ONE STEP BIOCONVERSION OF LIGNIN FROM RICE HUSK TO BIOVANILLIN BY *Phanerochaete chrysosporium*

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A thesis submitted in fulfilment of the
requirements for the award of the degree of
Doctor of Philosophy

Faculty of Science
Universiti Teknologi Malaysia

OCTOBER 2021

ACKNOWLEDGEMENT

First and Foremost, Alhamdulillah, I am grateful to Allah S.W.T. for the blessing and His willingness that has provided me with the infinite strength and patience to complete my PhD study. I would like to express my indefinite gratitude to my main supervisor, Assoc. Prof. Dr Madihah for her encouragement, constructive criticism, patience, guidance, helpful suggestion and strong motivation. She encouraged me to grow as an independent thinker, which improved not only my scientific thinking but also all aspects of my life. For everything you have done for me, I thank you. My infinite thanks also go to Dr Adibah who has always spoken kindly, encouraged me and give me mentally support especially during my toughest period.

My sincere thanks are first to all the members of Biorefinery Technology Research Laboratory, UTM for creating a healthy working environment in the lab, which has been unique in its own way. For this my thanks are due to Dr Atiqah, Dr Huszalina, Dr Ang, Dr Roslan, Dr Shankar, Dr Ibrahim, Husna, Zul, Shiffa, Fatimah, Akma, Aida, Yue Ming, Sazyani and Intan for their help, moral support, advice, toleration, cooperation, and sharing their hardship story and knowledge with me. They were good friends and always willing to help and give excellent suggestions whenever I am having a difficulty during my study period.

My thank you also dedicated to all Staffs of the Department of Bioscience, Faculty of Sciences, UTM especially to Pn Fatimah, Mr Afiezy, Mr Nazirul, Pn Farah and all other laboratory staff for their help to provide me analytical instruments and chemicals I need during my laboratory works. I am also obliged to express my big appreciation towards my beloved parents and my siblings for their enduring patience in waiting for me to complete my study, indefinite moral and financial supports. Last but not least, to all my friends who involved directly in my research work, heartfelt gratitude for all of you. My last appreciation goes to my best friends, Nurhalawiah and Fazilah who always lend their ears to me no matter what kind of situation they are in. To those who indirectly contributed to my works, your kindness means a lot to me. Thank you for everything. May God bless all of us.

ABSTRACT

Rice processing produces two major types of wastes which are rice husk and rice straw. The husk is the outer coat of paddy grain while the straw is the dry stalks of paddy. Burning in landfills, the common rice husk disposal method, is a cause of serious concern on the environment and human health. Despite the effort to fully utilize all the fractions of lignocellulosic biomass, most research only focuses on the cellulose and hemicellulose, whereas the lignin was left as worthless refuse. However, interest in lignin is growing for its abundant phenolic compounds and utilisation in aromatic chemicals such as biovanillin. The research aimed to investigate the potential production of biovanillin from rice husk lignin in submerged batch fermentation using *Phanerochaete chrysosporium* ATCC 24725. As a product of lignin degradation, ferulic acid is a precursor and intermediate in one-step biovanillin production in batch fermentation. Four types of pretreatments, namely physical, chemical, physicochemical, and enzymatic hydrolysis using cellulase from *Trichoderma virens* KR259658 were applied prior to recovering lignin. Out of twenty-one types of pretreatments, the microwave method without any chemical addition was chosen for optimization using the One Factor-at-a-Time method and Central Composite Design. Because microwave radiation can heat uniformly inside the sample, it breaks down the lignin and hemicellulose structure. Furthermore it destroyed the partial crystallised cellulose and degraded hemicellulose into reducing sugars. The microwave also saved the pretreatment time and increased the lignin accessibility to hydrolytic enzymes. Three parameters were studied namely microwave irradiation time, solid loading, and microwave power. At irradiation time of 16.57 min, solid loading of 9.66%, and microwave power of 664.23 Watt, the predicted lignin recovery was at 34.91%. The effect of different nitrogen sources in biovanillin production was investigated using the General Factorial Design. To further investigate the significant factors affecting the biovanillin production, 2-Level Factorial Design was carried out using the combination of diammonium tartrate, meat peptone, and five other factors, lignin concentration, the temperature of incubation, pH of the medium, agitation speed, and size of inoculum. Out of these seven factors, only three factors - organic source, pH of medium, and agitation speed have significant impact and were used in optimization. The maximum biovanillin production was performed with the media composition of 20.0 g/L of glucose, 1.8415 g/L of diammonium tartrate, 6.0 mM of meat peptone, 0.2 g/L KH_2PO_4, 0.013 g/L $CaCl_2.2H_2O$, 0.5 g/L $MgSO_4.7H_2O$, 0.3 g/L of lignin and 0.0025 g/L thiamine hydrochloride, and treatment conditions of temperature at 30.64 ºC, pH 4.0 and 199.61 rpm agitation speed. The predicted R_2 0.6583 is in reasonable agreement with the adjusted R_2 of 0.8016. The biovanillin produced after the optimized condition equals to 0.0122 g/L. In conclusion, this work has shown the potential utilization of lignin extracted from rice husk to produce biovanillin in submerged fermentation using *Phanerochaete chrysosporium*.

ABSTRAK

Pemprosesan padi menghasilkan dua jenis sisa utama iaitu sekam padi dan jerami padi. Sekam adalah kulit luar yang menyelaputi bijirin manakala jerami adalah batang kering padi. Pelupusan sekam padi melalui pembakaran di tapak pembuangan sampah memberi kesan ke atas alam sekitar dan kesihatan manusia yang perlu diberi perhatian serius. Walaupun dengan usaha menggunakan fraksi-fraksi lignoselulosa sepenuhnya, sebahagian besar penyelidikan hanya memfokus kepada penggunaan selulosa dan hemiselulosa manakala lignin disingkirkan sebagai bahan buangan yang tidak bernilai. Walau bagaimanapun, minat terhadap lignin semakin meningkat susulan kandungan sebatian fenolik yang tinggi dan berguna dalam penghasilan bahan kimia aromatik seperti biovanilin. Tujuan kajian ini adalah untuk menyiasat potensi penghasilan biovanilin daripada lignin sekam padi melalui penapaian terendam berkelompok menggunakan *Phanerochaete chrysosporium* ATCC 24725. Sebagai hasil degradasi lignin, asid ferulik menjadi pelopor dan perantara penghasilan biovanilin melalui kaedah selangkah di dalam penapaian berkelompok. Empat jenis rawatan digunakan untuk mendapatkan lignin iaitu fizikal, kimia, fisikokimia, dan hidrolisis enzim selulase daripada *Trichoderma virens* KR259658. Daripada dua puluh satu jenis rawatan yang dibuat, kaedah gelombang mikro tanpa bahan kimia telah dipilih untuk pengoptimuman menggunakan kaedah Satu Faktor-Pada-Satu-Masa dan Rekabentuk Tergubah Berpusat. Oleh kerana radiasi mikro boleh memanaskan sampel secara seragam, ia akan memecahkan struktur lignin and hemiselulosa. Tambahan pula, ia menghancurkan selulosa separa-hablur dan menguraikan hemiselulosa kepada gula penurun. Gelombang mikro juga menjimatkan masa rawatan dan meningkatkan serapan enzim hidrolitik. Tiga parameter diuji iaitu masa radiasi gelombang mikro, beban pepejal, dan kuasa gelombang mikro. Hasil ramalan lignin sebanyak 34.91% telah dicapai pada 16.57 min, beban pepejal 9.66% dan kuasa gelombang mikro 664.23 Watt. Kesan penggunaan sumber nitrogen yang berbeza terhadap penghasilan biovanilin telah dikaji menggunakan Rekabentuk Faktoran Umum. Untuk menyiasat faktor-faktor bererti dalam penghasilan biovanilin, Rekabentuk Faktoran Dua Aras telah dijalankan menggunakan gabungan diamonium tartrat, pepton daging, dan lima faktor lain iaitu kepekatan lignin, suhu eraman, pH media, kelajuan goncangan, dan saiz inokulasi. Daripada tujuh faktor, hanya tiga faktor - sumber organik, pH media dan kelajuan goncangan yang memberikan impak bererti seterusnya digunakan dalam pengoptimuman. Dalam kajian ini, biovanilin maksima terhasil dengan komposisi media 20.0 g/L glukosa, 1.8415 g/L diamonium tartrat, 6.0 mM pepton daging, 0.2 g/L KH_2PO_4, 0.013 g/L $CaCl_2.2H_2O$, 0.5 g/L $MgSO_4.7H_2O$, 0.3 g/L lignin dan 0.0025 g/L tiamin hidroklorida, dan keadaan rawatan pada 30.64 ºC, pH 4.0 dan kelajuan goncangan 199.61 rpm. Ramalan R_2 0.6583 adalah persetujuan yang munasabah dengan R_2 larasan iaitu 0.8016. Biovanilin yang dihasilkan selepas keadaan pengoptimuman adalah bersamaan 0.0122 g/L. Sebagai kesimpulan**,** kajian ini menunjukkan potensi penggunaan lignin yang diekstrak daripada sekam padi untuk menghasilkan biovanilin di dalam penapaian terendam dengan menggunakan *Phanerochaete chrysosporium.*

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LIST OF ABBREVIATIONS

SmF	-	Submerged Fermentation
CCD	-	Central Composite Design
RSM	-	Response Surface Methodology
OFAT		One Factor at a Time
BSA	-	Bovine Serum Albumine
GFD		General Factorial Design
CMC	-	Carboxymethylcellulose
2LFD		2 Level Factorial Design
CMCase	-	Carboxymethylcellulase
DNS	-	Dinitrosalicyclic acid
FPase	-	Filter Paperase
TPC	-	Total Phenolic Content
FTIR	-	Fourier Transform Infrared
HPLC	-	High Performance Liquid Chromatography
LiP	-	Lignin Peroxidase
MnP		Manganese Peroxidase
VP		Versatile Peroxidase
NaOH		Natrium Hydroxide
HCl		Hydrochloric acid
HNO_3		Nitric Acid
PDA		Potato Dextrose Agar
rpm		Rotation per Minute
pNPG		p-nitrophenyl β-D-glucoside
ABTS		2,2´-azino-bis (3-ethylbenzothiazoline)-6-sulphonic acid
GAE		Gallic Acid Equivalent
SEM		Scanning Electron Microscopy
H NMR		Hydrogen Nuclear Magnetic Resonance
C NMR		Carbon Nuclear Magnetic Resonance
MW		Molecular Weight
PTFE		Polytetrafluoroethylene

KH_2PO_4	Potassium Hydrogen Phosphate
$(NH_4)_2SO_4$	Ammonium Sulfate
$CaCl_2$	Calcium Chloride
$MgSO_4$	Magnesium Sulfate
$FeSO_4.7H_20$	Ferum Sulfate Heptahydrate
$MnSO_4.4H_2O$	Mangan (II) Sulfate tetrahydrate
$ZnSO_4.7H_2O$	Zinc Sulfate Heptahydrate
$CoCl.6H_20$	Cobalt (II) Chloride Hexahydrate
G:S: H	Guaiacyl: Syringyl: Hydroxyphenyl
OCH_3	Methoxy
OH	Phenoxyl
C=O	Carbonyl
CO_2	Carbon Dioxide
H_2O_2	Hydrogen Peroxide
MWL	Milled Wood Lignin
Fe	Ferum
Ca	Calcium
Na	Sodium
K	Potassium
Mg	Magnesium
FA	Ferulic Acid

LIST OF SYMBOLS

g	-	Gram
mM	-	Milimolar
w/v	-	Weight per Volume
v/v	-	Volume per Volume
W	-	Watt
ºC	-	Degree Celcius
g/L	-	Gram/Liter
µmole	-	Micromole
ml	-	Millilitre
psi	-	Pound per square inch
h	-	Hour
µm	-	Micrometre
kV	-	Kilo Volt
kg	-	Kilogram
mm	-	millimetre
MJ/Kg	-	Mega Joule per Kilogram
Spores/ml	-	Spores per millilitre
nm	-	Nanometer
M	-	Molar
µL	-	Microliter
µmol	-	Micromole
µg/ml	-	Micro gram per mililiter
cm	-	Centimetre
mg/ml	-	Miligram per mililiter
mg/g	-	Milligram per gram

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

1.1 Background of Research

Vanillin is a natural and widely used flavouring ingredient. It is an important ingredient in food, perfumes, and beverages, as well as an intermediary in the pharmaceutical industry. The majority of flavour compounds are always produced by the process of chemical synthesis, which can have adverse health and environmental impact. Because of the high demand for natural and healthy products, and also the fact that acid ferulic (FA) derived from plant materials may be used as a precursor for biovanilin synthesis, it is reasonably inexpensive as a natural product. This research work is the continuation worked by two doctorate students who successfully reported the utilisation of ferulic acid from lemongrass leaves hydrolysates to produce biovanillin by using the same microorganism. Their findings revealed the potential production of biovanillin by using the same strain of white-rot fungus but using a different substrate which was lemongrass leaves. Ferulic acid which recovered from liquid hot water treatment in the form of hydrolysate was used as a substrate for biovanillin production. Therefore, as a promising precursor in biovanillin production, this study was carried out to investigate the potential biovanillin production from ferulic acid which was expected to come from the lignin degradation process in submerged fermentation by *Phanerochaete chrysosporium* ATCC 24725. The lignin was recovered from rice husk is become one of the alternatives to achieve effective waste management during the rice milling process and also to prevent air pollution in the environment.

Rice (*Oryza sativa*) is the most important staple food for a large part of the world's human population, particularly in East and South Asia, Latin America, the Middle East, and the West Indies. Peninsular Malaysia uses about 300,500 ha of land and another 190,000 ha in East Malaysia just for paddy cultivation, with around

300,000 people were farmers (Adnan *et al.*, 2018). The cultivation of paddy has become the main food source for Malaysia with per capita consumption accounting for between 500 to 799 calories per day (Yusof *et al.*, 2019). Increasing food production means an increase in agricultural activities that generate a significant amount of waste. Other than creating rice grain during paddy processing, it also produces two main important types of residues which is husk and straw. For each kilogram of paddy grain produced, between 0.41 and 3.96 kg of rice straw will be generated, while rice husk accounts for between 20-33 % of the paddy weight equivalent to over 148 million metric tonnes of rice husk is produced globally (Menya *et al.*, 2018). In the perfect processing paddy milling, 1000 kg of paddy rice produces 200 kg of the husk, 80-110 kg of bran depending on the processing degree and 680-730 kg of white rice grain depending on the variety (Pode, 2016).

Rice husk mainly consists of three components which are cellulose, hemicelluloses, and lignin. Carbohydrate compositions of rice husk and rice straw were similar, but the contents of lignin differed significantly (Wu *et al.*, 2018). The amount of lignin in husk was in the range of 19.0 % to 35.3% while straw in the range of 5% to 27.7% (Abraham *et al.*, 2016; Dagnino *et al.*, 2018; Lubwama andYiga, 2018; Malik *et al.*, 2015; Shoaib *et al.*, 2018; Song *et al.*, 2019; Wu *et al.*, 2018). Both of these residues available abundantly during rice milling and are low in cost, however, higher lignin content in husk gives a significant reason to choose the husk instead of straw. Regarding this issue, research on the utilization of rice husk residues and turn it into value-added products are becoming an increased interest, especially in Malaysia.

Currently, 85% of the world's vanillin supply is produced from oil-derived guaiacol, with the remaining 15% obtained from softwood lignin. Natural vanillin, whose market value ranges from $1200 to $4000 per kg supplies just approximately 1% of the worldwide demand for vanillin. Ferulic acid fermentation is the only commercially feasible biocatalytic method for vanillin. which produce vanillin at $10 to $15 per kg. Vanillin may also be synthesised chemically, using coniferin, guaiacol, eugenol, ferulic acid, and lignin as potential starting ingredients (Mota *et al.*, 2016). Lignin is nature's renewable resource of aromatics, and the conversion of lignin to vanillin is incredibly attractive. However, vanillin production is directly connected to lignin resource and isolation technique, and lignin always generates a relatively low yield of vanillin. Therefore, the unique structure and abundance of aromatic compounds in the lignin structure become the potential marker to break down and produce natural precursors in biotechnologically biovanillin production based on microbial fermentation processes.

In the pulp and paper industry, lignin has traditionally been produced from the kraft and sulfite processes. To recover chemicals from the pulping process and provide process heat, the vast majority of lignin is burned and only 2% of all lignin is utilized for rather low-value applications (Wilson and Lee, 2014). This drive the common perception that lignin is just a waste product of the pulping process and, as a result, this has limited the research for value-added applications for lignin and lignin-based materials for quite some time. However, higher-value products are continually being found from time to time. The phenolic-rich compounds in its complex aromatic structure make lignin one of the potential sources for biovanillin production. Given the situation of a very large feedstock accessible at a low cost that could be used to fulfil the demand for a high-value product makes a biotransformed of lignin-to-vanillin is highly feasible. However, to preserve sustainability and a greener environment, such a process must produce a reasonably high yield of single monomeric products with no harmful secondary waste.

Nowadays, the research towards vanillin production using biological methods has become growing attention through the biochemical pathway of the conversion process remains the challenge in this present study. Thus far, extensive literature is available regarding the production of vanillin from ferulic acid recovered from different types of lignobiomass with the application of bacteria or fungus (Kaur and Chakraborty, 2013; Kaur *et al.*, 2013; Motedayen *et al.*, 2013; Patil andYadav, 2018; Paz *et al.*, 2018). The production of vanillin from the oxidation of lignin has also been demonstrated in several studies by using different sources of lignin (Aarabi *et al.*, 2017; Shakeri *et al.*, 2013; Tang *et al.*, 2015; Wang *et al.*, 2018). However, a strong oxidizing agent used such as hydrogen peroxide tends to over-oxidize the lignin during long reactions while nitrobenzene tends to create toxic by-products during the transformation process (Moodley *et al.*, 2012). The biotechnological approach offered several advantages over other techniques such as mild reaction conditions and less harm to the environment.

Therefore, this research is intended to apply the conversion of lignin recovered from rice husk into biovanillin by using *Phanerochaete chrysosporium* as the potential fungi in batch submerged fermentation. *Phanerochaete chrysosporium* had known as good ligninolytic properties (lignin peroxidase, manganese peroxidase and, laccase), fast growth, easy to handle and able to degrade lignin (Munir *et al.*, 2015). The variety of phenolic compounds, especially ferulic acid are produced from lignin degradation can be served as the intermediate and further utilize for conversion into biovanillin. Vanillin or 4-hydroxy-3-methoxy benzaldehyde, $C_8H_80_3$ is a phenolic aldehyde that consisting of three functional groups, aldehyde, phenol and ether. It is a plant secondary metabolite and it is the major component of vanilla extract from cured vanilla pods. It was isolated from vanilla beans in 1816 and its structure was determined in 1874 (Zheng *et al.*, 2007). Production of vanillin from the orchid plant is costly and time-consuming which can take several months and a slow rate. The yield of vanillin produced from 500 kg vanilla pods is only 1 kg and its global production is less than 1 % (Gallage *et al.*, 2014). The demand for vanillin is approximately 12,000 tons per year but unfortunately, only 1800 tons of natural vanillin is produced. Therefore, one of the alternative ways to fulfil the need of consumer demands on natural vanillin is to produce biovanillin from lignocellulosic biomass by application of biological and biorefinery knowledge.

1.2 Problem Statement

The increasing production of paddy has increased the production of waste especially rice husk. The open burning practice of rice husk by societies especially rice millers has given rise to several problems such as the greenhouse effect, air pollution, and health problem, especially among asthmatic people. Higher lignin content in rice husk with the richness content of phenolic compounds become an interesting environmental issue to be solved. Only 2% of lignin is applied in industries (Wang *et al.*, 2018) and the rest is typically burned for low-value heat for energy and power in pulping and biorefinery processes, leaving behind environmentally harmful aromatics (Bruijnincx and Weckhuysen, 2014; Meng *et al.,* 2019; Wang *et al.*, 2018). The study to explore the use of rice husk instead of other lignocellulosic waste like lemongrass was performed to ensure the sustainable source of the lignin prior to biovanillin production.

Vanillin is typically produced by chemical synthesis, with just a minor contribution from natural sources. The main disadvantage of chemical synthesis is that it is not environmentally friendly, and the required chemicals commonly emerge as harmful mixture that might interfere with fermentation processes. Although synthetic vanillin may be used in replace of natural vanillin, it is not considered a natural product, which has motivated researchers to look for alternative vanillin manufacturing techniques. Biotechnological approaches, such as microbial biotransformation, are one of the most promising methods for generating vanillin (Zamzuri *et al.*, 2014) Biotechnological approaches, such as microbial biotransformation, are one of the most promising methods for generating vanillin (Overhage *et al.* 2006). Because they are obtained from biological sources that are converted by living cells and enzymes, products of vanillin biotransformation have a high potential to be considered natural products. Aside from its high cost, natural vanilla bean production cannot meet the world's complete vanillin demand. More over 12,000 tonnes of vanillin are produced each year, with just 1% coming from *Vanilla planifolia* (Chattopadhyay *et al.*, 2018). The price demand between chemically synthesized and natural vanillin contribute to the growing interest in this study to explore the biotechnological routes in vanillin production. The price difference between natural and chemically produced vanillin, as well as increasing consumer demands for natural vanillin flavour, are the driving forces in this study to investigate the biotechnological methods for vanillin production.

1.3 Research Objectives

The objectives of this research study are as follows:

a) Analyze the composition of rice husk and screening the potential use of lignin obtained from physical, chemical, physicochemical, and enzymatic hydrolysis pretreatments for biovanillin production in submerged batch fermentation.

b) Optimised microwave pretreatment for lignin recovery using One Factor At-A-Time method (OFAT) and Central Composite Design (CCD).

c) Optimised the significant factors for biovanillin production by using a two-level factorial design (2LFD) and central composite design (CCD) in submerged batch fermentation.

1.4 Scope of the Research

This study focused on lignin recovery from rice husk by using different types of pretreatment methods from physical, chemical, physicochemical and enzymatic hydrolysis. The lignin recovered were then gravimetrically quantified and their functional group present in the structure was analyzed by using Fourier Transform Infrared Spectroscopy (FTIR).

All the extracted lignins were used in the screening of their potential for biovanillin production in submerged batch fermentation by using white-rot fungus, *Phanerochaete chrysosporium*. The pretreated lignin which produced the highest recovery of biovanillin was chosen and optimized using the One Factor at A-Time method and further proceed with Central Composite Design (CCD) of Response Surface Methodology (RSM). Only one type of pretreated lignin was chosen for the next stage in this research. The morphology structure of rice husk before and after the pretreatment was carried out by using Scanning Electron Microscopy.

The effect of nitrogen supply in submerged fermentation towards biovanillin production was carried out by using General Factorial Design (GFD). Organic and inorganic sources were supplied in submerged fermentation to understand the effect of two or more independent variables upon a single dependent variable with respect to the value of biovanillin produced. The result obtained from this part of the study was used to screening seven factors affecting biolignin production in Two-Level Factorial Design (2LFD). The significant factors were identified and proceed further in Central Composite Design (CCD) of Response Surface Methodology (RSM).

1.5 Thesis Organization

This thesis is presented in eight chapters and the content of each chapter is described as follows:

Chapter 1 presents the background of research, problem statement, the objective of the research and the outline of research in the scope of the study. The general introduction of the overall research and the objectives are stated specifically in this chapter. **Chapter 2** review the relevant works of literature of lignocellulosic biomass on its availability in Malaysia, their composition and its structure. The overview of rice husk as raw material and the potential of lignin as a substrate for biovanillin production are explained in this chapter. Different types of lignin recovered from different methods of extraction and its influential factors are also reviewed. Works of literature related to the application of lignin and biovanillin in different sectors of industry are briefly summarized. **Chapter 3** describes the general experimental procedures performed in this study. All common methods and procedures are placed in this chapter and be referred to the specific chapter, respectively.

Results and discussion are divided into three main chapters. **Chapter 4** reported the analysis of raw rice husk which including the chemical composition, ultimate composition and functional group confirmation by using Fourier Transform Infrared Spectroscopy (FTIR). The potential production of biovanillin from lignin in submerged fermentation using *Phanerochaete chrysosporium* was also described in detail in this chapter. The next part of this study was proceeding by selecting only one of the best pretreatment for lignin recovery by respecting biovanillin yield and the details were presented in **Chapter 5**. The optimization conditions of microwave pretreatment were carried out with respect to lignin recovery by using One Factor at a Time (OFAT) and Central Composite Design (CCD) of Response Surface Methodology (RSM). **Chapter 6** focused on the statistical screening of factors and optimization using 2 Level Factorial Design (2LFD) and Central Composite Design (CCD) of Response Surface Methodology (RSM) with respect to biovanillin yield in submerged fermentation. The potential sustainable production of biovanillin from lignin in industry also discussed in the end of this chapter.

The conclusion obtained from this research is summarized in **Chapter 7.** The problems remain and recommendations for future work are also discussed in this chapter.

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