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ONE-STEP BIOTRANSFORMATION OF FERULIC ACID INTO BIOVANILLIN USING RECOMBINANT Escherichia coli BL21 (DE3)

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By

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Vanillin is one of the most important flavour compounds used in foods, beverages, perfumes and pharmaceuticals industry through isolation from vanilla pods of *Vanilla planifolia* or chemical synthesis. An alternative biotechnology-based approach for biovanillin production is searched for due to the high price of natural vanillin that isolated from vanilla pods and rising on demand for naturally produced foods. Vanillin production through biotechnology routes is focused on the microbial bioconversion from precursors like ferulic acid and eugenol. However, the common problem regarding biovanillin production is the oxidation pathway of vanillin into vanillic acid. Thus, low vanillin is detected as the desired product. The objective of this study includes the construction of recombinant *Escherichia coli* that can be further utilized for biotransformation of ferulic acid into biovanillin by one step fermentation without further oxidation of vanillin into vanillic acid. The desired genes involving in biovanillin production, enoyl-CoA hydratase *(ech)* andferuloyl-CoA synthetase *(fcs)* are screened and isolated from locally isolated bacteria, later named as *Pseudomonas* sp. AZ10 UPM. It showed the highest degradation of ferulic acid as carbon source with the yield, $Y_{p/s}$ and productivity, P_r obtained were 1.08 mg/mg and 53.1 mg/L/h, respectively. Oxidation of vanillin into vanillic acid was observed lead to low vanillin production after 48 hours of incubation. Therefore, this strain was selected as the potential vanillin producer due to accumulation of vanillic acid at the end of fermentation process with assumption that vanillin was oxidized into vanillic acid. Further study was conducted on the isolation of biovanillin producing gene from *Pseudomonas* sp. AZ10 UPM. By using DNA walking strategy, full length of both enoyl co-A hydratase (*ech*) and feruloyl co-A synthetase (*fcs*) were successfully isolated. Analysis of the nucleotide sequences revealed the presence of an open reading frame of 906 bp with protein encoded 302 amino acids and 1869 bp with protein encoded 623 amino acids of *ech* and *fcs*, respectively. The deduced amino acids for *ech* and *fcs* about 62% and 98% homology with *Pseudomonas* sp., respectively. The recognition of GTG (Guanine-Thymine-Guanine) for both genes *ech* and *fcs* as start codon was assisted by the presence of Shine-Dalgarno sequence, which located at 8 bp for *ech* and 7 bp for *fcs* upstream the initiation codon. The pRSFDuet-1/*ech-fcs*

expression system has been constructed by cloning the full length of *fcs* and *ech* under the transcriptional control of T7 promoter of pRSFDuet-1 into an *E. coli* BL21 (DE3). In comparison to wild type, *Pseudomonas sp.* AZ10 UPM, this recombinant *E. coli* harbouring pRSFDuet-1/*ech-fcs* was able to produce vanillin in one step fermentation without further oxidation of vanillin into vanillic acid. In order to confirm the genes expression for vanillin production, fermentation was done in 2YT medium with supplementation of 0.1% (w/v) ferulic acid. From the observation, the recombinant *Escherichia coli* able to produce 165 mg/L vanillin by one step fermentation without further oxidation into vanillic acid. Furthermore, the recombinant shows the ability to convert agricultural waste containing ferulic acid into vanillin. It was able to convert 200 mg/L oil palm empty fruit bunches (OPEFB) alkaline hydrolysate into 27 mg/L vanillin with no vanillic acid detected as the oxidized product. In conclusion, this study was successfully developed a recombinant *Escherichia coli* BL21 (DE3) with plasmid harbouring key genes for biovanillin production which were *ech* and *fcs*. The recombinant was able to produce biovanillin in one step fermentation without further oxidation of vanillin into vanillic acid. In fact, it shows potential to utilize agricultural waste, OPEFB alkaline hyrdrolysate as natural source of ferulic acid for biovanillin production.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

BIOTRANSFORMASI ASID FERULIK MELALUI KAEDAH SATU LANGKAH KEPADA BIOVANILIN OLEH *Escherichia coli* BL21 (DE3) REKOMBINAN

Oleh

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Vanilin merupakan salah satu diantara sebatian perisa yang digunakan di dalam industri makanan, minuman, minyak wangi dan farmasi melalui pemencilan daripada pod vanila tumbuhan *Vanilla planifolia* atau sintesis kimia. Penghasilan vanilin secara alternatif berasaskan bidang bioteknologi sedang meningkat naik kerana harga vanilin yang tinggi apabila diesktrak dari pokok vanila semulajadi dan permintaan yang meningkat terhadap makanan yang diproses secara semulajadi. Penghasilan vanilin melalui kaedah bioteknologi memberi fokus terhadap penukaran mikrob daripada prekursor asid ferulik dan eugenol. Walaubagaimanapun, masalah yang biasa terjadi dalam penghasilan biovanilin ialah pengoksidaan vanilin kepada asid vanilik. Seterusnya menyebabkan vanilin tidak dapat dikesan sebagai produk yang dikehendaki atau hasil yang diperolehi adalah sangat rendah. Oleh itu, objektif kajian ini ialah untuk menghasilkan *E. coli* rekombinan yang mampu menggunakan asid ferulik bagi tujuan transformasi kepada biovanilin melalui kaedah satu langkah tanpa pengoksidaan vanilin kepada asid vanilik. Kajian ini adalah untuk menyaring dan mengasingkan gen yang di kehendaki bagi tujuan penghasilan biovanilin iaitu enoyl-CoA hydratase *(ech)* dan feruloyl-CoA synthase *(fcs)* daripada bakteria yang telah diasingkan, dan diberi nama sebagai *Pseudomonas* sp. AZ10 UPM. Ia menunjukkan tahap degradasi asid ferulik tertinggi sebagai sumber karbon. Pengoksidaan vanilin kepada asid vanilik dicerapi dan menyebabkan penghasilan vanilin sebagai produk yang dikehendaki menjadi rendah. Hasil degradasi asid ferulik, $Y_{p/s}$ dan produktiviti, P_r yang diperolehi masing-masing adalah 1.08 mg/mg and 53.1 mg/L/h. Hasil perolehan yang lebih tinggi berbanding kajian terdahulu berkaitan penukaran asid ferulik kepada asid vanilik dicerapi. Pengoksidaan vanilin kepada asid vanilik turut dilaporkan selepas tempoh 48 jam. Strain ini telah dipilih atas potensi penghasilan vanillin dengan andaian asid vanilik yang terkumpul adalah hasil daripada pengoksidaan vanilin. Kajian seterusnya melibatkan pemencilan gen daripada *Pseudomonas* sp. AZ10 UPM untuk penghasilan biovanilin. Dengan menggunakan strategi perjalanan DNA, ia telah berjaya mendapatkan jujukan lengkap bagi kedua dua gen iaitu enoyl co-A hydratase (*ech*) and feruloyl co-A synthetase (*fcs*). Analisis terhadap jujukan nukleotida menunjukkan

kehadiran satu rangka terbuka sepanjang 906 bp dengan jujukan peptide masing-masing sebanyak 302 asid amino dan 1869 bp berserta jujukan peptida sebanyak 623 asid amino untuk *ech* dan *fcs*. Asid amino *ech* dan *fcs* dapat disimpulkan mempunyai persamaan masing-masing sebanyak 62% dan 98% dengan *Pseudomonas* sp. Pengesanan GTG (Guanin-Thiamin-Guanin) sebagai fungsi pemula gen *ech* dan *fcs* adalah dibantu melalui kehadiran jujukan Shine-Dalgarno yang terletak di kedudukan 8 bp untuk gen *ech* and 7 bp bagi gen *fcs* di bahagian atas jujukan daripada fungsi pemula. Sistem ekspresi pRSFDuet-1/*ech-fcs* telah dibina melalui pengklonan jujukan lengkap *fcs* dan *ech* dibawah kawalan transkripsi pemula T7 yang terletak pada pRSFDuet-1 kepada *E. coli* BL21 (DE3). Jika dibandingkan dengan strain liar *Pseudomonas sp.* AZ10 UPM, *E. coli* rekombinan yang mengandungi pRSFDuet-1/*ech-fcs* ini mampu menghasilkan vanilin dalam satu langkah tanpa pengoksidaan vanilin kepada asid vanilik. Bagi tujuan pengesahan maklumat tentang ekspresi gen bagi penghasilan vanilin, proses fermentasi telah dijalankan di dalam media 2YT dengan tambahan 0.1% (w/v) asid ferulik. Berdasarkan pemerhatian, *E. coli* rekombinan mampu menghasilkan vanilin sebanyak 165 mg/L melalui kaedah satu langkah fermentasi tanpa berlaku pengoksidaan vanilin kepada asid vanilik. Malahan, rekombinan ini juga menunjukkan kebolehan menggunakan sisa pertanian kepada penghasilan vanilin. Ia mampu menukarkan 200 mg/L asid ferulik yang terkandung di dalam hidrolisat alkali buah tandan kosong kelapa sawit kepada 27 mg/L vanilin. Asid vanilik juga tidak dikesan sebagai produk pengoksidaan. Kesimpulannya, kajian ini berjaya menghasilkan *E. coli* rekombinan yang mempunyai plasmid berserta gen-gen penghasil biovanillin iaitu *ech* dan *fcs*. Rekombinan ini juga mampu menghasilkan biovanilin dalam satu langkah tanpa berlaku pengoksidaan vanilin kepada asid vanilik. Malahan, kajian menunjukkan rekombinan berpotensi untuk menggunakan sisa pertanian, hidrolisat alkali buah tandan kosong kelapa sawit sebagai sumber semulajadi asid ferulik untuk penghasilan biovanilin.

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

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% v/v	percentage volume per volume
% w/v	percentage weight per volume
FA	Ferulic acid
VA	Vanillic acid
Ech	enoyl co-A hydratase
Fcs	feruloyl co-A synthetase
G	Gram
g/L	gram per liter
mg/L	milligram per liter
HPLC	High Performance Liquid Chromatography
KH_2PO_4	Potassium dihydrogen Phosphate
L	Liter
LB	Luria-Bertani
M	Molar
Mg	Milligram
mL	Milliliter
mL/min	milliliter per minute
mM	Millimolar
NaCl	sodium chloride
NCBI	National Center for Biot*ech*nology Information
ORF	Open Reading Frame
SD	Shine-Dalgarno
PCR	Polymerase Chain Reaction
Rpm	rotation per minute
rRNA	ribosomal RNA
VP	Vanillin producing
TB	Terrific Broth
µL	Microliter
µg/mL	microgram per milliliter
µm	micrometer

CHAPTER 1

INTRODUCTION

1.1 Background of study

Vanillin is known as a major component that provides the taste and aroma for natural vanilla. Vanilla has been known for many years and used widely as flavouring agents worldwide. South and Central America were the first used this flavour before Europeans arrived in the sixteenth century (Lubinsky et al., 2006). This spice was brought back to Europe by the Spanish explorers which then became very popular there for flavouring of foods. Since then, vanilla became a well known flavouring material. Vanillin is obtained through vanilla beans extraction from Vanilla orchid of *Vanilla planifolia*, *Vanilla tahitiensis* and *Vanilla pompon*a.

Based on vanillin worldwide market, Priefert et al. (2001) reported that out of 10,000 tons of vanillin, only 0.5% of the vanillin is isolated from vanilla pods annually. Traditionally, the flavour of vanillin for vanilla ice cream, yoghurt and cakes was extracted from the tropical orchid pods. The strong demand and the fact that this flavouring compound is expensive move the trends towards alternative sources research. Li and Rosazza (2000) also mentioned that the estimated vanillin world consumption is 12,000 tonne per year. However, the extracted natural vanillin from vanilla pods is only 50 tons and the remaining supplies are fromlignin or guaiacol which is the chemically synthesized vanillin (Clark, 1990). Through a reaction with guaiacol and glyoxylate, the production of synthetic vanillin was accomplished. Besides that, Dignum et al. (2001) reported that cell culture of microorganism has been used in order to preserve a "natural" additive claim. The estimated synthetic vanillin production is about 13000 tons per year. Vanillin that is produced synthetically is usually produced from guaiacol which is a petrochemical product. Natural vanillin supplies only 2% of the vanillin market (Havkin-Frenkel and Belanger, 2008). Synthetic vanillin costs approximately USD $11-15 per kg. However, natural vanillin has a significantly higher price where vanillin produced from microorganism has a price of about USD $1,000 per kg (Korthou and Verpoorte, 2007; Converti et al., 2010).

Ander et al. (1980), Chatterjee et al. (1999) and Civolani et al.(2000) have been reported that in n order to produce vanillin in an environmental friendly way, microbial conversion has been proposed by using bacteria and fungi to utilize eugenol and ferulic acid as the substrates. Besides that, previous study has adding the knowledge regarding the coding genes of biovanillin producing enzymes from ferulic acid (Overhage et al., 1999; Narbad and Gasson, 1998; Venturi et al., 1998). The recombinant strains carrying target genes for vanillin production also created new opportunities for metabolic engineering routes to be developed. Okeke and Venturi (1999) mentioned that only a few literaturesreported on genetically engineered *E. coli* for the vanillin bioproduction and some of the reported work produced low amount of vanillin

(Achterholt et al., 2000). Previous study by Yoon et al. (2005a) has successfully inserted the *fcs* (feruloyl-CoA synthetase) and *ech* (enoyl-CoA hydratase/aldolase) genes from *Amycolatopsis* sp. strain HR104 and *Delftia acidovorans*. It further developed two recombinant *E. coli* strains under the control of the arabinose-inducible promoter P_{BAD} into the pBAD24 expression vector. The *E. coli* strain carrying the *Amycolatopsis* genes produced the highest vanillin production of 580 mg/L from 1 g/L ferulic acid under optimized growing-cell conditions. The recombinant *E. coli* was constructed by cloning the desired genes that responsible to convert ferulic acid into vanillin.

1.2 Problem statement

Recently, biovanillin production from biotechnology routes has been considered as a potential source of natural vanillin as certified by FDA. Thus, the increasing market request for natural bioflavours has created a great potential in exploring vanillin through biotechnological approaches. One of the approaches is the microbial production of vanillin from ferulic acid. It has been reported that ferulic acid is the attractive precursors for natural vanillin production by Rosazza et al. (1995).

The most available and promising substrates include, ferulic acid extracted from sugar beet pulp as reported by Lesage-Meessen et al. (1999), waste of rice bran oil processing (Zheng et al., 2007) glucose (Li and Frost, 1998; Hansen et al., 2013) and even lignin fragments (Havkin-Frenkel and Belanger, 2008). Separation technologies are maturing (Brazinha et al., 2011) and it is responsible for the industrial-scale production of vanillin from biotechnologies. These processes are still emerging technologies producing high-cost vanillin, which is suitable for the aroma and fragrances field for marketing reasons, but not for potential use in renewable resources-based polymers (Fache et al., 2015).

Furthermore, the increasing worldwide demand for natural vanillin is because of the increasing concerns regarding nutritional and health issue (Ashengroph et al., 2011; Zhao et al., 2006). In addition, there is greater preference for natural vanillin due to the presence of racemic mixtures in synthetic vanillin production (Rana et al., 2013). Therefore, biovanillin that regarded as natural and involved non-chemically process has been investigated by the researchers which can be further utilized in the next future.

Malaysia's economic growth is experiencing a strong development in new oil palm plantations and palm oil mills. Oil palm wastes are highly generated as this industry becomes bigger. It will later provide waste heavy loads thus create the problem of disposal difficulties and will increase the operation cost. Awalludin et al. (2015) describes that the oil palm waste has significant potential in various applications. Moreover, the waste utilization will help to minimize the impacts caused to the environment by recycling oil palm empty fruit bunch (OPEFB). This is due to the serious thought on sustainability of palm oil industry triggers ways on the alkaline treatment strategy for ferulic acid (FA) release from OPEFB fibres (Mohd Aanifah et

al., 2014). Therefore, the FA obtained from these fibers can be great potential to be utilized for vanillin production.

Pseudomonas sp. strains, has been described as the potential biovanillin producers as it has broad versatility in metabolic pathway. Apart from that, it has the ability to metabolize phenolic compound rapidly. Biotransformation of ferulic acid into vanillin has been reported in *Pseudomonas putida*. However, the major problem by using *Pseudomonas* sp. was the oxidation of vanillin into vanillic acid. Therefore, the amount of vanillin accumulated at the end of fermentation process was nearly undetectable (Mulheim and Lerch, 1999). As a result, it lowers vanillin production as the desired product. Moreover, Giraud et al. (2014) also described that the problem of ferulic acid conversion to vanillin is due to the vanillin degradation to vanillic acid and vanillyl alcohol and the low amount of vanillin detected is because of the oxidation or reduction process. Furthermore, the oxidation of vanillin was reported in the complex media (Panoutsopoulos and Beedham, 2005; Sachdev et al., 2008). Thus, a research is conducted in order to produce biovanillin through microbial fermentation without further oxidation of vanillin into vanillic acid.

1.3 Objectives

The general objective of this study was to develop recombinant *Escherichia coli* harbouring the biovanillin target genes for simple, faster and stable biovanillin production. This recombinant strain was then tested for its ability to utilize ferulic acid for production of biovanillin. In this work, the recombinant *E. coli* BL21 (DE3) strains transformed into pRSFDuet-1 vector with the insert of *ech* and *fcs* genes from locally isolated *Pseudomonas* sp. AZ10 UPM and named as *E. coli* BL2 (DE3)/ pRSFDuet-1/*ech-fcs*. This recombinant strain was later tested for gene expression and biovanillin production from synthetic ferulic acid and OPEFB alkaline hydrolysate.

The specific objectives of this study are:

1. To screen, isolate and identify local biovanillin producing bacteria

2. To isolate functional genes and construct recombinant *E. coli* for one step biotransformation of ferulic acid into biovanillin

3. To utilize OPEFB as an alternative substrate for biovanillin production using recombinant *E. coli.*

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