PRODUCTION OF NATURAL VANILLIN FROM *Cymbopogon citratus* USING *Phanerochate chrysosporium*

HUSZALINA BINTI HUSSIN

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SPECIALLY DEDICATED TO MY FAMILY & FRIENDS

"WORDS CAN NEVER EXPRESS MY GRATITUTES"

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ABSTRACT

Malaysia as one of the important agricultural countries in the world producing 70 million tonnes of lignocellulosic biomass. One of the abundant agricultural wastes which has high lignocellulose content is lemongrass leaves (*Cymbopogon citratus*). A total of 8,000 tonnes of dry leaves are produced annually from 1,150 hectares of *C. citratus* plantation in Malaysia. Parts of the leave wastes are burned for electric generation while the rest are left in the fields to decompose naturally. The use of lemongrass leaves as ruminant feedstock is, however, not favoured due to animal rejection against its aroma. *C. citratus* leaves have 58% hemicellulose and lignin content and are also rich in ferulic acid. Ferulic acid is the precursor for vanillin production. The *C. citratus* leave wastes that contain ferulic acid could be potentially used for vanillin production via microbial approach, which is currently less investigated. The main purpose of this research was to investigate the potential of a one-step natural vanillin production from *C. citratus* leaves hydrolysate by *Phanerochaete chrysosporium*, the white-rot fungi of basidiomycetes. The research work focused on the recovery of ferulic acid as well as the optimization of natural vanillin production from *C. citratus* leaves. Leaves within size of 125-249 µm appeared to be suitable for ferulic acid extraction, with 1.12g/L total recovery of ferulic acid by 55 minutes of boiling. A total of 27 strains of fungi were screened for natural vanillin and vanillic acid production. Among these fungi, *Phanerochaete chrysosporium* was chosen due to its high natural vanillin productivity. The effect of different nitrogen source in vanillin production was investigated using the General Factorial Design. A combination of ammonium chloride with yeast extract (ratio: 75:4) increased the vanillin production to 15-fold. To further optimize the process using 2-Level Factorial Design, the aforementioned nitrogen sources and 5 others independent variables (incubation temperature, pH, incubation time, agitation and inoculum size) were studied. The ammonium chloride concentration, temperature and pH were found to be the key factors for natural vanillin production and these factors were further examined using the Central Composite Design (CCD). The maximum vanillin production was observed with media composition of 1.0 g/L ferulic acid from hydrolysate of *C. citratus* leaves, 4.43 g/L ammonium chloride (inorganic nitrogen), 0.25 g/L yeast extract (organic nitrogen), 0.2 g/L KH_2PO_4, 0.013 g/L $CaCl_2.2H_2O$, 0.5 g/L $MgSO_4.7H_2O$ and 0.0025 g/L thiamin hydrochloride, at 36.3 °C and pH 6.84 under shaking condition at 150 rpm with inoculum size of 7 % (w/v). The generated model fitted well to the data set with R^2 of 0.9059. The actual optimum conditions yielded 0.20 g/L vanillin (17-fold higher than non-optimised condition). The actual experimental data showed 2-fold higher yield as compared to previous reported studies. Artificial Neural Network model were also found to provide accurate validation and prediction result based on feed-forward back propagation as similar to Response Surface Methodology (RSM). A network with one hidden layer and 25 neurons were found to be the most optimum model with the lowest root mean squared error (RMSE; 0.0002415), absolute average deviation (AAD; 0.0004358) and the highest R^2 (0.9024). In this study, it was confirmed that *C. citratus* leaves could serve as a promising alternative source for vanillin production with potential to scale-up for commercial production in Malaysia.

ABSTRAK

Malaysia merupakan salah satu negara pertanian yang penting di dunia, telah menghasilkan 70 juta ton biojisim lignoselulotik. Sejumlah 8,000 ton daun kering dihasilkan pada setiap tahun daripada 1,150 hektar ladang *C. citratus* di Malaysia. Daun serai (*Cymbopogon citratus*) adalah hasil sisa pertanian yang mengandungi kandungan lignoselulosa yang tinggi. Sebahagian daripada sisa daun dibakar bagi menghasilkan tenaga elektrik sementara bakinya dibiarkan berdegradasi secara semulajadi. Penggunaan daun *C. citratus* sebagai sumber makanan ternakan tidak diterima oleh haiwan kerana aromanya. Daun *C. citratus* mempunyai 58% kandungan hemiselulosa dan lignin dan juga kaya dengan asid ferulik. Asid ferulik merupakan prekursor bagi penghasilan vanilin. Sisa daun *C. citratus* yang mengandungi asid ferulik berpotensi digunakan untuk penghasilan vanilin melalui pendekatan mikrob, di mana hingga kini kurang kajian dilakukan. Tujuan projek penyelidikan ini adalah untuk mengkaji potensi penghasilan vanilin semulajadi menggunakan satu langkah daripada hidrolisat daun *C. citratus* oleh *Phanerochaete chrysosporium*, kulat putih *Basidiomycetes*. Projek penyelidikan ini menfokuskan kepada pengestrakan asid ferulik dan juga pengoptimuman penghasilan vanilin semulajadi daripada *C. citratus*. Kajian mendapati, daun di dalam lingkungan saiz 125-249 µm sesuai bagi pengekstrakan asid ferulik dengan jumlah keseluruhan pengestrakan asid ferulik 1.12g/L pada minit 55 pra-rawatan pendidihan. Sejumlah 27 strain kulat telah dikaji bagi penghasilan vanillin semulajadi dan asid vanilik. Di antara kulat-kulat ini, *Phanerochaete chrysosporium* telah dipilih berdasarkan kepada produktiviti penghasilan vanilin semulajadi yang tinggi. Pengaruh kepada perbezaan sumber nitrogen terhadap penghasilan vanilin semulajadi telah dikaji menggunakan Rekabentuk Faktorial Umum. Kombinasi ammonium klorida dan extrak yis pada nisbah 75:4 telah menunjukkan peningkatan penghasilan vanilin semulajadi sebanyak 15 kali ganda. Untuk pengoptimuman selanjutnya dengan menggunakan Rekabentuk Faktorial Tahap-2, sumber nitrogen yang dinyatakan di atas dan 5 lagi pembolehubah tak bersandar (suhu eraman, pH, tempoh masa eraman, pengadukan dan saiz inokulasi) telah dikaji. Kepekatan amonium klorida, suhu eraman dan pH telah didapati sebagai faktor kunci terhadap penghasilan vanilin semulajadi dan faktor-faktor ini telah dikaji selanjutnya menggunakan Rekabentuk Komposit Berpusat (RKB). Parameter-paramter yang ideal yang menghasilkan vanilin yang maksimum berlaku apabila 1.0 g/L asid ferulik daripada hidrolisat daun *C. citratus*, 4.43 g/L amonium klorida (nitrogen organik), 0.25 g/L ekstrak yis (nitrogen inorganik), 0.2 g/L KH_2PO_4, 0.0132 g/L $CaCl_2 2.H_2O$, 0.5 g/L $MgSO_4.7H_2O$ dan 0.0025 g/L tiamin hidroklorik digunakan pada 36.3 °C dan nilai pH 6.84 di bawah keadaan pengadukan 150 rpm dengan saiz inokulat 7 % (w/v). Model yang terhasil sangat sesuai dengan set data iaitu R^2 0.9059. Keadaan optima pada keadaan sebenar telah menghasilkan 0.20 g/L vanilin semulajadi (17 kali ganda lebih tinggi berbanding keadaan tanpa pengoptimuman). Data eksperimen pada keadaan sebenar menunjukkan 2 kali ganda lebih tinggi hasil berbanding kajian yang dilaporkan terdahulu. Model Rangkaian Neural Buatan memberikan keputusan ramalan dan pengesahan yang tepat berdasarkan propogasi berbalik suapan ke hadapan yang sama seperti Rekabentuk Faktorial Umum (RFU). Satu jaringan dengan satu lapisan tersembunyi dan 25 neuron merupakan model paling optimum iaitu pada keadaan ralat kuadrat purata asas (RKPA: 0.0002415), perbezaan ralat mutlak (PRM; 0.0004358) yang paling rendah dan pada R^2 (0.9024) paling tinggi. Dalam kajian ini, telah disahkan bahawa daun *C. citratus* mampu menyediakan satu sumber yang menjanjikan kaedah alternatif bagi penghasilan vanilin dan berpotensi untuk digunakan secara komersil pada skala-besar di Malaysia.

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LIST OF ABBREVIATION

2-LFD	-	Two-factorial design
ANOVA	-	Analysis of Variance
BSA	-	Bovine Serum Albumin
CCD	-	Central Composite Design
CMC	-	Carboxymethyl cellulose
CMCase	-	Carboxymethyl cellulase
DNS	-	Dinitrosalicyclic acid
FESEM	-	Field Emission Scanning Electron Microscope
FID	-	Flame Ionized Detector
FPase	-	Filter Paper culture enzyme
FTIR	-	Fourier Transform Infrared Spectroscopy
g	-	Gram
GFD	-	General Factorial Design
H_2SO_4	-	Sulphuric acid
HCl	-	Hydrochloric acid
H_2O_2	-	Hydrogen Peroxides
HNO_3	-	Nitric acid
HPLC	-	High Performance Liquid Chromatography
kDa	-	Kilo Dalton
L	-	Liter
min	-	Minute
mL	-	Milliliter
mm	-	Millimeter
MW	-	Molecular Weight

NaOH	-	Sodium hydroxide
N/A	-	Not applicable
nm	-	Nanometer
^{o}C	-	Degree Celsius
OPT	-	Oil Palm Trunk
PAGE	-	Polyacrylamide Gel Electrophoresis
PDA	-	Potato Dextrose Agar
pNPG	-	p-nitrophenyl β-D-glucoside
RID	-	Refractive Index Detector
RSM	-	Response Surface Methodology
SDS	-	Sodium Dodecyl Sulfate
U/g	-	Unit of enzyme per gram
v/v	-	Volume per volume
w/v	-	Weight per volume
μL	-	Micro liter
μm	-	Micro meter

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CHAPTER 1

INTRODUCTION

1.1 Background of problem

Over 200 billion million tonnes of agricultural wastes were produced globally and they increased rapidly throughout the years (Figoli *et al.*, 2016; Agamuthu, 2009). In Malaysia, 15% (1.2 million tonnes) of agricultural waste has been disposed either to landfills or by burning activity that could lead to environmental problems (Agamuthu, 2015; Goralski *et al*., 2015). Thus, the conversion of the lignocellulosic agriculture wastes into various value-added products can be a great idea to overcome this problem since they are considered as a promising natural, abundant and renewable feedstock. In addition, their physical and chemical properties made them potential biomass feedstock for secondary product in biofuel, macromolecules and aromatic compound production (Saini *et al.,* 2015; Ragauskas *et al.,* 2014; Zamzuri and Abd-Aziz, 2013; Chandra *et al.,* 2012).

As one of the world's largest producers and exporter of palm oil, cocoa and rubber, Malaysia creates a substantial amount of lignocellulosic agriculture wastes (Agamuthu, 2015). An estimation of 33 million tons of wastes are generated from the empty fruit bunch, fiber and shell itself (Abdullah and Sulaiman, 2013). Apart from the main plantation resources, herbs plants do contribute into Malaysia's economy

(Zakaria, 2015; International Trade Statistics Database, 2015). One of the most abundant and major herbs plant in the row is *Cymbobogon citratus* leaves (Agrofood Statistics and Department of Statistics, 2015; Ragauskas *et al.*, 2014; Nambiar and Matela, 2012).

The total production of *Cymbopogon. citratus* leaves in Malaysia was 12, 562 tonnes. 70% of the production of the *C. citratus* leaves, which is about 8800 tonnes of *C. citratus* leaves, was discarded as waste (Ministry of Agriculture and Agro-Based Industry report, 2015). The waste of *C. citratus* are partially dried in the fields and a fraction is burned to generate steam for stripping while the rest is left in the fields to naturally degrade (Syed *et al*., 2016; Machado *et al*., 2015; Amenaghawon *et al*., 2014). The usage as ruminant feedstock is however not a favor due to animal rejection against harsh or brittle texture of the leaves that can injure an animals' mouth (Kaur and Dutt, 2013; Guretzky *et al*., 2011; Ball *et al.*, 2001). *Cymbopogon citratus* leaves consisted of 28.5-47% (w/v) of hemicelluloses, 29.9-35% (w/v) cellulose and 7-11% (w/v) lignin (Joshua *et al.*, 2012; Nambiar and Matela 2012; Rolz, 1986). *C. citratus* leaves have approximately 58% of hemicellulose and lignin contents which related to rich of ferulic acid source. Although the utilization of *C. citratus* has been widely used in various fields, *C. citratus* leaves is an underexploited with ferulic acid as precursor for natural vanillin production. The idea of conducting the project is to generate side-income to farmers and promote Bioeconomic National Transformation Programme agenda for high-income nation by 2020. The renewable product and their conversion by available lignocellulosic resource into food, feed stock, and healthcare wellness products via innovative and efficient of green, eco-friendly technologies should be emphasised.

Vanillin is the characteristic aroma component of the vanilla pod and frequently used for the production of flavors for foods and fragrances for perfume. The compounds practically prolonged the food life (Vandamme, 2003). At present, "artificial" or "nature-identical" vanillin is mostly produced from petrochemicals like guaiacol and from lignin. Due to the increasing demand for healthy and natural food, there is a growing interest to produce vanillin from natural raw materials by biotransformation, which can then be regarded as a "natural" aroma chemical. This process is possible through microbial transformation when consortium of microorganisms and fungi are used to induce the production of these flavour compounds into optimum final yield (Lomascolo *et al.,* 1999).

Over the period of 2014 to 2019, flavors market will contribute a 5.5 billion dollar turnover and 4.86% growth rate which covers about one fourth of the global food additives market, and it is believed that this will continuously increase in proportion to the world demand of flavours (Sarethy and Pan, 2017). Flavouring compounds are known to be produced naturally from vanilla pod, chemically synthesized vanillin derived from lignin or fossil hydrocarbon and recently through bioconversion process, which is more eco-friendly towards environment (Leffingwell and Leffingwell, 2015; Gallage and Moller, 2015; Dal Bello, 2013; Havkin-Frenkel and Belanger, 2010; Brochado *et al.*, 2010).

1.2 Objectives

The main aim of the research is to develop environmental friendly bioconversion with significant yield of a one-step conversion of natural vanillin from *Cymbopogon citratus* leaves using *Phanerochaete chrysosporium*. In order to achieve the aim, the following studies have been conducted on;

1. Effect of chemical, physical, physicochemical and enzymatic pretreatments of *Cymbopogon citratus* leaves on the recovery of ferulic acid and reducing sugar
2. Isolation of fungi for a one-step conversion of natural vanillin production in batch culture

3. Screening and optimizing the significant factors of natural vanillin production using two level factorial design and central composite design (CCD) in batch culture

1.3 Limitation of consideration

In particular, this PhD work focused on the one step bioconversion of *C. citratus* leaves hydrolysate (containing ferulic acid) to natural vanillin production by selected fungi in which finally the *Phanerochaete chrysosporium* was employed. Recovery of ferulic acid made from *Cymbopogon citratus* leaves. Eventhough two common types of *Cymbopogon* available in Malaysia namely, *Cymbopogon citratus* and *Cymbopogon nardus*, yet *Cymbopogon citratus* leaves were chosen. This is because *Cymbopogon nardus* stalk and leaves are extracted for its essential oils in ingredient of therapeutic remedy. *Cymbopogon citratus* on the other hands, are abundance and feasible in terms of the production. The stalk of *Cymbopogon citratus* is mainly for cooking and flavouring, yet the leaves are discarded. There is eleven physical and chemical factors that involved in the production of natural vanillin. Due to time limitation, only seven major parameters were selected from previous studies for optimum production of natural vanillin. The selection parameters are inoculum size, pH value, supplement medium composition of inorganic and organic nitrogen source, incubation temperature, inoculum as well as incubation duration time. There are also many statistical approaches that can be used for statistical analysis. Plackett-Burman design is one of the choice but require more than 20 factors to fit a design. The one-factor-at-a-time (OFAT) is normally give accurate results but rather irrelevant with unnecessary experiment design that consume a lot of time and cost. The Response Surface Methodology (RSM) statistical approach of Design Expert ® version 6.04 is chosen. This is because RSM has better approach in identifying the ideal combination of factors with any levels that can affect the process and indicates the optimal value of the selected parameters. Artificial Neural Network (ANN) is chosen or further validation and prediction because it does not need any mathematical model. One of advantages of an ANN is that it learns from examples and recognizes patterns in a series of input and output data without any prior assumptions about their nature and interrelations. There are many types of learning algorithms in the literature which can be used for training of the network which enable the network to model nonlinear and complex functions. However, feed-forward neural network is chosen as it could provide an accurate prediction even with a single hidden layer. The appropriate selection of propagation learning algorithm can be adopted for the training of all the ANN with high rate of successful data modelling. Due to time constraints, Genetic Algorithms could not be performed due to time limitation and require selection of larger class of evolutionary algorithms.

1.4 Organization of research

The research is initiated with screening of selected fungi through quantitative and qualitative analyses to identify the isolate that was capable to transform ferulic acid to natural vanillin through liquid-state fermentation (*C. citratus* leaves hydroysate), *Phanerochaete chrysosporium* was finally selected into further steps of optimization. The wild type strain is able to use ferulic acid as carbon source (Karode *et al.,* 2013; Barbosa *et al.,* 2008). The experimental activities focused on the recovery of ferulic acid as well as optimization of natural vanillin production. The aim was thus to develop environmentally friendly bioconversion protocols based on the combination of enzymatic and physicochemical (thermal/boiling) pretreatment of *C. citratus* leaves. The methods were used in order to obtain a high recovery of ferulic acid and sugar in hydrolysate. There is evidence that purification of ferulic acid from the carbohydrate occurring in the hydrolysate might be necessary to avoid increase in oxido-reductive enzymes activity that converts natural vanillin into side-products, thus reducing the natural vanillin molar yield (Dal Bello, 2013). The natural vanillin production from *Phanerochaete chrysosporium* was optimized using statistical approaches. The best nitrogenous supplement in the basal medium was determined using general factorial design (GFD). The two-level factorial design (2LFD) was used to select the most significant parameters that influenced the natural vanillin production, and lastly CCD was used to determine their optimal values. Then followed by Artificial Neural Network (ANN) using a multi-layer perceptron (MLP) based feed-forward back propagation learning algorithm to validate datasets obtained during the natural vanillin production and predict value of sample as well. The thesis comprises of 7 chapters (including the present chapter), contents of which are briefly outlined below:

In chapter 2, a comprehensive review of literature on lignocellulosic biomass and agriculture waste residue as well as several important aspects of classification, type and processing methods of vanilla or natural vanillin. This including the physical and chemical factors that influence the natural vanillin production. The major findings of significant parameters in obtaining optimum natural vanillin production are also emphasised.

In chapter 3, the general research methodology repeatedly use throughout the study is presented in great depth.

In chapter 4, the biomass of lignocellulosic composition, phenolic compounds profile and reducing sugar analysis in raw *C. citratus* leaves were highlighted for substrate selection suitability. Then describe the chemical, physical, physicochemical and enzymatic pretreatment of the *C. citratus* leaves by measuring ferulic acid and phenolic compounds recovery, physical modification and total reducing sugar analysis as well.

Chapter 5, the isolation and screening of potential fungi amongst the local isolates and commercial strains that capable in a one-step conversion of natural vanillin production is presented.

Chapter 6, the statistical optimization of medium and physical factors including incubation temperature, inoculums sizes, pH and ratio of selected supplement medium composition that had strongly influenced the submerged liquid fermentation of vanillin production is described.

Chapter 7 summarizes the major finding of the thesis and gives some suggestions for further research.

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