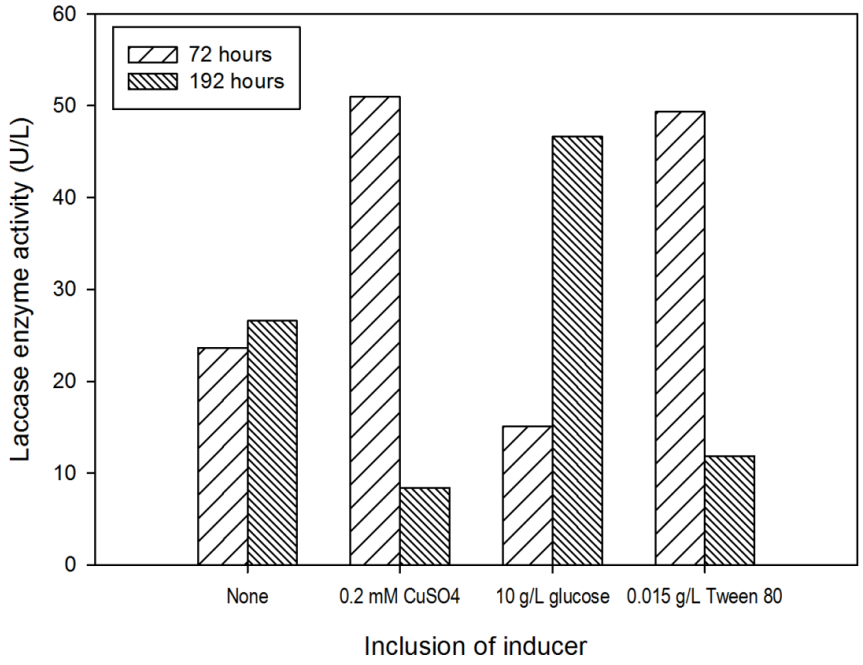


Figure 6.7: Effect of copper sulphate, glucose and Tween 80 on laccase enzyme production after 72 and 192 hours of fermentation.

The findings from the time profile study show that *P. sajor-caju* exhibited maximum laccase enzyme activity after three days of fermentation. Like the other fungus of the same genus – *Pleurotus ostreatus* (Mansur et al., 2003), the laccase enzyme production system of *Pleurotus sajor-caju* appeared to be constitutive as the enzyme was produced even in the absence of inducers. Nevertheless, the supplementations of copper sulphate, glucose and Tween 80 in solid-state fermentation of rice husk have shown significant enhancing effect on the fungal laccase enzyme production. Thus, all the inducers were varied in concentration for the subsequent optimization study of solid-state fermentation.

6.1.3 Optimization of Solid-State Fermentation

The optimization of solid-state fermentation was conducted using central composite experimental design with four parameters at five levels. The parameters considered are fermentation duration (X'_1), copper sulphate (X'_2), glucose (X'_3), and Tween 80 concentrations (X'_4). Based on the findings from the previous section, the low and high levels of the parameters were determined (Table 6.1). The design matrix of central composite experimental design including the response (Y') is tabulated in Table 6.2, where Y' is the specific laccase enzyme activity of the crude enzyme filtrate. The specific enzyme activity instead of laccase activity was used in the response for reporting enzyme production yield. The highest specific laccase enzyme activity (0.96 U/mg) was obtained at fermentation conditions with supplementation of 0.4 mM copper sulphate, 20 g/L glucose, 0.02 g/L Tween 80, and fermentation for 120 hours. The least specific laccase enzyme activity (0.02 U/mg) was observed when no copper sulphate was supplemented while the other parameters remained the same.

Considering the large ratio of the maximum response (0.96 U/mg) to the minimum response (0.02 U/mg), the response (Y') was transformed by taking logarithm to the base of 10 prior to statistical analysis of variance (ANOVA). The ANOVA of the reduced quadratic model is summarized in Table 6.3, and it confirmed the significance and goodness of fit of the model. The expression of reduced model in actual terms is shown in equation 6.1, where Y' represents the specific laccase enzyme activity as a function of fermentation duration (X'_1), copper sulphate (X'_2), glucose (X'_3), and Tween 80 concentrations (X'_4).

Table 6.1: Low and high levels with alpha values of CCD

Parameter	Unit	Low level	High level	Low Alpha	High Alpha
Fermentation duration (X'_1)	h	84	156	48	192
Copper sulphate (X'_2)	mM	0.2	0.6	0	0.8
Glucose (X'_3)	g/L	10	30	0	40
Tween 80 (X'_4)	g/L	0.01	0.03	0	0.04

The reduced model explained ~80% of the variability in the optimization of fungal solid-state fermentation of rice husk for laccase production (determination of coefficient, R^2 = 0.80; adjusted-R^2 = 0.70). Compared with the non-reduced model, the predicted-R^2 value of the reduced model has improved considerably. This makes the model fit for predicting specific laccase enzyme activity produced in solid-state fermentation. From the optimization study, the concentration of copper sulphate (X'_2) had the most significant effect on the specific laccase enzyme activity, which agreed with earlier discussion in which copper is one of the components that constitutes the laccase enzyme, and its presence in the fermentation substrate induces the fungus to express and produce more laccase enzyme (Palmieri et al., 2000). Although the inclusion of Tween 80 (X'_4) was shown to promote laccase enzyme activity production, its effect is comparatively smaller than the inclusion of glucose (X'_3) in the optimization of solid-state fermentation.

Table 6.2: Design matrix of CCD and the response

Run	Block	Parameter				Response
		X'_1 (h)	X'_2 (mM)	X'_3 (g/L)	X'_4 (g/L)	Y' (U/mg)
1	1	84	0.2	10	0.03	0.56
2	1	156	0.2	10	0.01	0.48
3	1	84	0.6	10	0.01	0.70
4	1	156	0.6	10	0.03	0.32
5	1	84	0.2	30	0.01	0.40
6	1	156	0.2	30	0.03	0.33
7	1	84	0.6	30	0.03	0.67
8	1	156	0.6	30	0.01	0.45
9	1	120	0.4	20	0.02	0.90
10	1	120	0.4	20	0.02	0.96
11	2	84	0.2	10	0.01	0.25
12	2	156	0.2	10	0.03	0.13
13	2	84	0.6	10	0.03	0.52
14	2	156	0.6	10	0.01	0.21
15	2	84	0.2	30	0.03	0.08
16	2	156	0.2	30	0.01	0.11
17	2	84	0.6	30	0.01	0.24
18	2	156	0.6	30	0.03	0.74
19	2	120	0.4	20	0.02	0.20
20	2	120	0.4	20	0.02	0.39
21	3	48	0.4	20	0.02	0.29
22	3	192	0.4	20	0.02	0.23
23	3	120	0	20	0.02	0.02
24	3	120	0.8	20	0.02	0.17
25	3	120	0.4	0	0.02	0.35
26	3	120	0.4	40	0.02	0.32
27	3	120	0.4	20	0	0.63
28	3	120	0.4	20	0.04	0.48
29	3	120	0.4	20	0.02	0.22
30	3	120	0.4	20	0.02	0.14

$$\begin{aligned}\mathrm{Log}_{10}(Y') = {} & 0.19 - 0.006X'_1 + 2.81X'_2 - 0.05X'_3 - 42.47X'_4 - 4.33X'^{2}_2 \\ & + 705.21X'^{2}_4 + 0.0003X'_1X'_3 + 0.04X'_2X'_3 + 36.08X'_2X'_4\end{aligned} \quad (6.1)$$

Table 6.3: Analysis of variance (ANOVA) of reduced quadratic model for optimization of solid-state fermentation

Source	Sum of Squares	Degree of freedom	Mean Square	*F*-Value	*P*-value
Block	0.97	2	0.49		
Model	2.10	9	0.23	8.17	<0.0001
X'_1	0.035	1	0.035	1.22	0.2837
X'_2	0.66	1	0.66	22.98	0.0001
X'_3	0.016	1	0.016	0.55	0.4681
X'_4	6.875E-5	1	6.875E-5	2.406E-3	0.9614
X'^2_2	0.85	1	0.85	29.83	<0.0001
X'^2_4	0.14	1	0.14	4.95	0.0391
$X'_1X'_3$	0.13	1	0.13	4.63	0.0452
$X'_2X'_3$	0.092	1	0.092	3.24	0.0888
$X'_2X'_4$	0.083	1	0.083	2.92	0.1049
Residual	0.51	18	0.029		
Lack of Fit	0.45	15	0.030	1.47	0.4232
Pure Error	0.062	3	0.021		
Corrected Total	3.59	29			

The specific laccase enzyme activity exhibited a quadratic dependence on both copper sulphate (X'_2) and Tween 80 (X'_4) concentrations (refer Table 6.3). The illustrations of quadratic pattern of these parameters are shown in the one-factor plots in Figure 6.8. The supplementation of copper sulphate as a significant parameter for laccase enzyme production conforms to the findings of Rajendran and co-workers (2011). They reported that laccase enzyme production by *Pycnoporus sanguineus* exhibited a quadratic dependency on the amount of copper sulphate. Similarly, the fungal laccase enzyme was shown to increase proportionally to the copper concentration in this study. When copper concentrations were raised beyond the optimal concentration, laccase production by *P. sanguineus* was declined. The high copper concentrations impose oxidative stress on the fungus and trigger the fungal defence mechanism, which suppresses the fungal laccase production (Fernandez-Larrea and Stahl, 1996). Specific laccase enzyme activity also has quadratic dependency on Tween 80 concentration. Similar findings were reported by Patel and colleagues (2009) whereby laccase enzyme activity

increased with the supplementation of Tween 80 up to an optimal concentration. After which, the production of laccase enzyme declined.

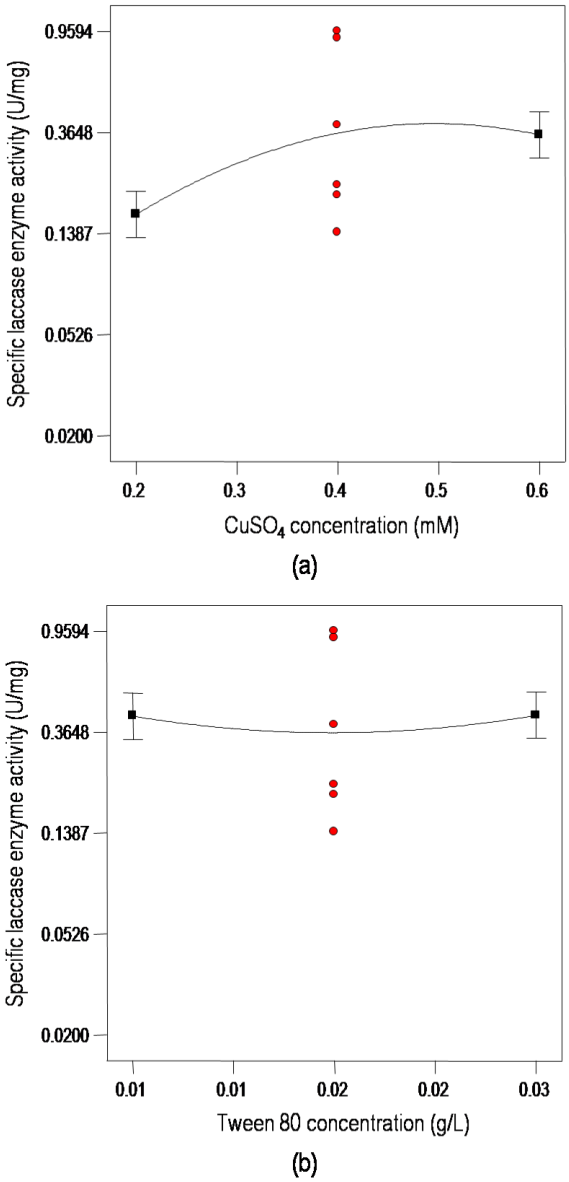


Figure 6.8: One factor plots of (a) copper sulphate and (b) Tween 80 loadings showing the quadratic dependency of specific laccase enzyme activity (the other parameters are set at centre points).

In the optimization of solid-state fermentation, three significant interacting terms namely fermentation duration versus glucose concentration ($X'_1X'_3$), copper sulphate versus glucose concentrations ($X'_2X'_3$) and copper sulphate versus Tween 80

concentrations ($X'_2X'_4$) were investigated. The contour plots of the two most significant interacting terms, *i.e.* $X'_1X'_3$ and $X'_2X'_4$ are represented in Figure 6.9. The interacting term $X'_1X'_3$ exhibits an inversely proportional relationship as depicted in Figure 6.9a. Interestingly, the highest enzyme activity was obtained at lower glucose concentrations. The supplement of high glucose concentration benefits specific laccase enzyme production only at a longer fermentation duration. The other interacting terms involving copper sulphate and Tween 80 concentrations ($X'_2X'_4$) demonstrated that the synergistic effect of the inducers was more clearly observed at the optimal copper sulphate loading region (between 0.45 and 0.55 g/L copper sulphate as shown in Figure 6.9b) and not below or above the optimal copper sulphate loading.

The numerical optimization based on the reduced model suggested an optimal solid-state fermentation condition – 84 hours of fermentation duration, 0.5 mM copper sulphate, 10 g/L glucose, and 0.01 g/L Tween 80, with a predicted specific laccase activity of 0.67 U/mg. The suggested condition resembles other solid-state fermentations for laccase enzyme production, except a higher copper sulphate loading is applied. Although high copper sulphate loading (more than 0.28 mM) was reported to impose oxidative stress to some fungi, *P. sajor-caju* seems to perform well under high copper concentration. Thus, it can be deduced that the threshold of copper concentration leading to oxidative stress is species specific. For verification, triplicate runs of solid-state fermentation were conducted at the optimal condition. The specific laccase enzyme activity detected was 0.5 ± 0.1 U/mg, which was equivalent to 68 U/L enzyme activity. The resulting actual specific laccase activity was very close to the predicted value, and the enzyme activity obtained is comparable to the 71 U/L enzyme activity reported by an optimized submerged fermentation with synthetic medium using *P. sanguineus* (Rajendran et al., 2011). The slight difference observed in the actual and

predicted values might be attributed by the complexity of biological system, the lignocellulosic substrate used, and the batch-to-batch variation of solid-state fermentation.

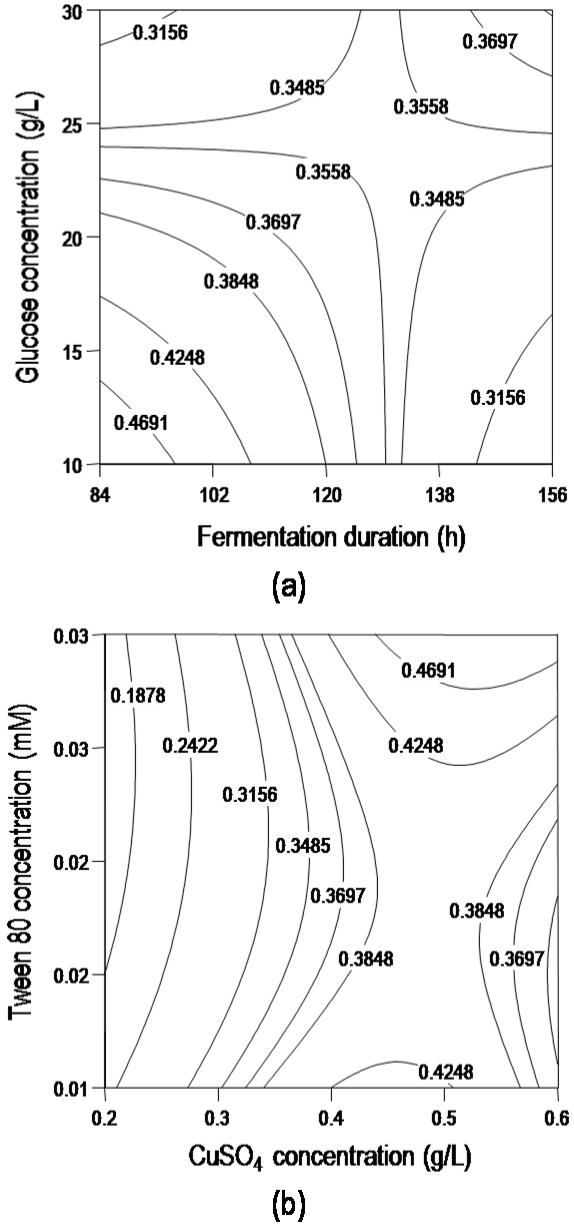


Figure 6.9: Contour plots of the interacting parameters (a) fermentation duration versus glucose concentration, and (b) copper sulphate versus Tween 80 concentrations (the other parameters are set at centre points).

6.1.4 Modelling of Laccase Enzyme Production

To have better understanding on the principles of laccase enzyme production in fungal solid-state fermentation, modelling of the enzyme production was performed. With the aid of response surface methodology, an optimal condition with respect to the maximum specific laccase enzyme activity was obtained. The response surface methodology develops optimization model via empirical approach using various mathematical and statistical techniques. The modelling of laccase enzyme production was considered only at the increasing phase in this study because solid-state fermentation is usually discontinued at maximum enzyme activity before the decay phase of enzyme production.

Several attempts were made to measure fungal growth in terms of total protein and relative glucosamine contents to determine the maximum biomass concentration (X_m) and specific growth rate (μ_m) for the solid-state fermentation. The total protein content of the crude enzyme filtrate decreased since the beginning until ~18-hour of the fermentation, after which the total protein content remained constant at a relatively low level (Figure 6.10a). On the other hand, the relative glucosamine content of the fungus did not change significantly during the course of the fermentation (Figure 6.10b). This might be due to the relatively short fermentation duration for any significant changes in fungal glucosamine content to be detected (Sugama and Okazaki, 1979).

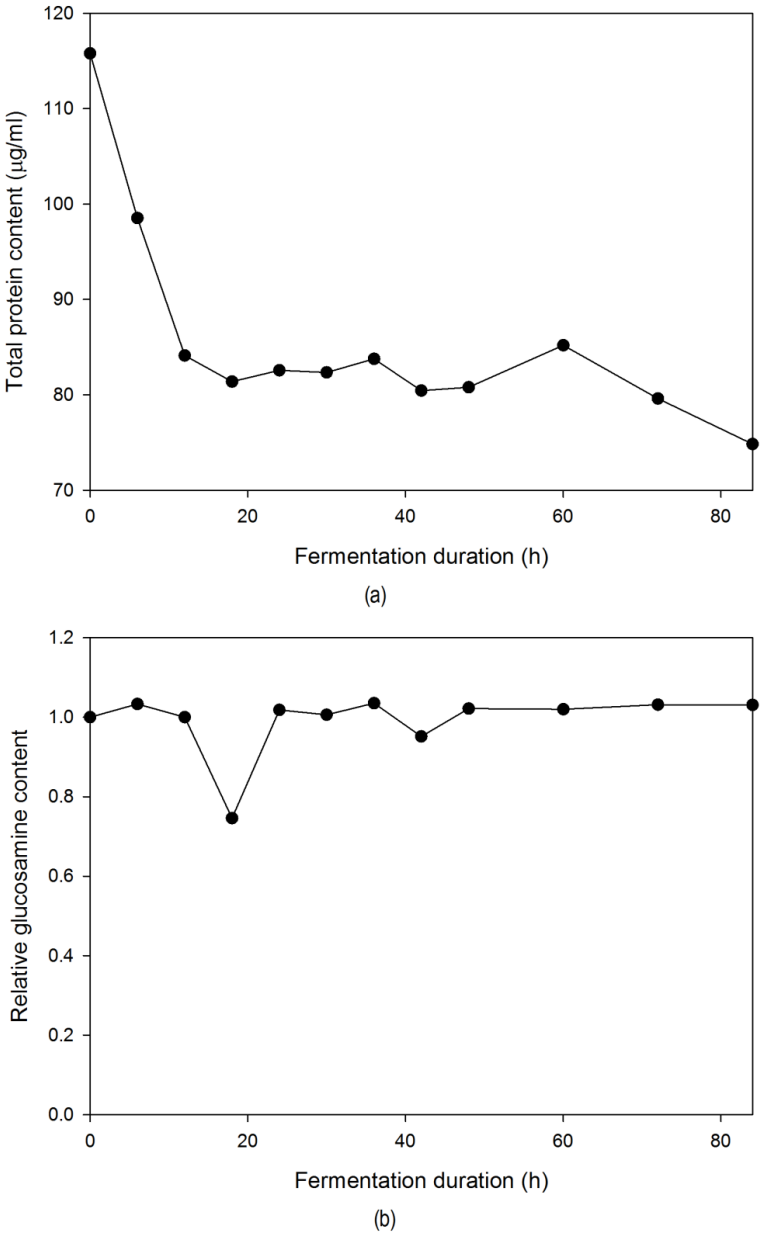


Figure 6.10: Fungal biomass of solid-state fermentation in terms of (a) total protein and (b) relative glucosamine contents.

The mentioned approaches could not represent the fungal growth accurately. Hence, the rate constant for enzyme production (k_v), maximum biomass concentration (X_m), and maximum specific growth rate (μ_m) values were adopted from a solid-state fermentation study conducted by Al-Asheh and Duvnjak (1994). The constants are appropriate and could be used as initial estimates for solving the model equation coefficients of this study. The logistic equation for biomass growth (equation 2.7) was solved by iteration

with the logistic equation for enzyme production (equation 2.9**)** to determine the minimum of the constrained nonlinear multivariable functions. The model-estimated fungal growth curve is shown in Figure 6.11. The fitted model generates estimated constant values: (i) enzyme production rate constant (k_v) = 0.343 units/g moist rice husk/h; (ii) maximum biomass concentration (X_m) = 0.078 g/g moist rice husk; (iii) maximum specific growth rate (μ_m) = 0.022 h^{-1}. The enzyme production rate constant of this study was close to the rate constant of another fungal solid-state fermentation study that is ~0.5 units/g moist substrate/h, and the estimated maximum specific growth rate was slightly lower when the maximum biomass concentration value at 0.078 g/g moist rice husk (Al-Asheh and Duvnjak, 1994). The experimental data for laccase enzyme production and the data predicted using equation 2.11 for the increasing phase of enzyme production demonstrated that the suggested logistic model fitted the empirical data reasonably well (Figure 6.12). There is merely ~10% calculated root-mean-square (RMS) error between the experimental and predicted values. This indicated that the logistic model is accurate for investigating the correlation between fungal growth and enzyme production during the course of solid-state fermentation. Comparing Figure 6.11 and Figure 6.12, laccase enzyme activity of *P. sajor-caju* was in linear relationship with the biomass production implies that laccase enzyme is constitutively produced as the fungus colonized during the solid-state fermentation.

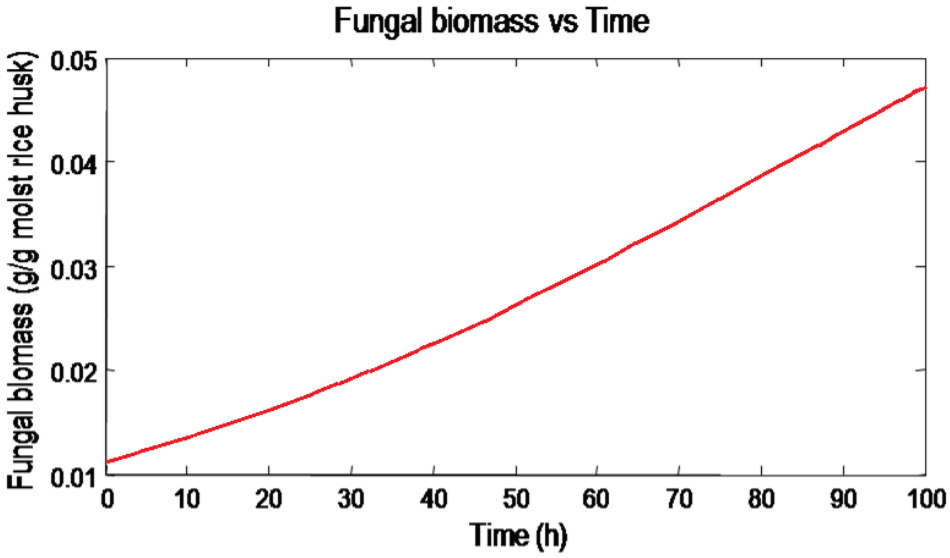


Figure 6.11: Model-estimated fungal growth curve for optimized solid-state fermentation (using initial fungal biomass, X_0 = 0.01114 g/g moist rice husk).

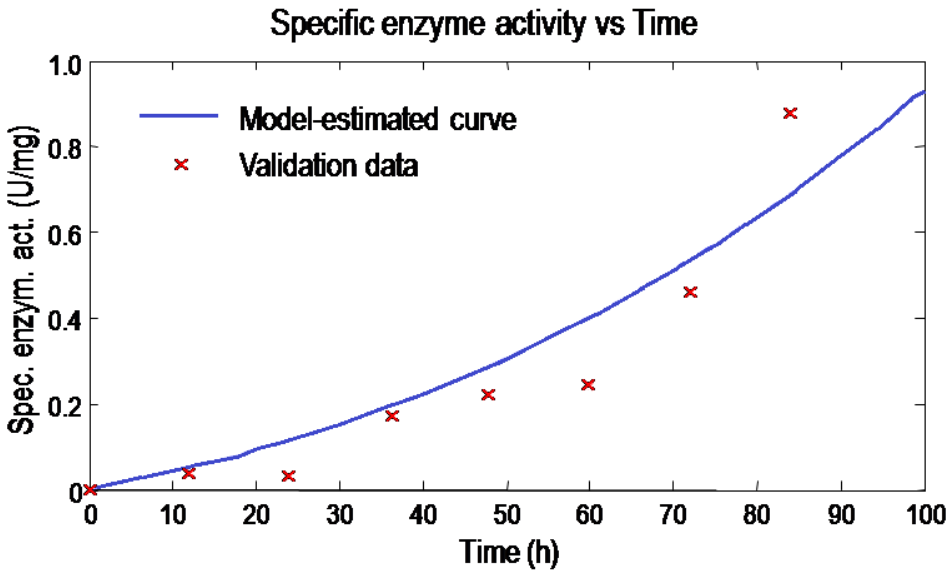


Figure 6.12: Experimental and model-estimated laccase enzyme production kinetics plots under optimized solid-state fermentation condition.

6.1.5 Characterization of Laccase Enzyme

To verify the activity of laccase enzyme produced in solid-state fermentation, the crude laccase enzyme was partially purified to approximately 16-fold. It was used in the

subsequent characterization studies to examine the specific enzyme activity of the laccase on selected substrates, and also to determine the molecular mass of the enzyme.

Laccase enzymes isolated from different fungal sources vary in their abilities in degrading guaiacol, 2,6-dimethoxyphenol (DMP) and 2,2'-azino-bis (3-ethylbenzothiazoline)-6-sulphonic acid (ABTS). In this study, specific enzyme activities of the partially purified laccase on several substrates were determined, and the results are shown in Table 6.4. The laccase enzyme degrades ABTS, DMP and guaiacol in descending order and the findings are consistent with the other studies reported (Kiiskinen et al., 2002; Xiao et al., 2003). Generally, specific laccase enzyme activity of the substrates investigated in this study is lower than the values reported in the literatures. The discrepancy between the specific laccase activities might be due to different enzyme preparations used in the assay. In this study, partially purified enzyme was used, whereas purified enzymes were reported in the other studies. The laccase enzyme did not show RBB-R decolourization activity under the condition used in the characterization of the current study, despite that fungal laccase has been reported to decolourize RBB-R with an efficiency of more than 80% (Khammuang and Sarnthima, 2009).

Table 6.4: Specific enzyme activity of partial purified laccase on different substrates

Substrate	**Specific activity (U/mg)**
ABTS	13.2
Guaiacol	0.7
RBB-R	0
DMP	12.4

The protein SDS-PAGE demonstrated that the partially purified laccase enzyme appeared as a single band in the gel, and its molecular mass was estimated to be 60 kDa compared with the standard molecular weight markers (Figure 6.13). The molecular masses of the enzyme fall within the range (60 to 100 kDa) of laccase enzymes isolated from various fungi. Table 6.5 compiles the molecular masses of laccase enzymes purified from fungi. The difference in molecular mass of these laccase isozymes could be due to the degree of glycosylation of the enzymes.

Table 6.5: Molecular mass of some purified fungal laccases

Organism	**Laccase isozyme**	**Molecular Mass (kDa)**	**References**
Pleurotus ostreatus V-184	LCC1	60	Mansur et al. (2003)
	LCC2	65	
	LCC3	80	
	LCC4	82	
Trametes sp. AH28-2	Laccase A	62	Xiao et al. (2003)
	Laccase B	74	Xiao et al. (2004)
Trametes versicolor 951022	Laccase	97	Han et al. (2005)
Daedalea quercina CCBAS528	Laccase	69	Baldrian (2004)
Basidiomycete PM1 (CECT2971)	Laccase	64	Coll et al. (1993)
Melanocarpus albomyces VTT D-96490	Laccase	80	Kiiskinen et al. (2002)
Cladosporium cladosporioides NCIM1340	Laccase	71.2	Halaburgi et al. (2011)
Ceriporiopsis subvermispora CZ-3	L1	71	Fukushima and Kirk (1995)
	L2	68	

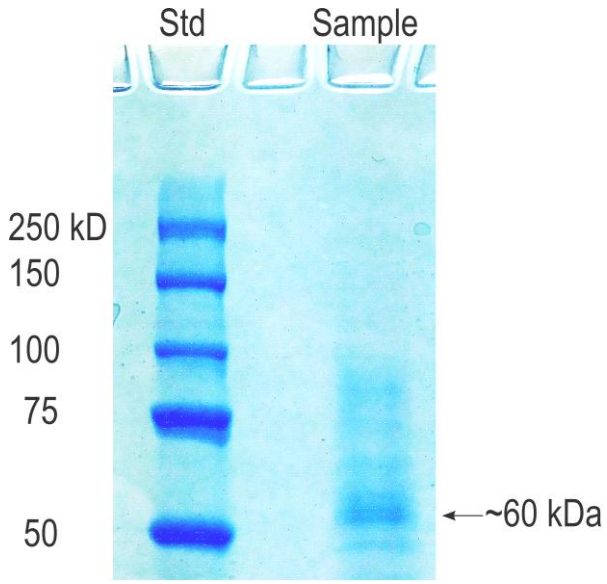


Figure 6.13: SDS-PAGE gel image showing partially purified laccase of *P. sajor-caju*.

6.1.6 Concluding Remarks

In this study, a novel and user-friendly inoculum preparation method – cellophane film culture (CFC) technique was developed. The method was verified and validated of its capability in producing actively growing fungal inoculum feasible to be used in solid-state fermentation. Besides, the technique requires less stringent aseptic operating condition, while presents a lower risk of contaminations compared to liquid culture and agar plugs approaches. With the developed cellophane film culture (CFC) method, the solid-state fermentation was optimized, and copper sulphate loading was found to have the most significant effect among the parameters investigated. In the optimization study using central composite experimental design, the optimized condition obtained was: fermentation duration, 84 hours; copper sulphate concentration, 0.5 mM; glucose concentration, 10 g/L; and Tween 80 concentration, 0.01 g/L. Despite slight discrepancy was observed between the actual (0.5 U/mg) and predicted (0.67 U/mg) responses, the reduced model can be used in the prediction of new solid-state fermentation with satisfactory accuracy. In addition, the modelling of laccase enzyme production using the logistic model has demonstrated a good fit between the

experimental and predicted data with a mere 10% root-mean-square (RSM) error. This signified that the model is suitable for studying enzyme production in solid-state fermentation systems. The laccase enzyme produced by *P. sajor-caju* has a molecular mass of 60 kDa, and its ability in degrading ABTS, DMP and guaiacol demonstrates its potential application in bioremediation of industrial effluents containing phenolic compounds.

CHAPTER 7

CONCLUSIONS AND RECOMMENDATIONS

7.1 Conclusions

Laccase is a versatile enzyme that can be used in various industries like paper and pulp, textile, petrochemical, polymer synthesis, bioremediation of contaminated site, wine and beverage, etc. However, one of the limitations to large-scale application is the lack of capacity to produce large volume of active enzyme at affordable prices. Therefore, a fungal solid-state fermentation scheme, which utilize rice husk as substrate, was proposed. The conclusions from the research findings are as follow:

i. Assessments of rice husk pretreatment using chemicals and ionic liquids were conducted. From the assessments, the dilute hydrochloric acid (HCl) pretreatment was found to be effective in pretreating rice husk. The pretreated rice husk showed desired structural and chemical characteristics for the subsequent fungal solid-state fermentation. Besides, the optimization of rice husk pretreatment with response surface methodology resulted in a relatively mild pretreatment condition (HCl loading, 0.5% (w/v); heating temperature, 125^{o}C; duration, 1.5 hours) that is economically feasible to be carried out in a bigger scale.

ii. Ligninolytic enzyme activity screenings were carried out for *Pycnoporus sanguineus*, *Phlebia radiata* and *Pleurotus sajor-caju*. Under the screening conditions, all the fungus showed varying extent of guaiacol oxidation and/or RBB-R decolourization activities. The selected fungus, *P. sajor-caju*, exhibited both guaiacol oxidation and RBB-R decolourization activities at room

temperature as well as 35^{o}C. Also, the fungus is reported to be able to strive on rice husk and give good biological efficiency. These findings demonstrated the potential of *P. sajor-caju* for the subsequent solid-state fermentation.

iii. A novel inoculum preparation method – the cellophane film culture (CFC) technique for solid-state fermentation was developed. With the verified and validated inoculum preparation method, solid-state fermentation of pretreated rice husk for the production of laccase enzyme using *P. sajor-caju* was optimized and resulted in an optimized fermentation condition of 84 hours fermentation, 0.5 mM copper sulphate, 10 g/L glucose, and 0.01 g/L Tween 80. Copper sulphate that induces laccase enzyme production was found to be the most significant parameter in the solid-state fermentation. Furthermore, the increasing phase of laccase production was successfully modelled using logistic model with only 10% root-mean-square (RSM) error between the empirical and predicted data. From the characterization studies, the partially purified laccase of *P. sajor-caju* has a molecular mass of ~60 kDa, and it can degrade ABTS, DMP and guaiacol like other fungal laccases.

Incorporating the dilute hydrochloric acid pretreatment of rice husk and the optimization of solid-state fermentation using the white-rot fungus *P. sajor-caju*, the proposed solid-state fermentation process is feasible for application in the production of laccase enzyme using the lignocellulosic rice husk. The process is practical both in cost and operation as it involves relatively low cost substrate and chemical reagents.

7.2 Novelties and Implications of Study

Some significant novelties and implications of this research project are highlighted below:

i. Rice husk that contains relatively high cellulose and hemicellulose is rarely used in fermentation for bioconversion due to its recalcitrant structure. The research addressed the potential application of pretreated rice husk as substrate in solid-state fermentation to produce laccase enzyme.

ii. The study has comprehensively examined and compared the effect of various pretreatment reagents on rice husk and its suitability for solid-state fermentation. In addition to the widely employed acid and alkali, the application of ionic liquids in dissolution pretreatment of rice husk was investigated. These findings are helpful in selecting suitable reagent for the pretreatment of lignocellulosic biomass to improve the microbial substrate digestibility in solid-state fermentation.

iii. A qualitative method for fungal ligninolytic enzyme activity screening was developed. This screening method is user-friendly and it can be used to estimate fungal growth and enzyme activity. With the application of this method, large number of fungi could be screened concurrently in a relatively short duration.

iv. To further improve the solid-state fermentation process, a new inoculum preparation method – cellophane film culture (CFC) technique was developed. The method is suitable for preparing actively growing fungal inoculum and overcome limitations present in the existing inoculum preparation methods.

7.3 Recommendations

All the scopes outlined in the study have been accomplished, some potential areas in solid-state fermentation and its associated upstream and downstream processes for possible future studies are recommended as below:

i. To research and refine on the isolation and purification protocols. These protocols are crucial steps in enzymology as they are important in producing good quality enzymes for subsequent studies. The costs of enzyme isolation and purification constitute a major portion of the commercial enzyme price. Therefore, more cost-effective isolation and purification protocols would benefit large-scale applications of the enzyme.

ii. To investigate the industrial applications of laccase enzyme, such as bioremediation of contaminated sites and decolourization of colour effluents. Besides, exploration of the new applications for laccase enzyme such as applications in medical or food industries are beneficial as this enzyme might be useful to replace some hazardous chemicals in the process.

iii. To characterize the purified laccase in a broader perspective, such as examine its molecular and structural components, substrate specificity and inhibition studies and etc. as to elucidate the mechanism of reactions catalyzed by the enzyme. Moreover, it would also provide a platform for engineering of the protein into a more potent and versatile enzyme for industrial applications.

iv. To study the fungal solid-state fermentation process in larger laboratory scales, which involves the design of bioreactor that overcomes mass transfer limitations

and eases the downstream processing of the end-product. The knowledge obtained has significant contribution to the application of solid-state fermentation process at pilot and industrial scales.

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APPENDIX A

Standardization of Working *α*-Amylase for NDF Determination

Heat-stable *α*-amylase Type XII-A from *Bacillus licheniformis* (Catalogue number A3403) stock solution was purchased from Sigma-Aldrich. The heat-stable *α*-amylase stock solution was standardized so that two additions of 2 ml removed raw corn starch from 0.5 g ground hominy corn (AOAC, 2005). The volumes of dosage examined were in the geometric progression of 0.025 ml from 0.100 to 0.250 ml of heat-stable *α*-amylase stock solution. A mixture of 0.5 g ground hominy corn and 50 ml of ND solution was refluxed for 10 minutes. Two equal doses of stock were added to the mixture, one dose was added at the moment when the mixture started to boil, and allowed to reflux for 60 minutes; the other dose was added after reflux and allowed to react for 60 seconds. The mixture was filtered through cheesecloth into 100.0 ml beaker and placed in a cold water bath for about 5 minutes before moved to a room temperature tempering bath. Next, 0.5 ml Burke's iodine solution was added and the colour of the solution was viewed after 90 seconds and before 120 seconds has elapsed. The efficacy of raw corn starch hydrolysis was evaluated using the scale tabulated in Table A1. The lowest dose that gave pale yellow colour represented the volume of heat-stable *α*-amylase stock solution used to make working solution. Blank (control) was prepared by adding two doses of 0.1 ml heat-stable *α*-amylase stock solution to 40 ml of ND solution. Prepared working solution was stored refrigerated and used within five days from the day of preparation.

Table A2 showed the colour indication of raw corn starch solution reacted with two dosages of varied volume *α*-amylase stock solution after addition of Burke's iodine

solution. Two dosages of the lowest amount of volume α-amylase stock solution (0.225 ml) that gave pale yellow colour was used to prepare α-amylase working solution.

Table A1: Colour indication of solution showing adequacy of α-amylase enzyme

Colour	Indication
Purple	Not adequate enzyme
Amber	Not adequate enzyme
Yellow	Adequate enzyme

Table A2: Adequacy of α-amylase stock solution for raw corn starch hydrolysis

Volume of α-amylase Stock solution (ml)	Colour	Indication
0.100	Amber	Not adequate enzyme
0.125	Light amber	Not adequate enzyme
0.150	Light amber	Not adequate enzyme
0.175	Light amber	Not adequate enzyme
0.200	Light amber	Not adequate enzyme
0.225*	Pale yellow	Adequate enzyme
0.250	Pale yellow	Adequate enzyme

* Volume used to prepare α-amylase working solution.

The total volume of α-amylase working solution used in the addition of two dosages of 2 ml of working solution for each test was calculated. The volume of α-amylase stock solution added twice, each before and after reflux, determined from the standardization was 0.225 ml. A guide on the calculation of α-amylase working solution is given below,

The total volume of α-amylase working solution = (n) x 4 ml

The volume of α-amylase stock solution = (n) x 2 x 0.225 ml

The reverse osmosis water used for dilution = (n) x [4 – (2 x 0.225)] ml

, where n is the number of tests.

The adequacy of α-amylase working solution prepared was confirmed by repeating standardization procedure using 0.5 g ground hominy corn with two dosages of 0, 2 and

4 ml working solution, each before and after reflux. The lower volume of α-amylase working solution was selected when there is no appreciable difference in colour between dosages. The outcome was tabulated in Table A3.

Table A3: Adequacy of α-amylase working solution dosage volume

Volume of α-amylase working solution (ml)	**Colour**	**Indication**
0 *	Purple	Not adequate enzyme
2	Pale yellow	Adequate enzyme
4	Pale yellow	Adequate enzyme

* Control of experiment.

Preparation of Hominy Corn for Standardization of Working α-Amylase

Hominy corn is dried corn kernel that has been treated with alkali (Sanderson et al., 1978). The hominy corn was prepared in the laboratory according to the procedure outlined by Sanderson *et al.* (1978). Dried corn (dried condiments, First Choice Brands) was purchased from a local supermarket. The corn was washed and decanted dry to remove foreign particles. Then, approximately 200 g of dried corn was boiled and stirred in lime water containing 20 g/L calcium hydroxide (Sigma-Aldrich) for 15 minutes. Subsequently, it was allowed to soak in the lime water for one hour. After the soaking, the lime-treated corn was washed several times to remove excess lime. The corn was boiled again in 500 ml water for 30 minutes and allowed to soak for another 30 minutes. Then, the corn hulls, tip caps, and germs were removed. The remaining endosperm of corn was boiled in water for 15 minutes and allowed to cool to room temperature. The corn was rinsed with cool water and dried in an incubator (JEIO TECH, model IB-10) at 50^{o}C until constant weight was achieved. The drying was done in mild temperature in incubator to avoid the content deterioration of the hominy corn. Figure A1 illustrates dried hominy corn produced in the laboratory before grinding to pass through a 500 μm sieve and stored in a dry cabinet prior to use.

Figure A1: Dried hominy corn for standardization of α-amylase activity for neutral detergent fibre determination.

APPENDIX B

Construction of Glucose Standard Calibration Curve

The calibration curve was constructed from standards prepared from anhydrous D-glucose (Fisher Scientific). Glucose standards were made from serial dilutions of 1.0 mg/ml glucose stock solution as shown in Table B1.

Table B1: Ratio of glucose stock to water in the preparation of standards

Concentration of glucose standard (mg/ml)	Volume of glucose stock (ml)	Volume of water (ml)
1.00	10.00	0.00
0.75	7.50	2.50
0.62	6.25	3.75
0.50	5.00	5.00
0.37	3.75	6.25
0.25	2.50	7.50

The absorbance data for the standard calibration curve was closely fit to a calculated best-fit line with a coefficient of determination (R^2) for the straight line fit close to one (Figure B1). The concentration of reducing sugar in the samples was determined from the calibration curve. The reducing sugar concentration of the samples must fall within the lowest and highest value of the calibration curve. It is advised to measure the glucose standards and samples in a single determination to minimize errors caused by experimenter and reagents.

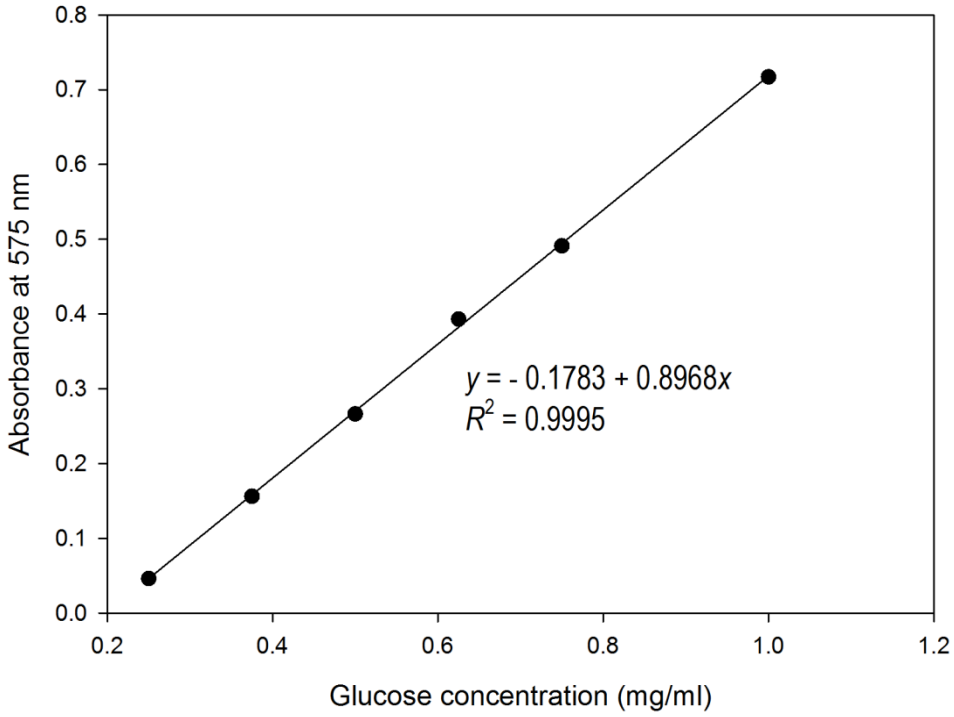

Figure B1: Glucose standard calibration curve.

APPENDIX C

Protocol for Total Soluble Protein Determination

The following protocol for total soluble protein determination was adapted from the technical bulletin of Total Protein Kit, Micro Lowry, Peterson's Modification (Sigma-Aldrich, USA) (Publication reference LCM/GL/MAM 10/03).

Stepwise protocol for total soluble protein determination without protein precipitation is outlined below:-

i. 'Standards' were prepared by diluting the 400 µl/ml protein standard solution in water to a volume of 1 ml in appropriately labelled test tubes according to Table C1. A test tube was labelled 'Blank' and was added 1 ml of deionised water. Test tubes were labelled 'Sample', and 1 ml of enzyme sample was added to the appropriately labelled test tube.

Table C1: Ratio of BSA stock solution to water in the preparation of standards

Protein concentration (µg/ml)	Volume of BSA stock (ml)	Volume of water (ml)
250	0.62	0.38
200	0.50	0.50
150	0.37	0.63
100	0.25	0.75
50	0.12	0.88
25	0.06	0.94
12.5	0.03	0.97

ii. Then, 1 ml of the Lowry reagent solution was added to 'Standard', 'Blank' and 'Sample' tubes, and mixed well. The solutions were allowed to stand at room temperature for 20 minutes.

iii. With rapid and immediate mixing, 0.5 ml of the Folin and Ciocalteu's Phenol reagent working solutions was added to each tube. The colour was allowed to develop for 30 minutes.

iv. After incubation, the solutions were transferred to a cuvette and the absorbance of the 'Standard' and 'Sample' tubes versus the 'Blank' were measured. Prior to the assay, the absorption wavelength between 500 and 800 nm of the enzyme sample was screened. The maximal absorption wavelength of the enzyme sample was found to be 765 nm (Figure C1), and it was used in total soluble protein determination. The absorbance readings were completed within 30 minutes.

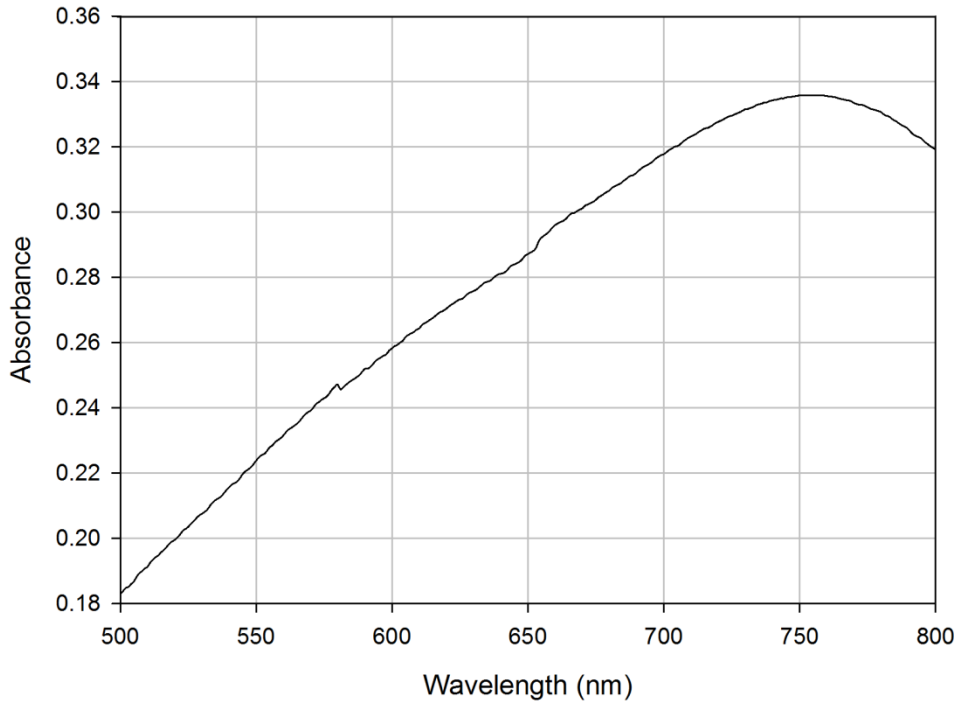

Figure C1: Absorption profile of the crude enzyme filtrate at wavelengths between 500 and 800 nm.

v. The absorbance values of the 'Standards' versus their corresponding protein concentrations was plotted in a calibration curve. The calibration curve was

constructed from ‘Standards’ prepared from bovine serum albumin (BSA, Sigma-Aldrich). At BSA protein concentrations between 12.5 and 250 μg/ml the absorbance data closely fit to a hyperbolic curve with a coefficient of determination (R^2) for the fitted line close to 1 (Figure C2). The protein concentration of the ‘Samples’ were determined from the calibration curve.

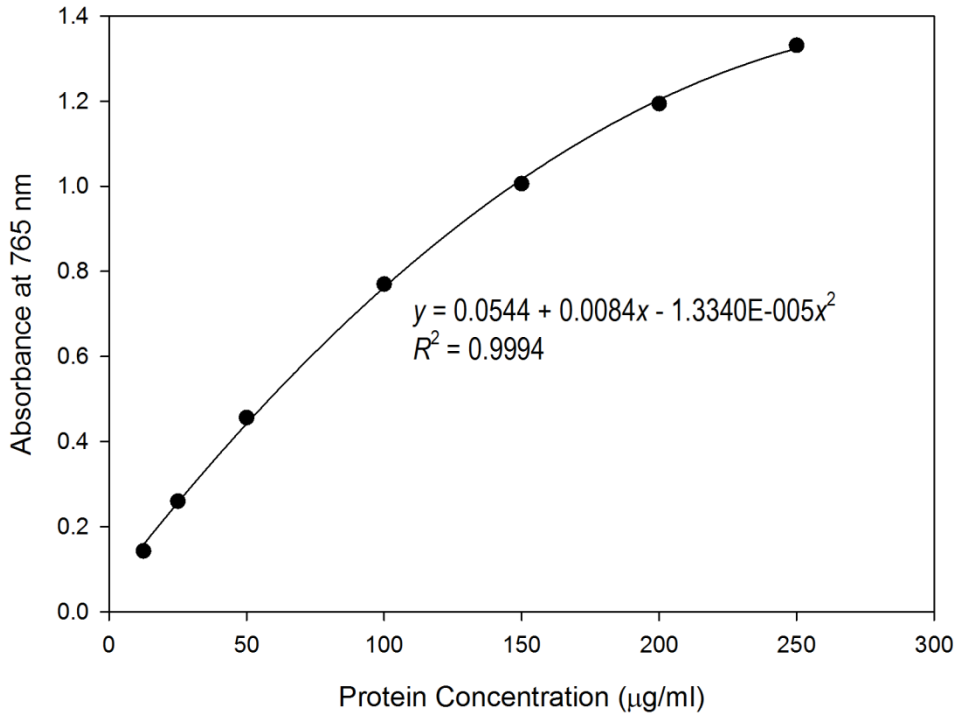

Figure C2: Protein standard calibration curve.

APPENDIX D

Protocol for Staining SDS-PAGE Gel

The following protocol for staining sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel of the partial purified laccase sample was adapted from the user guide of SimplyBlue™ SafeStain (Comassie G-250 stain, Novex® by Life Technologies, USA) (Publication part number IM-6050; Revision date 13th February 2012).

Stepwise protocol for staining SDS-PAGE gel is outlined below:-

i. The gel was rinsed 3 times for 5 minutes with 100 ml deionised water to remove SDS and buffer salts that interfere with binding of the dye to protein. The deionised water was discarded after each rinse.

ii. The gel was stained with SimplyBlue™ SafeStain using volume enough to cover the gel. The gel was stained for 1 hour at room temperature with gentle shaking. After incubation, the stain was discarded.

iii. The gel was washed with 100 ml of deionised water for 2 hours. Then, about 20 ml of 20% sodium chloride (NaCl) was added to the deionised water, and the gel was continued to wash for additional 1 hour.

iv. The second wash was applied to the gel for the clearest background. The gel was washed with 100 ml of deionised water for 1 hour. The proteins appear blue, and there is a small amount of dye in the deionised water that is in equilibrium with the dye bound to the proteins.