

BIOVANILLIN PRODUCTION FROM LEMONGRASS LEAVES  
HYDROLYSATES BY *Phanerochaete chrysosporium* ATCC 24725 IN BATCH  
CULTURE

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UNIVERSITI TEKNOLOGI MALAYSIA

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## ABSTRACT

Biovanillin is one of fungi secondary metabolites, that is widely used as aromatic and flavour compound with high fiscal value. The use of vanillin as flavour for various products is its foremost application in food industries. The global market demand for natural vanillin as flavour stands for less than one percent (1%) of its market demand annually. However, most of the flavour compounds are normally obtained through the process of chemical synthesis, which could cause health problem and environmental hitches. Demand for natural and healthy products coupled with the fact that acid ferulic (FA) extracted from plant materials can be a precursor for biovanillin production makes it relatively inexpensive as a natural product. The research was aimed to extract FA from lemongrass leaves (LGL), which was used as precursor for one-step biovanillin production by *Phanerochaete chrysosporium* (ATCC 24725) in batch culture. Initially, optimization of the LGL pretreatment practices using liquid hot water with sodium bisulfite (0.5% w/v) towards the release of the FA was investigated with central composite design (CCD). The optimized results produced 0.750 g/L as the highest FA released from the lemongrass leaves hydrolysates (LLH). Considerable alterations of the major LGL contents were observed during the pretreatment process, which increased the cellulose content by 39%. The Fourier transform infrared (FTIR) and field emission scanning electron microscopic (FESEM) analyses confirmed that the lignin which serves as the shielding layer from the LGL components became fragmented, thus decreasing the lignin content by 46%. The total reducing sugar production with enzymatic saccharification using enzymes cocktail (celluclast and viscozyme, 1 % v/v each) improved by up to 8.4-folds as compared to the direct enzymatic saccharification without removing the LGL extracts. Screening of significant factors for biovanillin production using 2-level Factorial Design showed that the biovanillin production processes was affected by the interactive effects of initial FA concentration, incubation temperature, incubation time and initial pH. The highest biovanillin production (0.093 g/L with molar yield 23 %) in shake flasks using the CCD was determined with FA (0.5 g/L), temperature (35 °C), time (72 h), and initial pH (6.0). Application of both pH and dissolved oxygen control strategies in 2 litre stirred tank bioreactor had increased the biovanillin production by 1.41 and 1.53-folds as compared to the optimized experiment using the shake flasks. The evaluation of kinetics from the two-phase pH control strategy demonstrated the performance of *P. chrysosporium* with the highest specific growth rate ( $\mu$ ) of 0.056 h<sup>-1</sup>, with an increase in the yield coefficient of biomass formation  $Y_{x/s}$  (0.5191 g/g) and maximum cell concentration  $X_{max}$  (13.0 g/L) by 1.03 and 1.05-folds as compared to one-phase of pH control, respectively. Performance of the kinetics using two-phase dissolved oxygen (DO) control strategy has shown that 80 % saturations of DO during active growth phase with 40 % saturations during production phase were highly essential for enhancement of biovanillin production from LLH by *P. chrysosporium* using 2 litre stirred tank bioreactor. LGL residue which contained FA can be used as a precursor to produce biovanillin by natural means via one-step bioconversion process with *P. chrysosporium* in batch culture using 2 litre stirred tank bioreactors.

## ABSTRAK

Biovanillin merupakan salah satu metabolit sekunder kulat, yang digunakan sebagai sebatian aromatik dan perasa dengan nilai fiskal yang tinggi. Penggunaan vanillin sebagai perasa untuk pelbagai produk adalah aplikasi terpenting dalam industri makanan. Permintaan pasaran global untuk vanillin semula jadi sebagai perasa adalah kurang dari satu peratus (1 %) dari permintaan pasarnya setiap tahun. Namun, sebilangan besar sebatian perasa biasanya diperoleh melalui proses sintesis kimia, yang boleh menyebabkan masalah kesihatan dan persekitaran. Permintaan untuk produk semulajadi yang sihat serta fakta bahawa asid ferulik (FA) yang diekstrak daripada bahan tumbuhan boleh menjadi bahan pendahulu bagi pengeluaran biovanilin menjadikannya secara relatif murah sebagai produk semulajadi. Penyelidikan ini bertujuan untuk mengekstrak FA dari daun serai (LGL), yang digunakan sebagai substrat untuk pengeluaran biovanillin satu langkah oleh *Phanerochaete chrysosporium* (ATCC 24725) dalam kultur kelompok. Pada peringkat awal, pengoptimuman prarawatan LGL menggunakan air panas dengan natrium bisulfit (0.5 % w/v) untuk meningkatkan pengeluaran FA dikaji dengan reka bentuk komposit pusat (CCD). Hasil FA yang optimum sebanyak 0.750 g/L merupakan FA tertinggi yang dikeluarkan dari proses hidrolisis daun serai (LLH). Perubahan besar kandungan LGL utama diperhatikan semasa proses prarawatan, dengan peningkatan kandungan selulosa sebanyak 39 %. Analisis Fourier transform infrared (FTIR) dan elektron mikroskopik pengimbasan emisi medan (FESEM) telah mengesahkan bahawa lignin yang berfungsi sebagai lapisan pelindung dari komponen LGL dipecahkan dan ini terbukti dengan penurunan kandungan lignin sebanyak 46 %. Peningkatan penghasilan gula secara keseluruhan semasa sakarifikasi enzimatik menggunakan enzim koktail (celluclast dan viscozyme, masing-masing 1 % v/v) adalah 8.4 kali ganda berbanding dengan sakarifikasi enzimatik secara langsung dengan tanpa mengeluarkan ekstrak LGL. Penyaringan faktor-faktor penting untuk pengeluaran biovanilin menggunakan Reka Bentuk Faktor 2 Peringkat menunjukkan bahawa penghasilan biovanilin dipengaruhi oleh kesan interaktif kepekatan FA awal, suhu pengeraman, jangkamasa pengeraman dan pH awal. Penghasilan biovanillin tertinggi sebanyak 0.093 g/L dengan hasil molar 23 % dalam kelalang penggongcang menggunakan CCD menunjukkan bahawa FA (0.5 g/L), suhu (35 °C), jangkamasa pengeraman (72 jam), dan pH awal (6.0). Aplikasi strategi pengawalan pH dan oksigen terlarut dalam bioreaktor tangki pengaduk 2 liter telah meningkatkan pengeluaran biovanillin sebanyak 1.41 dan 1.53 kali ganda berbanding eksperimen yang dioptimumkan kelalang penggongcang. Penilaian kinetik dari strategi kawalan pH secara dua fasa menunjukkan peningkatan prestasi *P. chrysosporium* bagi kadar pertumbuhan spesifik tertinggi ( $\mu$ ) 0.056 h<sup>-1</sup>, hasil pembentukan biomassa  $Y_{x/s}$  (0.5191 g/g) dan kepekatan sel maksimum  $X_{max}$  (13.0 g/L) sebanyak 1.03 dan 1.05 kali ganda berbanding kawalan pH satu fasa. Prestasi kinetik menggunakan strategi kawalan oksigen terlarut (DO) dua fasa telah menunjukkan bahawa 80 % oksigen terlarut tepu semasa fasa pertumbuhan aktif dengan 40 % oksigen terlarut tepu semasa fasa penghasilan sangat penting untuk peningkatan pengeluaran biovanilin dari LLH oleh *P. chrysosporium* menggunakan bioreaktor tangki pengaduk bersaiz 2 liter. Residu LGL yang mengandungi FA berpotensi untuk digunakan sebagai substrat untuk menghasilkan biovanillin dengan cara semula jadi melalui proses biopenukaran satu langkah oleh *P. chrysosporium* dalam kultur kelompok menggunakan bioreaktor tangki pengaduk 2 liter.

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## LIST OF ABBREVIATIONS

ATCC	-	American Type Culture Collection
2-LFD	-	Two-Level Factorial Design
ANOVA	-	Analysis of Variance
CCD	-	Central Composite Design
DNS	-	Dinitrosalicylic Acid
DOT	-	Dissolved Oxygen Tension
FA	-	Ferulic Acid
FESEM	-	Field Emission Scanning Electron Microscope
FS	-	Faculty of Science
FTIR	-	Fourier Transform Infrared Spectroscopy
g	-	Gram
G	-	Growth Phase
H <sub>2</sub> SO <sub>4</sub>	-	Sulphuric Acid
HCl	-	Hydrochloric Acid
HNO <sub>3</sub>	-	Nitric Acid
HPLC	-	High Performance Liquid Chromatography
L	-	Litre
LLH	-	Lemongrass Leaves Hydrolysates
LGL	-	Lemongrass Leaves
mL	-	Millilitre
mm	-	Millimetre
MW	-	Molecular Weight
NADPH <sub>2</sub>	-	Nicotinamide Adenine Dinucleotide Hydrogen Phosphate
NaOH	-	Sodium Hydroxide
PDA	-	Potato Dextrose Agar
P	-	Production Phase
RSM	-	Random Surface Methodology
v/v	-	Volume Per Volume

w/v	-	Weight per Volume
$\mu\text{L}$	-	Micro Litre
$^{\circ}\text{C}$	-	Degree Celsius
$\mu$	-	Specific Growth rate
$\mu_{\text{max}}$	-	Maximum Specific Growth rate
$P_{\text{max}}$	-	Maximum Product
$X_{\text{max}}$	-	Cell Biomass Concentration
$t_d$	-	Doubling Time
$Y_{x/s}$	-	Yield Coefficient of Biomass Formation Related to Substrate Utilization
$Y_{p/s}$	-	Yield Coefficient of Biovanillin Formation Related to Substrate Utilization
$Y_{p/x}$	-	Yield Coefficient of Biovanillin Formation Related to Cell Biomass Concentration

## LIST OF SYMBOLS

$\pm$	-	Plus, or Minus
$\leq$	-	Less than or Equal to
$\geq$	-	Greater or Equal to
-	-	Minus
=	-	Equal to
%	-	Percent

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# CHAPTER 1

## INTRODUCTION

### 1.1 Research Background

Biovanillin is a secondary metabolite, which is used as flavouring component present in natural vanilla (Gallage *et al.*, 2018; Pérez-Rodríguez *et al.*, 2016). The use of vanillin as flavour for production of various products was its foremost application in food industries, particularly beverages and dairy products (Chakraborty *et al.*, 2017a). However, researchers had discovered its potential applications in other industries like cosmetics, agriculture, drugs, and household (Galadima *et al.*, 2019a; Priefert *et al.*, 2001; Zamzuri and Abd-Aziz, 2013). The production of biovanillin through biotechnological processes was based on microbial conversions of natural precursors including ferulic acid, vanillic acid, phenolic stilbenes, eugenol, aromatic amino acids and isoeugenol (Chen *et al.*, 2016; Hua *et al.*, 2007a; Lesage-Meessen *et al.*, 1999; Ramachandra Rao and Ravishankar, 2000; Stentelaire *et al.*, 2000). These precursors could be obtained by pretreatment from agro-based residues like sugar beet pulp, maize stalk, wheat straw, rice bran, sugarcane bagasse, maize bran, oil palm empty fruit bunch (Galadima *et al.*, 2019b; Zheng *et al.*, 2015; Zheng *et al.*, 2007b; Zulkarnain *et al.*, 2018). Therefore, the bioconversions of the precursors to biovanillin could be performed through the biotechnological process by applying plant cells, fungi, bacteria, microalgae, genetically modified microorganisms or de novo biosynthesis (Gallage and Moller, 2015; Hua, *et al.*, 2007a; Overhage *et al.*, 2003; Priefert, *et al.*, 2001; Zheng, *et al.*, 2007b). Due to the lower yield of the biovanillin because of high toxic effects of the other precursors during fermentation process, as well as chemical similarity of ferulic acid (FA) to vanillin, the use of FA obtained from agricultural residues was established among the best routes for biovanillin production (Galadima, *et al.*, 2019a; Hua, *et al.*, 2007a; Patil and Yadav, 2018; Tilay *et al.*, 2010; Zheng, *et al.*, 2007b).

Ferulic acid (FA) is among the readily available hydroxycinnamates that exist in the cell wall of various plants (Hua, *et al.*, 2007a; Ishii, 1997; Yan *et al.*, 2016; Zheng, *et al.*, 2007b). It was discovered to be existed freely in plants, and linked covalently between arabinoxylans and lignin fragments by ester and ether linkages, which require application of certain pretreatment techniques to cleave the linkages before it could be released and used for the biotransformation processes (Hua, *et al.*, 2007a; Johnson *et al.*, 1996; Saulnier *et al.*, 2001; Saulnier and Thibault, 1999; Zheng, *et al.*, 2007b). There are many reports on the application of various pretreatment techniques to facilitate the release of the FA from different plant residues (Galadima, *et al.*, 2019a; Mirghani *et al.*, 2012; Zulkarnain, *et al.*, 2018). However, very limited literature is available on the utilization of statistical software to optimize the release of the FA from lemongrass leaves. Therefore, among the objectives of this research is to explore the application of central composite design to optimize the release of the FA from lemongrass leaves.

Majority of the available literature on the application of different microorganisms for the transformation of ferulic acid to biovanillin have confined on using two-step bioconversion processes with certain plant residues as FA sources (Galadima, *et al.*, 2019a; Motedayen *et al.*, 2013; Nazila *et al.*, 2013; Zulkarnain, *et al.*, 2018). The two-step bioconversion process was discovered to have some hitches like time mismanagement, formation of side-products, vulnerable to contaminations and labour intensive (Galadima, *et al.*, 2019b; Hua, *et al.*, 2007a; Hussin *et al.*, 2015). However, one-step bioconversion process is rather time efficient, lesser labour intensive, lower level of side-products formation with minimal risk of contamination (Galadima, *et al.*, 2019a; Hua, *et al.*, 2007a; Hussin, *et al.*, 2015). But there are very limited reports on application of the one-step bioconversion process for biovanillin production using lemongrass leaves. Also, there are no literatures on the application of pH and dissolved oxygen control strategies toward biovanillin production by *Phanerochaete chrysosporium* ATCC 24725 using 2 litre stirred tank bioreactor. The *P. chrysosporium* ATCC 24725 was reported with capability of liberating enzymes like laccases, lignin peroxidase and manganese peroxidase with high affinity to degrade lignin (which serves as a barrier) from the biomass, utilizing nitrogen and

carbon that were used as substrates for growth and metabolism (Ürek and Pazarlioğlu, 2005). Despite having high affinity to attack lignin by fungi, they also have higher tolerance to the toxic effect of the ferulic acid when compared to the other microorganisms like bacteria.

Therefore, this research has been tailored to explore the application of one-step bioconversion process towards biovanillin production using lemongrass leaves. The research further employed the application of pH and dissolved oxygen control strategies toward biovanillin production by *P. chrysosporium* ATCC 24725 using the 2-litre stirred tank bioreactor. Various problems that have instigated the purposes of this research were described below.

## **1.2 Statement of Problems**

In Malaysia, there are more than 250 acres of farmlands for lemongrass production, which are responsible for producing over 12,000 tonnes of dry lemongrass leaves annually (Hussin, *et al.*, 2015). From the total dry leaves of the lemongrass generated, only about 300 tonnes could be transformed into value-added products. More than 8,000 tonnes are being disposed on farmlands (Hussin, *et al.*, 2015). These disposed wastes of the lemongrass leaves could only be left on the farmlands to deteriorate naturally, because they could not be used as feedstuffs to feed animals due to the attribute of their aroma (Hussin, *et al.*, 2015; Kaur and Dutt, 2013). Alternatively, they could be destroyed by burning using conventional technique, however this would pose some challenges pertinent to environmental pollution (Hussin, *et al.*, 2015; Norli *et al.*, 2017). Research have discovered that various agro-based residues including lemongrass leaves consisted of ferulic acid, but the major challenge is with the best method of pretreatment to be adopted for its normal release.

The global market demand for fragrances and flavours is incessantly escalating (Batista, 2014; Dal Bello, 2013; Gallage and Moller, 2015). Majority of flavouring

composites are normally obtained through the process of chemical synthesis with a minute involvement of natural means (Bomgardner, 2016; Gallage and Moller, 2015; Krings *et al.*, 1993). The foremost downside of chemical synthesis is that the method is not welcomed environmentally as well as the needed compounds often emerged as adverse mixtures that could affect fermentation processes (Krings, *et al.*, 1993). The chemical means of flavour production suffers quite a lot of weaknesses including increasing effects on environment (Dal Bello, 2013). Furthermore, the compounds produced chemically are tagged “artificial products” or “nature identical”, which lessened their economic significance (Bomgardner, 2016; Dal Bello, 2013). Also, the natural vanillin extracted out of vanilla stands for less than one percent (1 %) of the market demand annually, and the demand for natural and healthy products is incessantly escalating.

However, in order to overcome these challenges and make the bioconversion routes viable economically, it became highly essential to get a precursor which is relatively near to vanillin and at the same time cost-efficient and easily accessible. Flavours obtained via biological means are termed “bioflavours” which are relatively abundant, cost-efficient and readily available (Dal Bello, 2013; Gallage, *et al.*, 2018). The application of biotechnology as imminent basis of biovanillin production was established promising using fungi via biotransformation of FA utilizing agricultural residues including lemon grass leaves (Galadima, *et al.*, 2019b; Hussin, *et al.*, 2015), and the process is universally accepted as natural (Bomgardner, 2016; Dal Bello, 2013). Interestingly, various plant materials has been discovered to consist ferulic acid, which has been extracted and utilized in many different fields (Batista, 2014; Kumar and Pruthi, 2014; Pérez-Rodríguez, *et al.*, 2016). Although, there are various literatures that have reported the bioconversion of FA obtained from different plant residues into biovanillin (Converti *et al.*, 2010; Karode *et al.*, 2013; Makela *et al.*, 2015; Motedayen, *et al.*, 2013; Pérez-Rodríguez, *et al.*, 2016; Thibault *et al.*, 1998; Zulkarnain, *et al.*, 2018), very limited literature is available on the utilization of lemongrass leaves as main source of FA for biovanillin production. Hence, this research has been focused on optimization of the release of FA from lemongrass leave hydrolysates, which was ultimately used for biovanillin production by *P. chrysosporium* ATCC 24725 using 2-

litre stirred tank bioreactor. Detail description of the objectives of this research is highlighted below.

### 1.3 Research Objectives

The research gave more concern towards production of biovanillin from lemon grass leave hydrolysates by *P. chrysosporium* ATCC 24725 in 2 litre stirred tank bioreactor with the aim to improve the production. Therefore, the main objectives of this research include.

- (a) To analyse the products from physico-chemical pretreatment and enzymatic saccharification of lemongrass leaves
- (b) To optimize ferulic acid (FA) release via physico-chemical pretreatment technique using Central Composite Design (CCD).
- (c) To screen and optimize the biovanillin production in shake flasks using 2 level factorial design (2-LFD) and central composite design (CCD) respectively.
- (d) To evaluate pH and Dissolved Oxygen control strategy for One-step biovanillin production from lemongrass leaves hydrolysates in batch culture using 2 litre stirred tank bioreactor.
- (e) To compare the biovanillin production by *Phanerochaete chrysosporium* ATCC 24725 in shake flasks and 2 litre stirred tank bioreactor.

## 1.4 Research Scope

The research gave more priority on the production of biovanillin with lemongrass by *P. chrysosporium* ATCC 24725 using fermentation processes in batch culture. The whole research was broadly segmented into different parts. Initially, this research was confined on the optimization of the FA release by physico-chemical pretreatment technique using Central Composite Design (CCD). The optimized FA released from the lemon grass hydrolysates was used in designing the subsequent experiments. The potentials of the lemongrass leaves were explored by determining the contents of cellulose, hemicellulose and lignin. The glucose contents from lemongrass leaves were equally assessed through enzymatic saccharification. The efficiency of the pretreatment technique was evaluated with Fourier-Transform Infrared Spectroscopy (FTIR) and field emission scanning electron microscope (FESEM). The parameters that were found to influence the biovanillin production were screened using 2-level factorial design to get the significant few ones, which were subsequently used to optimize the biovanillin production using CCD.

The research further employed the use of various pH control strategies toward biovanillin production in batch culture using 2 litre stirred tank bioreactor. pH of the culture medium was controlled by single-phase at 6.5, 6, 5.5, 5.0, 4.5, 4.0, 3.5 and without any pH control (initial pH of 6.0). The two-phase control of the culture pH towards biovanillin production in 2 litre stirred tank bioreactor with *P. chrysosporium* ATCC 24725 was also performed by strategic monitoring of the pH at both growth and production phases. Furthermore, the influence of dissolved oxygen on biovanillin production with *P. chrysosporium* ATCC 24725 in 2 litre stirred tank bioreactor by monitoring the dissolved oxygen during the fermentation processes was equally conducted. The fermentation kinetics of the biovanillin production from both shake flasks and the 2 litre stirred tank bioreactor were ultimately evaluated.

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## LIST OF PUBLICATIONS

### Journal with Impact Factor

1. **Galadima**, A. I., Salleh, M. M., Hussin, H., Safri, N. M., Noor, R. M., Chong, C. S., & Naser, M. A. (2019). One-Step Conversion of Lemongrass Leaves Hydrolysate to Biovanillin by *Phanerochaete chrysosporium* ATCC 24725 in Batch Culture. *Waste and Biomass Valorization*, 1-14. <https://doi.org/10.1007/s12649-019-00730-w>. (Q2, IF: 2.358)
2. **Galadima**, A. I., Salleh, M. M., Hussin, H., Chong, C. S., Yahya, A., Mohamad, S. E., & Al-Junid, A. F. M. (2019). Biovanillin: production concepts and prevention of side product formation. *Biomass Conversion and Biorefinery*, 1-21.. <https://doi.org/10.1007/s13399-019-00418-0>. (Q2, IF: 2.326).
3. **Galadima**, Ahmed Ibrahim. Madihah Md Salleh. Huszalina Hussein. Chong Chun Siong. Adibah Yahaya. Shaza Eva M. Suraini A. Nor Nadiah Mohamad Yusof. Muhammad. Feisal Merican Al-Junid: pH control strategy for biovanillin production from lemongrass leave hydrolysate using *Phanerochaete chrysosporium* ATCC 24725 in Batch Culture. *Processes* (Q2, IF:1.963). **Accepted for Publication.**

### Conference Proceedings

1. **Galadima**, Ahmed Ibrahim, Madihah Md Salleh, Huszalina Hussein, Norulsazyani Mohd Safri, Chong Chun Siong, Adibah Yahaya, Shaza Eva M, Suraini Abdul-Aziz., Nor Nadiah Mohamad Yusof, Muhammad Abu Naser, Amir Feisal Merican Al-Junid. Optimization of Ferulic Acid Recovery from Lemongrass Leave Hydrolysate Using Central Composite Design. Asian Federation of Biotechnology (AFOB, 2018). Pullman hotels and Resorts Kuching, Serawak, Malaysia.
2. Huszalina H, **Galadima** I, Sazyani S., Madihah M.S, Chong Chun Siong. Adibah Yahaya. Shaza Eva M. Suraini A. Nor Nadiah Mohamad Yusof. Muhammad Abu Naser. Amir Feisal Merican Al-Junid. Utilization of Lemongrass Biomass for Biovanillin Production by *Phanerochaete chrysosporium*. 13<sup>th</sup> Asian Congress on Biotechnology 2017. Bioinnovation and Bioeconomy. Pullman Khon Kaen Raja Orchid Hotel, Khon Kaen, Thailand.
3. **Galadima**, Ahmed Ibrahim. Madihah Md Salleh. Huszalina Hussein. Chong Chun Siong. Adibah Yahaya. Shaza Eva M. Suraini Abdul-Aziz. Potential Application of Lemongrass leaves for one stage fermentation of Biovanillin Production by *Phanerochaete chrysosporium* ATCC 24725. AFOB International Symposium, 2017 and 11<sup>th</sup> AFOB Board Meeting (2017).