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UTILISATION OF ALKALINE HYDROLYSATE OF OIL PALM EMPTY FRUIT BUNCH FOR BIOVANILLIN PRODUCTION

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Master of Science

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UTILISATION OF ALKALINE HYDROLYSATE OF OIL PALM EMPTY FRUIT BUNCH FOR BIOVANILLIN PRODUCTION

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Chairman : Suraini Abd. Aziz, PhD

Faculty : Biotechnology and Biomolecular Sciences

Interest in biovanillin production via biotechnological approach resulted from the expensive price of natural vanillin extracted from vanilla pod. Biovanillin is classified as natural flavour, thus offers a great potential to be commercialised to meet high demand of vanillin for worldwide user. Utilisation of lignocellulosic biomass as alternative substrate provides advantages on biovanillin production due to its abundancy and low cost. Therefore, this study aims to evaluate the potential of alkaline hydrolysate of OPEFB as an alternative substrate for biovanillin production via two-step bioconversion. However, 8 phenolic compounds were detected in the alkaline hydrolysate of OPEFB, namely; *p*-hydroxybenzoic acid, vanillic acid, vanillin, syringic acid, syringaldehyde, *p*-coumaric acid, *p*-hydroxybenzaldehyde and ferulic acid, with the highest concentration was *p*-hydroxybenzoic acid. Thus, a model of formulated phenolic compounds was used to study the effect of those phenolic compounds towards vanillic acid production which known as primary intermediate compound in two-step bioconversion as a response in the two-level factorial design. Prior for two-step bioconversion process, the potential fungi were screened on their abilities to produce vanillic acid and biovanillin. *Aspergillus niger* ATCC6257 and *Aspergillus niger* EFB1 were used for vanillic acid production while the *Phanerochaete chrysosporium* UIA and *Phanerochaete chrysosporium* EFB1 were used for biovanillin production. For vanillic acid production using glucose (before the addition of ferulic acid), *A. niger* ATCC6257 produced 51.3% molar yield of vanillic acid, indicates 0.18 fold increase of vanillic acid than *A. niger* EFB1 (43.6%). Whereas, *P. chrysosporium* UIA showed 30.6% molar yield of biovanillin, indicates 0.37 fold increase than *P. chrysosporium* EFB1 which recorded 22.4% molar yield of biovanillin. Hence, *A. niger* ATCC6257 and *P. chrysosporium* UIA were selected to be used for two-step bioconversion of alkaline hydrolysate of OPEFB to biovanillin. The results showed 41% and 39% molar yield of vanillic acid and

biovanillin, respectively, were able to be produced from two-step bioconversion of alkaline hydrolysate of OPEFB. On the other hand, only ferulic acid, *p*-hydroxybenzoic acid and *p*-coumaric acid are significant factors ($p<0.05$) in two-level factorial model. Ferulic acid plays a great factor in producing vanillic acid, whereas the interaction with *p*-hydroxybenzoic, and *p*-coumaric acid show negative effect that inhibit vanillic acid production. In overall, alkaline hydrolysate of OEPFB exhibited as potential alternative substrate for biovanillin production via two-step bioconversion.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Master Sains

PENGGUNAAN HIDROLISAT BERALKALI TANDAN KOSONG KELAPA SAWIT UNTUK PENGHASILAN BIOVANILIN

Oleh

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Minat terhadap penghasilan biovanilin melalui pendekatan bioteknologi adalah disebabkan oleh harga vanilin asli yang diekstrak dari vanila pod yang tinggi. Biovanilin diklasifikasikan sebagai perasa asli maka ia mempunyai potensi yang baik untuk dikomersialkan bagi memenuhi permintaan yang tinggi dari pengguna seluruh dunia. Penggunaan biomass lignosellulosa sebagai substrat alternatif memberikan kebaikan bagi penghasilan biovanilin dalam jumlah yang banyak serta kos yang rendah. Justeru itu, kajian ini bertujuan untuk menilai potensi hidrolisat beralkali tandan kosong kelapa sawit (OPEFB) sebagai substrat alternatif untuk penghasilan biovanilin melalui biopenukaran dua peringkat. Walaubagaimanapun, 8 kompaun fenolik telah dikenalpasti dalam hidrolisat beralkali OPEFB, iaitu, asid *p*-hidroksibenzoik, asid vanilik, vanilin, asid siringik, siringaldehid, asid *p*-kumarit, asid *p*-hidrosibenzaldehid dan asid ferulik, dengan kepekatan tertinggi kompaun fenolik dalam hidrolisat beralkali OPEFB adalah asid *p*-hidroksibenzoik. Oleh itu, sebuah model formulasi kompaun fenolik telah digunakan untuk mengkaji kesan kompaun fenolik tersebut terhadap penghasilan asid vanilik yand dikenalpasti sebagai kompaun perantaraan dalam biopenukaran dua peringkat sebagai respon dalam dua peringkat rekabentuk faktorial. Sebelum proses biopenukaran dua peringkat, fungi yang berpotensi telah disaring kebolehannya untuk menghasilkan asid vanilik and biovanilin. *Aspergillus niger* ATCC6257 dan *Aspergillus niger* EFB1 telah digunakan bagi penghasilan asid vanilik manakala *Phanerochaete chrysosporium* UIA dan *Phanerochaete chrysosporium* EFB1 telah digunakan bagi penghasilan biovanilin. Bagi penghasilan asid vanilik menggunakan glukosa (sebelum penambahan asid ferulik), *A. niger* ATCC6257 menghasilkan 51.3% molar hasil asid vanilik, menunjukkan kenaikan 0.18 kali ganda asid vanilik daripada *A. niger* EFB1 (43.6%). Walaubagaimanapun, *P. chrysosporium* UIA menunjukkan 30.6% molar hasil biovanilin, menandakan kenaikan 0.37 kali ganda biovanilin

berbanding *P. chrysosporium* EFB1 yang merekodkan 22.4% hasil molar biovanilin. Oleh itu, *A. niger* ATCC6257 dan *P. chrysosporium* UIA telah dipilih untuk digunakan dalam biopenukaran dua peringkat menggunakan hidrolisat beralkali OPEFB kepada biovanilin. Keputusan menunjukkan 41% and 39% molar hasil asid vanilik dan biovanilin, masing-masing berjaya dihasilkan daripada biopenukaran dua peringkat menggunakan hidrolisat beralkali OPEFB. Disebaliknya, hanya asid ferulik, asid *p*-hidroksibenzoik dan asid *p*-kumarit merupakan faktor penting ($p<0.05$) dalam dua peringkat model faktorial. Asid ferulik memainkan faktor penting dalam penghasilan asid vanilik manakala interaksi bersama asid *p*-hidroksibenzoik dan asid *p*-kumarit menunjukkan kesan negatif yang menghalang penghasilan asid vanilik. Secara keseluruhannya, hidrolisat beralkali OPEFB berpotensi sebagai subtrat alternatif untuk penghasilan biovanilin melalui biopenukaran dua peringkat.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

HMF	5-hydroxymethyl furfural
ABE	Acetone-Butanol-Ethanol
ADF	Acid Detergent Fibre
ADL	Acid Detergent Lignin
ANOVA	Analysis of Variance
FAOSTAT	Food and Agriculture Organization of the United Nations Statisctic Division
FA	Ferulic acid
FTIR	Fourier Transform Infrared Spectroscopy
HPLC	High Performance Liquid Chromatography
KOH	Potassium hydroxide
MF	Mesocarp Fibre
NaOH	Sodium hydroxide
NDF	Neutral Detergent Fibre
OPEFB	Oil Palm Empty Fruit Bunch
OPF	Oil Palm Frond
OPKS	Oil Palm Kernel Shell
OPT	Oil Palm Trunk
POME	Palm Oil Mill Effluent
PCA	*p*-coumaric acid
PHBA	*p*-hydroxybenzoic acid
PHA	Polyhydroxyalkanoates
SEM	Scanning Electron Microscopy
$NaHSO_3$	Sodium bisulfite
SD	Syringaldehyde
SA	Syringic acid
SSF	Two-stage simultaneous fermentation
VA	Vanillic acid

CHAPTER 1

INTRODUCTION

In the recent years, nations are started to emphasise their attentions on the issues of sustainable development and environmental conservation. The impact of this phenomenon has called attention to the need to utilise renewable and abundant resources such as lignocellulosic biomass into high value-added product (Kumar *et al.*, 2009). Lignocellulosic biomass, mostly derived from agricultural residue, forestry waste and municipal solid waste (Anwar *et al.*, 2014). Regrettably, there are still many lignocellulosic biomass are either left at the plantations or being disposed by burning which could lead to environmental problems (Iqbal *et al.*, 2013). Thus, the conversion of lignocellulosic biomass 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 being recognised as potential feedstock for valued-added product synthesised via biotechnological approaches (Malherbe & Cloete, 2002).

Being one of the largest palm oil exporters in the world, Malaysia generated a large amount of lignocellulosic biomass from palm oil industry annually. One of them is oil palm empty fruit bunch (OPEFB). It is reported that approximately 46 million dry tonnes of OPEFB are produced in 2010 and the capacity predicted to escalate to 49 million dry tonnes by 2020 which made it as one of the main by-products generated from palm oil industry (National Innovation Agency Malaysia, 2013). Therefore, the use of OPEFB as a starting material offers a great potential for value-added products such as bioenergy (Geng, 2013; Ibrahim *et al.*, 2012; Han *et al.*, 2011), enzymes (Ibrahim *et al.*, 2013), activated carbon (Alkhatib *et al.*, 2011; Hameed *et al.*, 2009) and food additives (Aanifah *et al.*, 2014).

Since OPEFB is made up of complex structure between cellulose, hemicellulose and lignin, thus, pretreatment are needed to degrade the OPEFB structure in order to get target product from this biomass (Harmsen *et al.*, 2010). In general, researchers are commonly focusing on the degradation of cellulose and hemicellulose of lignocellulosic biomass for further utilisation into fermentable sugars but less attention has been paid to the recovery of lignin and other phenolic compounds from the lignocellulosic biomass (Tang *et al.*, 2014). Hence, this study focuses on the use of alkaline hydrolysate of OPEFB which comes from lignin degradation as an alternative substrate for biovanillin production. On the other note, various pretreatment strategies are available in order to degrade the lignin structure of lignocellulosic biomass, one of the common methods is using alkaline pretreatment. The degradation of lignin resulted in the release of wide range of compounds including sugars, weak acids, furan derivatives and phenolic compounds (Jönsson & Martín, 2016; Jönsson *et al.*, 2013; Misson *et al.*, 2009). Among the phenolic compounds derived from lignocellulosic biomass, ferulic acid and vanillic acid are the most highlighted compounds as starting materials for biovanillin production (Converti *et al.*, 2010; Walton *et al.*, 2003).

Vanillin or 4-hydroxy-3-methoxy benzaldehyde is a plant secondary metabolite which consists of functional groups of aldehyde, ether and phenol. Naturally, vanillin is extracted from vanilla pod which obtained from the tropical vanilla orchid, principally *Vanilla planifolia* and to a lesser extent, *V. tahitiensis* and *V. pompona*. Vanillin acts as one of the major aromatic components in natural vanilla which contributes to the pleasant smell of vanilla (Zamzuri & Abd-Aziz, 2012). Vanillin is frequently used as flavouring in the worldwide market due to its microbial and antioxidant properties. It is estimated around 60% of vanillin flavouring used in the beverages, food and confectionery industry, 33% in the perfume and cosmetic industry and 7% in the pharmaceutical industry (Priefert *et al.*, 2001).

The production of vanillin is estimated to be approximately 12 000 tonnes per year, with less than 0.5% of the production are come from natural vanillin (Yoon *et al.*, 2005). Meanwhile the remainders of vanillin production are come from synthetic vanillin which synthesised via chemical process using eugenol, coniferin and guaiacol as starting materials (Havkin-frenkel & Belanger, 2011). Synthetic vanillin is preferred by the industries due to fact that the chemical process is easier and cheaper than natural vanillin (Muheim & Lerch, 1999; Asther & Lomascolo, 1998). In addition, limited supply of vanilla pod and the tedious process during extraction of natural vanillin from vanilla pod contribute to the higher price of natural vanillin. However, recently a great interest shown by the consumers towards natural based-products has stimulated the search for alternative production of vanillin via biotechnological approaches, called as biovanillin. According to European Economic Community (EEC) legislation, biovanillin can be considered as natural, hence can substitute the synthetic vanillin in the market (Gioia *et al.*, 2007). Previously, several researchers have proposed the idea of using lignocellulosic biomass such as corn cob (Torres *et al.*, 2009), brewer's spent grain (Mussatto *et al.*, 2007), sugarcane bagasse and maize bran (Tilay *et al.*, 2008) as target substrate for biovanillin production. Thus, utilisation of OPEFB as a starting material can be a great alternative for biovanillin production.

In short, this research work aims to evaluate the potential of alkaline hydrolysate of OPEFB as an alternative substrate for biovanillin production using ferulic acid as precursor via two-step bioconversion. However, a series of phenolic compounds can also be found in the alkaline hydrolysate of OPEFB aside than ferulic acid. Hence, a model of formulated phenolic compounds that mimic the real alkaline hydrolysate of OPEFB are used to evaluate the effect of major phenolic compounds in the alkaline hydrolysate of OPEFB towards vanillic acid production which known as the primary intermediate compound in two-step bioconversion. The objectives of this study can be classified into three parts as follow:

1. To determine phenolic compounds in alkaline hydrolysate of OPEFB.
2. To utilise alkaline hydrolysate of OPEFB as potential substrate for two-step bioconversion of biovanillin.
3. To evaluate the effect of major phenolic compounds in formulated alkaline hydrolysate of OPEFB model towards vanillic acid production using two-level factorial design approach.

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