PRODUCTION OF VANILLATE FROM LIGNIN DERIVATIVES USING GENETICALLY MODIFIED *Escherichia coli*

Mr. Jedsadakorn Ninrat

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Biotechnology
FACULTY OF SCIENCE
Chulalongkorn University
Academic Year 2022

การผลิตวานิลเลตจากอนุพันธ์ลิกนินโดยใช้ *Escherichia coli* ที่ถูกดัดแปรพันธุกรรม

นายเจษฎากร นิลรัตน์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ ไม่สังกัดภาควิชา/เทียบเท่า
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2565

Thesis Title	PRODUCTION OF VANILLATE FROM LIGNIN DERIVATIVES USING GENETICALLY MODIFIED*Escherichia coli*
By	Mr. Jedsadakorn Ninrat
Field of Study	Biotechnology
Thesis Advisor	Professor Dr. ALISA VANGNAI

Accepted by the FACULTY OF SCIENCE, Chulalongkorn University in Partial Fulfillment of the Requirement for the Master of Science

..... Dean of the FACULTY OF SCIENCE
(Professor Dr. POLKIT SANGVANICH)

THESIS COMMITTEE

..... Chairman
(Associate Professor SUPAART SIRIKANTARAMAS)
..... Thesis Advisor
(Professor Dr. ALISA VANGNAI)
..... Examiner
(Dr. SUPAWIN WATCHARAMUL)
..... External Examiner
(Assistant Professor Dr. Noppon Lertwattanasakul)

เจษฎากร นิลรัตน์ : การผลิตวานิลเลตจากอนุพันธ์ลิกนินโดยใช้ *Escherichia coli* ที่ถูกดัดแปรพันธุกรรม . (PRODUCTION OF VANILLATE FROM LIGNIN DERIVATIVES USING GENETICALLY MODIFIED*Escherichia coli*) อ.ที่ปรึกษาหลัก : ศ. ดร.อลิสา วังใน

กรดวานิลลิกเป็นสารประกอบสำคัญถูกนำไปใช้ในอุตสาหกรรมอาหาร เครื่องดื่ม น้ำหอม และยา รักษาโรค กรดวานิลลิกถูกสร้างได้ในวิถีเมแทบอลิซึมของพืช จุลินทรีย์ธรรมชาติบางชนิดและจุลินทรีย์ดัดแปรพันธุกรรม อย่างไรก็ตามกรดวานิลลิกจะถูกสลายต่อในการเจริญของเซลล์จุลินทรีย์ ซึ่งทำให้กรดวานิลลิกมีการสะสมน้อยและไม่เพียงพอต่อการนำไปใช้ประโยชน์ ดังนั้น สำหรับการผลิตกรดวานิลลิกเพื่อการใช้ในปริมาณมากในภาคอุตสาหกรรมต้องอาศัยจุลินทรีย์ตัวเร่งปฏิกิริยาที่มีประสิทธิภาพและกระบวนการผลิตทางเทคโนโลยีชีวภาพที่มีประสิทธิภาพ เพื่อใช้สารอนุพันธ์ชีวมวลจากวัตถุดิบการเกษตรโดยเฉพาะส่วนของลิกนินเป็นสารตั้งต้น กรดเฟอรูลิกเป็นสารอนุพันธ์ลิกนิน ถูกนำไปใช้เป็นสารตั้งต้นในการผลิตกรดวานิลลิก มีการรายงานวิถีสังเคราะห์วานิลลินซึ่งเป็นสารตั้งต้นของกรดวานิลลิกจากกรดเฟอรูลิก โดยใช้เอนไซม์ซึ่งต้องอาศัยการทำงานร่วมกับโคเอนไซม์ ในงานวิจัยนี้ได้พัฒนาวิถีสังเคราะห์กรดวานิลลิกในเชื้อแบคทีเรียเจ้าบ้าน *E. coli* ซึ่งประกอบด้วยยีน 4 ชนิด คือ *comt padC vdh* และ *ado* ที่เข้ารหัสให้เอนไซม์ที่มีประสิทธิภาพทำงานโดยไม่ต้องอาศัยโคเอนไซม์ ผลการศึกษาสภาวะที่เหมาะสมในการผลิตกรดวานิลลิกในระดับฟลาสก์ พบว่า มีประสิทธิภาพการผลิตกรดวานิลลิก ร้อยละไบโอคอนเวอร์ชัน 96 ± 2 จากกรดเฟอรูลิกความเข้มข้น 0.194 กรัมต่อลิตร (1 มิลลิโมลาร์) เป็นสารตั้งต้นด้วยการเลี้ยงเชื้อแบบปราศจากการเจริญที่ค่าความขุ่น เท่ากับ 40 ในสารละลายบัฟเฟอร์ทริสไฮโดรคลอริกที่ค่าความเป็นกรดด่าง เท่ากับ 8 กรดวานิลลิกความเข้มข้นสูงที่สุดที่ผลิตได้ มีค่าเท่ากับ 0.95 ± 0.002 กรัมต่อลิตร 4.92 ± 0.01 มิลลิโมลาร์ ร้อยละไบโอคอนเวอร์ชัน 58 ± 1 จากกรดเฟอรูลิกความเข้มข้น 1.94 กรัมต่อลิตร (10 มิลลิโมลาร์) ภายในเวลา 48 ชั่วโมง การผลิตกรด วานิลลิกจากกรดคาเฟอิกด้วยวิธีการผลิตทางชีวภาพแบบ 2 ขั้นตอน พบว่า มีประสิทธิภาพการผลิตกรดวานิลลิก 0.168 ± 0.003 กรัมต่อลิตร 1.00 ± 0.02 มิลลิโมลาร์ ร้อยละไบโอคอนเวอร์ชัน 100 ± 2 ภายในเวลา 48 ชั่วโมง จากกรดคาเฟอิกความเข้มข้น 0.180 กรัมต่อลิตร (1 มิลลิโมลาร์) เป็นสารตั้งต้น ผลการศึกษาสภาวะที่เหมาะสมในการผลิตกรดวานิลลิกในระดับถังหมักขนาด 5 ลิตร พบว่ามีประสิทธิภาพการผลิตกรดวานิลลิก ร้อยละไบโอคอนเวอร์ชัน 74 ± 4 0.121 ± 0.006 กรัมต่อลิตร และ 0.72 ± 0.04 มิลลิโมลาร์ ภายในเวลา 67 ชั่วโมง จากกรดเฟอรูลิกความเข้มข้น 0.194 กรัมต่อลิตร (1 มิลลิโมลาร์) เป็นสารตั้งต้น ดังนั้น เชื้อ *E. coli* ดัดแปรพันธุกรรมนี้ จึงมีศักยภาพในการนำไปขยายการผลิตกรดวานิลลิกและสามารถพัฒนาเพื่อใช้ในระดับอุตสาหกรรมต่อไปได้

สาขาวิชา	เทคโนโลยีชีวภาพ	ลายมือชื่อนิสิต .....
ปีการศึกษา	2565	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

6171931723 : MAJOR BIOTECHNOLOGY
KEYWORD: BIOTECHNOLOGICAL PROCESS, VANILLIC ACID, LIGNIN DERIVATIVES, OPTIMIZATION, SCALE UP, FERMENTATION
Jedsadakorn Ninrat : PRODUCTION OF VANILLATE FROM LIGNIN DERIVATIVES USING GENETICALLY MODIFIED*Escherichia coli* . Advisor: Prof. Dr. ALISA VANGNAI

Vanillic acid is an important compound used in food, beverage, fragrance, and pharmaceutical industries. Vanillic acid can be produced by using either natural plant, native, and engineered microorganisms. However, the industrial scale production of vanillic acid compound still difficult due to low yielding production which can be improved by metabolic engineering of bacterial cells. Hence, the efficient biocatalyst, engineering, and biotechnology process can be a promising strategy to enhance vanillic acid production from lignin derivatives and strengthen the large-scale production for industrial approach. Among several lignin-derived compounds, ferulic acid is a widely used substrate for vanillic acid production. Recently, the Coenzyme A-independent production of vanillin from ferulic acid has been reported. To further develop this pathway for vanillic acid production, herein, the synthetic pathway consisting of four genes which are *comt, padC, vdh,* and *ado* was constructed and expressed in the recombinant *E. coli* strain BL21(DE3). Such strain showed an effective vanillic acid production with 96 ± 2 % vanillic acid yield when the growth-independent conversion was conducted with an initial OD_{600} of 40 in 100 mM Tris-HCl buffer pH 8, and ferulic acid 0.194 g/L (1 mM) as the substrate. The maximum production of vanillic acid was 0.95 ± 0.002 g/L (4.92 ± 0.01 mM, 58 ± 1 % molar yield) from 1.94 g/L (10 mM) of ferulic acid within 48 hours in shake flask scale. A two-step bioconversion process was established to produce vanillic acid from caffeic acid. The production of vanillic acid reached 0.168 ± 0.003 g/L (1.00 ± 0.02 mM, 100 ±2 % molar yield) from 0.180 g/L of caffeic acid (1 mM) within 48 hours. For the production of vanillic acid in 5-L fermenter, the maximum vanillic acid of 0.121 ± 0.006 g/L (0.72 ± 0.04 mM, 74 ± 4 % molar yield) was achieved from 0.194 g/L of ferulic acid within 67 hours. This study suggests this recombinant *E. coli* might be a practical strain for scaling up vanillic acid production and further development for industrial application.

Field of Study:	Biotechnology	Student's Signature .....
Academic Year:	2022	Advisor's Signature .....

ACKNOWLEDGEMENTS

I would like to deepest my gratitude to my advisor, Professor Dr. Alisa Vangnai for excellent instruction, guidance, encouragement, attention, and support throughout this thesis. Importantly, I am most grateful her teaching me, not only the research methodologies but also guidance in career path of my life. Without this kindness and understanding, this thesis could not be accomplished. Besides, I would like to give a deep thank to Dr. Panaya Kotchaplai. This thesis would not have been completed without all the support and opinion from her. Furthermore, I would like to thanks to all staff members of room 604/1 and all of my friends for their friendships, kind assistance and support. Finally, I would like to express my lovely family for their infinite love, willpower, support, understanding, encouragement, and everything giving to my life.

Jedsadakorn Ninrat

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

CHAPTER I

INTRODUCTION

1.1 Statement of purposes

Vanillic acid is an important phenolic compound. It is widely used in various applications of food, beverages, and pharmaceutical formulations (Lesage-Meessen *et al*., 1996; Narbad & Gasson, 1998; Muheim & Lerch, 1999; Plaggenborg et al., 2006; Baqueiro-Peña et al., 2010). The biological approaches i.e. natural and engineered microorganisms for the production of vanillic acid from ferulic acid have been reported. These include *Pseudomonas fluorescens* strain BF13 (Di Gioia et al., 2011), *Amycolatopsis* sp. strain HR167 (Achterholt et al., 2000), *Streptomyces halstedii* (Brunati et al., 2004), and *Halomonas elongata* (Abdelkafi et al., 2006). However, one impediment of the industrial-scale bioproduction is that vanillic acid can be further metabolized rapidly by most of the natural vanillic acid-producers (Peng et al., 2002; Sainsbury et al., 2013).

Recently, the effective synthetic pathway in *Escherichia coli* containing enzymes catalyzing the bioconversion of lignin-derivatives to vanillin production has been reported. This pathway consists of phenolic acid decarboxylase (Pad) that converts ferulic acid to 4-vinylguaiacol and aromatic dioxygenase (Ado) that converts 4-vinylguaiacol to vanillin (Ni et al., 2018). Since vanillin can be oxidized to vanillic acid by vanillin dehydrogenase (Vdh) (Nishimura et al., 2018), using the *pad-ado-vdh* system is a promising alternative for vanillic acid production. Therefore, the amount of vanillic acid for industrial application can be elucidated by biotechnological process and highly efficient biocatalyst from ferulic acid or lignin derivatives compounds. In this thesis, the construction of expression vector for vanillic acid biosynthesis was developed. The optimization of vanillic acid production was conducted in shake flask and 5-L fermenter.

1.2 Objectives of this research

The aim of the present study was to develop vanillic acid biosynthesis in *E. coli* BL21 (DE3) and optimization of vanillic acid production from caffeic acid in 5 L fermenter scale.

1.3 Conceptual framework of this research

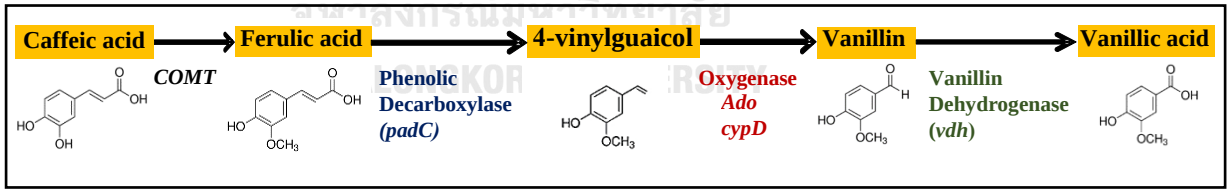
The conceptual framework was shown in Figure 1

1.3.1 The construction of expression vector for vanillic acid biosynthesis in *E. coli* BL21(DE3) host

This pathway consists of caffeic acid O-methyltransferase (*COMT*) converting caffeic acid to ferulic acid, phenolic acid decarboxylase (*Pad*) that converts ferulic acid to 4-vinylguaiacol, aromatic dioxygenase (*Ado*), Cytochrome P450s (*cypD*) that converts 4-vinylguaiacol to vanillin and vanillin dehydrogenase (*vdh*) that oxidizes vanillin to vanillic, respectively.

1.3.2 The optimization and improvement of vanillic acid production in flask and 5 L fermenter scale

1.3.1 Construction of a synthetic metabolic pathway



1.3.2 Bioconversion and optimization for vanillic acid production

Flask scale and 5 L fermenter scale	
1.	Type of inducer
2.	Inoculum percentage

Figure 1.1 The conceptual framework of this research

CHAPTER II

THEORY AND LITERATURE REVIEW

2.1 Lignocellulosic biomass and lignin valorization

Lignocellulosic biomass is a major component of plants, abundantly found in agricultural wastes and residues (Brar et al., 2016; Isikgor & Becer, 2015). Lignocellulosic biomass mainly consists of three compartments i.e. cellulose (30%-60%), hemicellulose (20%-40%) and lignin (10%-25%) (Horn et al., 2012; Nanda et al., 2014; Nanda et al., 2013; Quiroz-Castañeda & Folch-Mallol, 2013). Composition of lignocellulosic biomass depends on types of agricultural wastes/residues (Bayer et al., 2014).

Biomass becomes an important resource for sustainable bio-based economy. Which cellulose and hemicellulose are the polymers of sugar units, lignin is a polymer of aromatic compounds (Pérez et al., 2002). Unlike cellulose and hemicellulose of which their pretreatment and valorization have been extensively studied and successfully demonstrated, the lignin bioconversion and utilization technologies are still challenging (Kumar et al., 2020). Normally, lignin is composed of three different building blocks: coniferyl alcohol (G-aromatics), sinapyl alcohol (S-aromatics) and *p*-coumaryl alcohol (H-aromatics) as shown in Figure 2.2

The biological valorization of lignin, the process converting lignin into manufacture high-value chemicals, fuels, and renewable products, has been studied (Beckham et al., 2016). Recently, the genetic engineering tools for bacteria have efficiently converted lignin-derived aromatic and their intermediates to defined chemical, such as protocatechuate, catechol, and gallate, which is referred to as a ‘biological funnel’ pathway (Linger et al., 2014; Borchert et al., 2022). Biological funnel in microbes is the metabolization of heterogenous lignin derivatives from depolymerized lignin to generating valuable aromatic chemicals (Liu et al., 2022). Aromatic fine chemicals were produced from biological funnel of lignin-derived compounds by bacteria are shown in Table 1.1.

Protocatechuic acid (PCA) is precursor for synthesize valuable pharmaceutical and important aromatics such as gallic acid. The inactivation of PCA-degrading pathways in ligninolytic microbes could increase the production of PCA. Nguyen et al. (2021) reported the synthesize of protocatechuic acid from lignin-derived aromatics by engineered *Pseudomonas putida* KT2440, thereby knockout protocatechuate 3,4-dioxygenase (the aromatic ring opening gene) and express the formaldehyde metabolize pathway and aldehyde dehydrogenase. Gallic acid was achieved from protocatechuic acid or *p*-hydroxybenzoic acid by the recombinant *Escherichia coli* and *Rhodococcus opcacus via* the hydroxylation process (Cai et al., 2021; Fu et al., 2021).

Vanillin has been obtained from ferulic acid using *P. putida* KT2440 by inactivating vanillin dehydrogenase (*vdh)* and overexpressing the feruloyl coenzyme-A synthetase (*fcs*) and enoyl-coenzyme-A hydratase/aldolase (*ech*) gene (Graf and Altenbuchner, 2014).

The inactivation of *p*-hydroxybenzoate-3-hydroxylase and benzoyl-CoA ligase and expression of *p*-hydroxycinnamoyl-CoA synthetase II in *Burkholderia glumae* BGR1 achieved *p*-Hydroxybenzoic acid from *p*-coumaric acid as a carbon source (Jung et al., 2016).

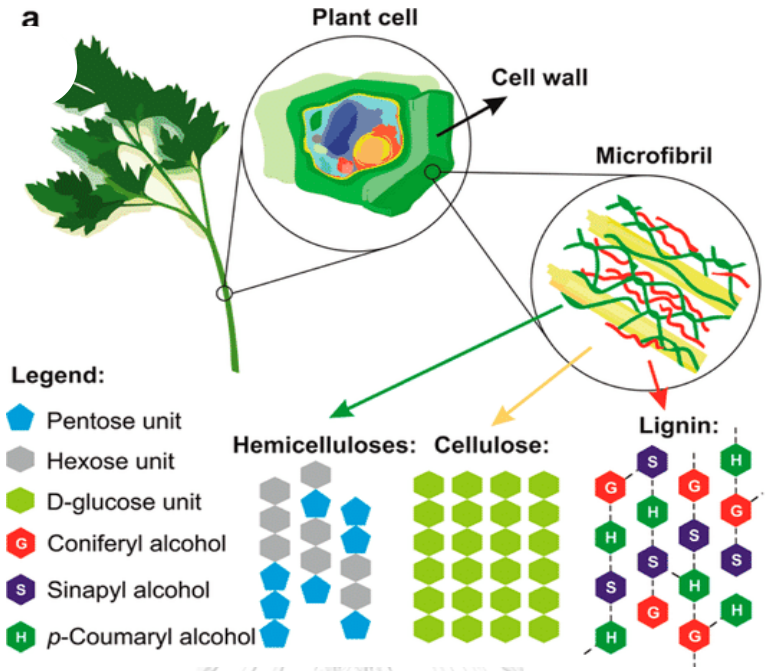


Figure 2.1 Component of lignocellulosic biomass (Xu et al., 2019)

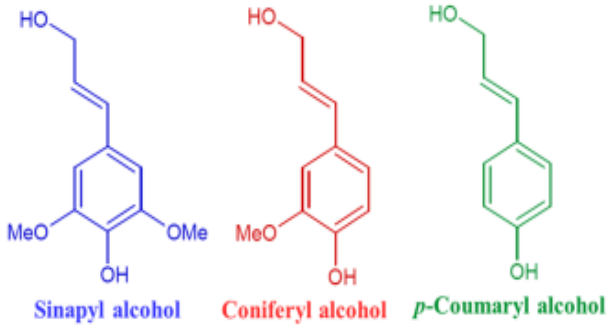


Figure 2.2 Building units of lignin (Zhang et al., 2018)

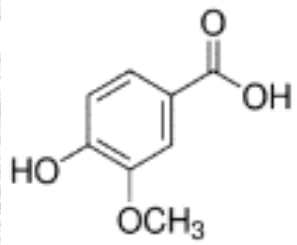
Table 2.1 Bacterial valorization of lignin-derived compounds into aromatic fine chemicals (Liu et al., 2022)

Substrate	***Strain***	**Products**	**Product titer**	**Fermentation**	**Gene manipulation**	**References**
Ethanol-assisted depolymerized lignin	*P. putida* KT2440	PCA	6.73 mg/L	Batch	Deleting pcaGH	(Nguyen et al., 2021)
Vanillin	*E. coli*	Catechol	8 mg/L	Batch	Overexpressing CouP, LigV, LigM and Aro under ADH7 promoter	(Wu et al., 2018)
Alkaline pretreated liquor	*R. opacus*	Gallic acid	0.40 g gallic acid/g APL	Batch	Integrating biological hydroxylation, *O*-demethylation, and aryl side-chain oxidation	(Cai et al., 2021)
Ferulic acid and *p*-coumaric acid	*E. coli*	Gallic acid	19.57 mM gallic acid from 20 Mm FA; 19.96 mM gallic acid from *p*-coumaric acid	Fed-batch	Overexpressing FCS, ECH, HFD1, VanAB, and PobA^{Y385F}; overexpressing FCS, ECH, HFD1, HpaBC, and PobA^{Y385F}	(Fu et al., 2021)
Ferulic acid	*P. putida* KT2440	Vanillin	1.31 g/L	Batch	Deleting *vdh*, molybdate transporter gene; overexpressing *fcs*, *ech* gene	(Graf & Altenbuchner, 2014)
p-Coumaric acid	*B. glumae* BGR1	*P*-Hydroxybenzoic acid	2.73 g/L	Batch	Deleting *phb3h*, *bcl* gene and overexpressing *phcs* II	(Jung et al., 2016)
Vanillin	*E. coli*	Vanillic acid	400 mg/L	Batch	Overexpressing CouP, LigV, LigM and Aro driven by ADH7 promoter	(Wu et al., 2018)
p-Coumaric acid	*E. coli*	*p*-Hydroxystyrene	266 mM/L	Packed-bed reactor	Overexpressing phenolic acid decarboxylase from *B. amyloliquefaciens*	(Jung et al., 2013)

2.2 Vanillic acid and applications

Vanillic acid or 4-hydroxy-3-methoxybenzoic acid (VA) is a vanillin derivative which can be found at high concentration in vanilla beans (Sostaric *et al*., 2000). It appears as a white/yellow powder or crystalline with a good creamy odor (Wang *et al*., 1996). The physical and chemical properties of vanillic acid are as shown in Table 2.2. Vanillic acid is widely used as a flavor/odor agent in various applications of food and beverages. It has been licensed as a food additive (JECFA no. 959) with no safety concern by the joint FAO/WHO Expert Committee on Food Additives (Lesage-Meessen et al., 1996; Narbad & Gasson, 1998; Gitzinger et al., 2012; Muheim & Lerch, 1999; Plaggenborg et al., 2006; Baqueiro-Peña et al., 2010). Vanillic acid has been associated with pharmacological activities such as inhibiting snake venom activity (Dhananjaya et al., 2009; Dhananjaya et al., 2006), anticancer (Vetrano *et al*., 2005), neuroprotective effects (Ahmadi *et al*., 2021), antibacterial (Qian *et al*., 2019) and anti-inflammatory (Itoh *et al*., 2009). Vanillic acid can be used as a precursor for the synthesis of biobased polymers such as poly(ethylene vanillate) or PEV which exhibited the similar to poly(ethylene terephthalate) or PET (Gioia et al., 2016; Wilsens et al., 2015) and active pharmaceutical ingredients such as etamivan, flecainide, brovanexine, vanitiolide, etc. (Satpute *et al*., 2019). Due to the pharmacological actions of vanillic acid along with its the characteristic vanilla flavor, and industrial applications, these increase the demand for vanillic acid (Fiormarkets report, 2022). The global market size of vanillic acid has been forecasted to reach USD 1189.1 million by 2026 from USD 1239.8 million in 2019 (Memafn report, 2022).

Table 2.2 Chemical and physical properties of vanillic acid (Murga et al., 2004)

Property	Vanillic acid
CAS registry number	121-34-6
Chemical formula	$C_8H_8O_4$
Chemical name	4-hydroxy-3-methoxybenzoic acid
	4-Hydroxy-3-methoxybenzoate
	3-Methoxy-4-hydroxybenzoic acid
	4-Hydroxy-3-methoxy-benzoate
	p-Vanillate
	p-Vanillic acid
Chemical structure	
Molecular weight (g/mol)	168.148
pKa at 25 °C	4.51 (at 25 °C)
Physical appearance	White to light yellow powder or crystals
Melting point	211.5 °C
Boiling point	257.07°C
Solubility in water (g/100 g H_2O)	18.6 mg/mL
	1.5 mg/mL at 14 °C

2.3 Vanillic acid production

2.3.1 The production of vanillic acid by chemical synthesis

The first chemical process of vanillic acid synthesis can be achieved by employing silver oxide method with the oxidation of vanillin (Pearl, 1946). For the silver oxide method, the reaction is started by mixing vanillin, silver oxide and sodium hydroxide at 55°C to produce sodium vanillate (Figure 2.3I). Then, sodium vanillate is hydrolyzed with hydrochloric acid, resulting in vanillic acid (Figure 2.3II). However, there are two limitations from this method: (i) use of high value vanillin as a substrate; and (ii) use of the harsh reaction conditions.

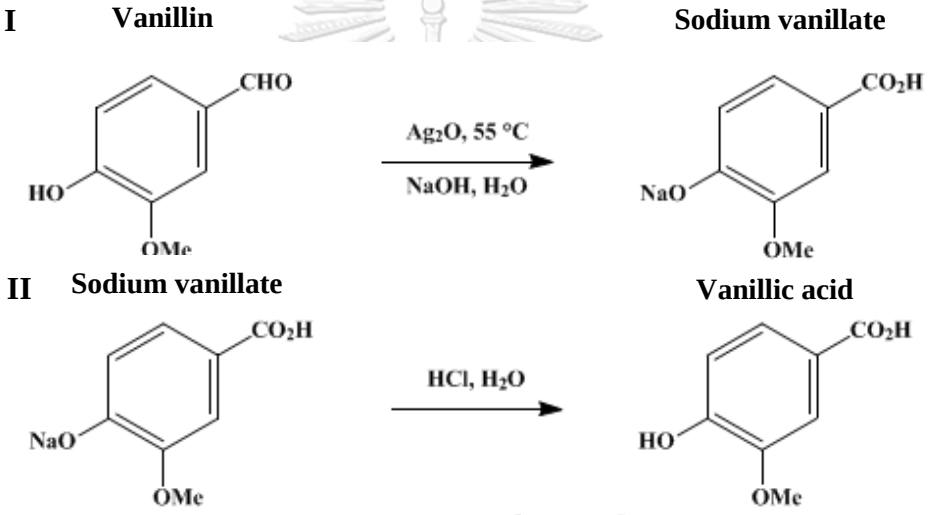
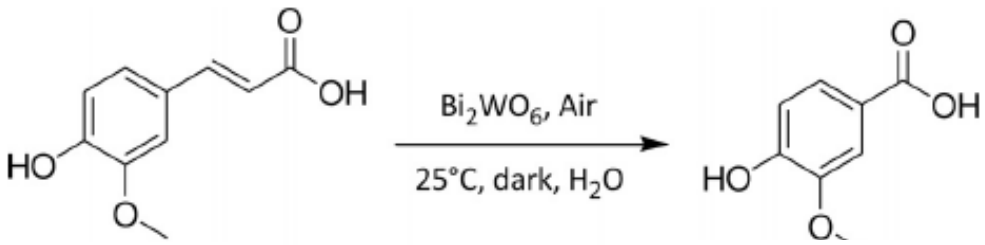


Figure 2.3 The synthesis of vanillic acid from vanillin by silver oxide method (Pearl, 1946)

The other route of chemical synthesis of vanillic acid was reported by Delisi *et al*., 2016, they synthesized vanillic acid in high yield (60%) after 86 h of rection time by air oxidation of ferulic acid in presence of russellite/bismuth tungstate (Bi_2WO_6). Bi_2WO_6 (1.5 g/L) and the aqueous solution of ferulic acid (0.6 mM) were taken in tightly closed tube under stirring in the dark at room temperature (Figure 2.4).



Ferulic acid **Vanillic acid**

Figure 2.4 Production of vanillic acid by catalytic oxidation of ferulic acid in presence of Bi_2WO_6 (Delisi et al., 2016).

2.3.2 The production of vanillic acid via biological synthesis

Vanillic acid was produced from biotechnological approaches, used in production of near natural bio-vanillin.(Barghini et al., 1998; Muheim & Lerch, 1999). When compared with a chemical approach, biological production has the advantage of less harmful to environment, having an impact in the price (Krings and Berger, 1998; Kaur and Chakraborty, 2013). Vanillic acid production by several microorganisms have been reported. *Pseudomonas* group is one of the well-studied microorganisms for vanillic acid production. Zamzuri et al. (2014) reported the bioconversion of ferulic acid to vanillic acid, with the yield of 1.08 mg/mg, by the soil-isolate *Pseudomonas* sp. AZ10 (Zamzuri et al., 2014). Upadhyay et al. (2019) used the engineered *P. putida* KT2440 for producing vanillic acid from corn bran-derived ferulic acid. They knocked out the *van*AB gene encoding for vanillate-O-demethylase that converted vanillic acid to metabolism. They found the resting *P. putida* KT2440 mutant cells resulted in more than 95±1.4% molar yield of vanillic acid production within 24 h. Vanillic acid was obtained from eugenol by using *P. resinovorans* SPR1, 1.1 g/L of vanillic acid (molar yield of 44%) were produced after 60 h biotransformation (Ashengroph et al., 2011). The production of vanillic acid from isoeugenol was reported by (Furukawa et al., 2003), the conversion 98% yield of vanillic acid was obtained by the resting cells of *P. putida* I58 after 40-min incubation. Abdelkafi et al. (2006) synthesized vanillic acid from

ferulic acid by *Halomonas elongata* strain Mar, the production of vanillic acid (with 86% yield) was obtained within 6 h using 5 mM of ferulic acid as a substrate (Abdelkafi et al., 2006). The vanillic acid of 2.2 g/L or 95% yield was obtained from 2 g/L of vanillin by *Ochrobactrum anthropic (Girawale et al., 2022).* By using *Streptomyces sannanensis* MTCC 6637 which possesses CoA-dependent vanillic acid production, the 48% of vanillic acid yield (0.49 g/L) was obtained from 1 g/L (5 mM) of ferulic acid in 20 days (Ghosh et al., 2007). The resting cells of *Halomonas elongata* DSM 2581, under hypersaline conditions, produced 0.7 g/L (3.95 mM) or 40% yield of vanillic acid from 1.94 g/L of ferulic acid within 10 hours (Abdelkafi et al., 2008).

2.4 Bacterial membrane transporter for uptake of lignin-derived aromatic compounds

The porins and channels for uptake lignin-derived aromatic compounds were investigated in the outer membrane (OM) of gram-negative bacteria. The substrate-specific channel OpdK is involved in the transport of vanillate/vanillin across the outer membrane in *Pseudomonas aeruginosa* (Biswas et al., 2008). The benzoate-specific porin (BenF, PP_1383) was asscociated with ferulic acid uptake in *P. putida* KT2440 (D'Arrigo et al., 2019).

The inner membrane transport of lignin derived monomers in bacteria consist of major facilitator superfamily (MFS) transporter, ATP-binding cassette (ABC) transporter, and tripartite ATP-independent periplasmic transporter (TRAP-T).

The MFS comprise the largest superfamily of secondary active transporters in all organisms. The MFS transporters are categorized into the aromatic acid/H+ symporter (AAHS) family and metabolite/H+ symporter (MHS) family. PcaK transporter (AAHS family), a 4-hydroxybenzoate permease in *P. putida* PRS2000, uptakes protocatechuate and 4-hydroxybenzoate into the cells (Nichols & Harwood, 1997);(Harwood et al., 1994). The inhibition of PcaK in *P. putida* PRS2000 affected this strain could not uptake and grown on protocatechuate and 4-hydroxybenzoate, this indicating that PcaK is the primary

transporter of these substrates (Harwood et al., 1994). The PcaK homologs have been reported in several bacterial strains, these include PcaK permeases in *Acinetobacter baylyi* ADP1 (Pernstich et al., 2014), *Corynebacterium glutamicum* (Chaudhry et al., 2007), *Pseudomonas putida* KT2440 (Wada et al., 2021), and *Sphingobium* SYK-6 (Mori et al., 2018). The reconstituted PcaK of *Acinetobacter baylyi* ADP1 in proteoliposome was shown to uptake of vanillate, protocatechuate, salicylate, 3-hydroxybenzoate (3-HBA) and 2,4-dihydroxybenzoate (2,4-DHB) (Pernstich et al., 2014). CgVanK is exclusive for vanillate transport in *C. glutamicum* ATCC 13032 (Chaudhry et al., 2007). PcaK and VanK of *Sphingobium* SYK-6 have been shown to import vanillate and protocatechuate (Mori et al., 2018). The PcaT metabolite/H+ symporter (MHS family) was associated in the uptake of ferulate in *P. putida* KT2440 (D'Arrigo et al., 2019).

An ABC transporter (CouPSTU) and a TRAP transporter (TarPQM) in *Rhodopseudomonas palustris* were identified that showed high-affinity binding of CouP to lignin-derived phenylpropanoids (coumarate, ferulate, caffeate, and cinnamate) (Bisson et al., 2022);(Salmon et al., 2013).

2.5 Vanillic acid and vanillin production pathway in native and engineered bacteria

Several microbial pathways for vanillic acid and vanillin production have been reported as shown in Figure 2.5. The well-studied and most reported pathway for ferulic acid conversion to vanillic acid is via CoA-dependent pathway which is reported in *Amycolatopsis sp*. HR167, *Streptomyces sp*. strain V-1, *Pseudomonas* strains and the engineered *E. coli* (Overhage et al. 1999, Achterholt, et al. 2000, Yoon et al. 2005, Plaggenborg et al. 2006, Barghini et al. 2007, Yang et al. 2013). In this coenzyme A-dependent, non-β-oxidative pathway, the two critical enzymes i.e. feruloyl-CoA synthetase and enoyl-CoA hydratase/aldolase are encoded by *fcs* and *ech*, respectively (Figure 5A). Firstly, the activation of ferulic acid to feruloyl-CoA is catalyzed by Fcs. Feruloyl-CoA is then hydrated and cleaved,

obtaining vanillin and acetyl-CoA. Vanillin was converted to vanillic acid by vanillin dehydrogenase (vdh). Owing to this pathway being well-studied, the CoA-dependent pathway are highly employed in the microbial metabolic engineering for vanillic acid production (Yoon et al., 2005, Barghini et al., 2007, Chakraborty et al., 2017, Jang et al., 2017). However, one drawback of this pathway is the requirement of ATP and CoA which may increase the cost of vanillic acid bioproduction (Furuya et al. 2014). Lee et al (2009) attempted to enhance vanillin production by introducing gltA encoding citrate synthase of which regenerate CoA from Acetyl CoA, favoring vanillin production (Lee et al. 2009).

Ni et al. (2018) constructed a coenzyme-independent pathway for vanillin production (Ni et al., 2018). The two-steps reaction consists of carboxylase and oxygenase. In this pathway, phenolic acid decarboxylase encoded by Pad rapidly convert ferulic acid to 4-vinylguaiacol of which further converted to vanillin by aromatic dioxygenase (Ado) (Ni et al. 2018).

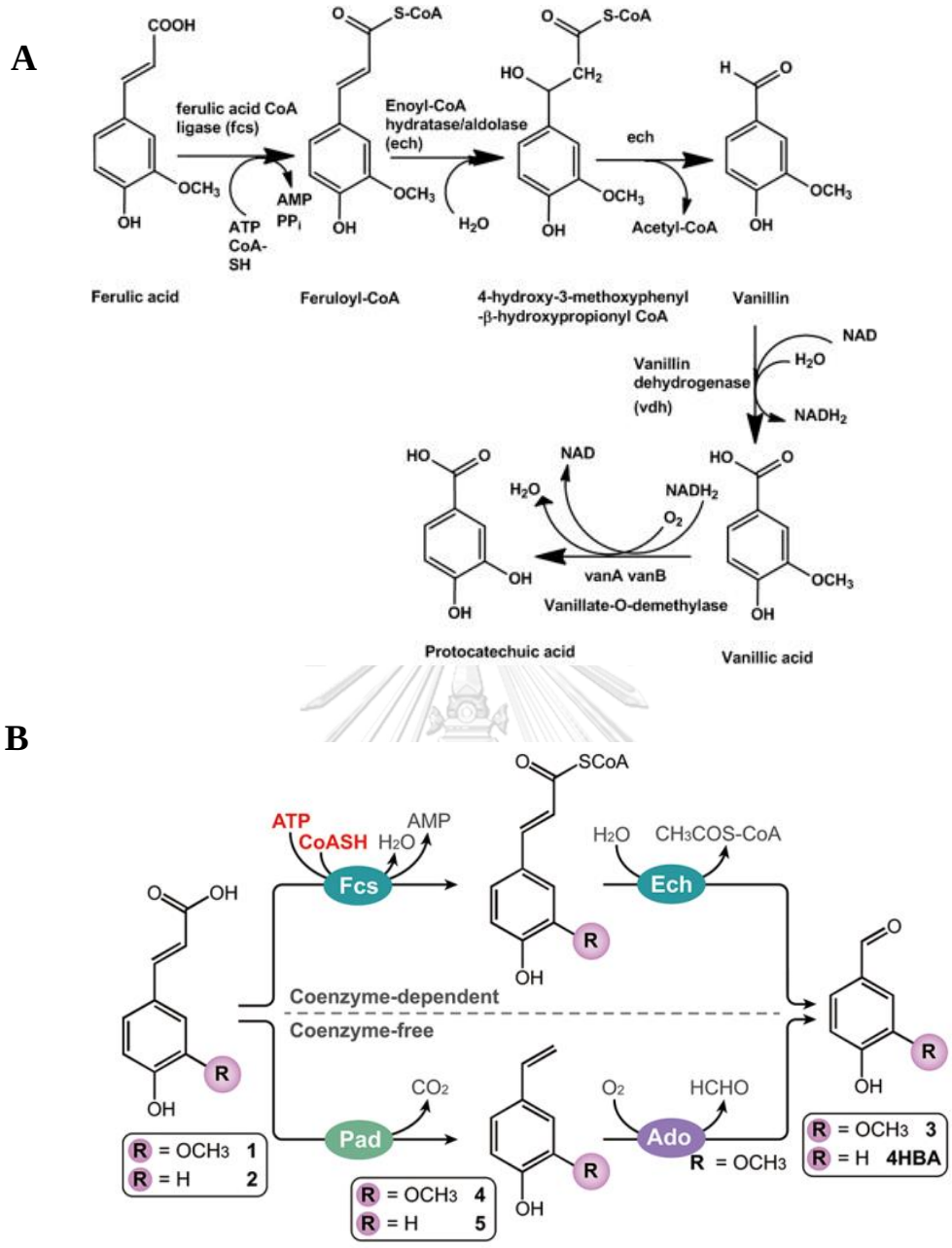


Figure 2.5 The major pathways for vanillin and vanillic acid production from ferulic acid. (A) CoA-dependent pathway from *Amycolatopsis sp.* 39116 (Gallage and Moller, 2015) and (B) CoA-dependent pathway and CoA-independent pathway from engineered *E. coli* BL21(DE3) (Furuya et al., 2014; Ni et al. 2018).

4HBA = 4-hydroxy benzaldehyde, (1) = ferulic acid, (2) = *p*-coumaric acid, (3) = vanillin, (4) = 4-vinylguaiacol, and (5) = 4-vinylphenol.

Table 2.3 Biosynthesis of vanillic acid from different phenolic compounds

Microorganism used	Substrate	Substrate concentrations (g/L)	Vanillic acid (g/L)	Reaction time	Yield (%)	Reference
Streptomyces setonii	Ferulic acid	8	0.2	26 h	2.4	(Muheim & Lerch, 1999)
Psychrobacter sp. Strain CSW4	Isoeugenol	1	0.128	30 h	13	(Ashengroph et al., 2012)
Recombinant *P. fluorescens* BF13 via random transposon mutagenesis (Tn*Mod*-Okm)	Ferulic acid	1	0.112	24 h.	16	(Civolani et al., 2000)
Amycolatopsis HR167	Ferulic acid	1	0.29	4.5 h.	29	(Achterholt et al., 2000)
Trichosporon asahii MP24	Isoeugenol	5	1.8	48 h	36	(Ashengroph & Amini, 2017)
First ferulic acid addition to *Aspergillus niger* K8 and its supernatant to *Phanerochaete*	Ferulic acid	0.3	0.117	36 h.	37	(Motedayen et al., 2013)
Halomonas Elongata DSM 2581	Ferulic acid	1.94	0.66	10 h	40	(Abdelkafi et al., 2008)
Pseudomonas resinovorans SPR1	Eugenol	2.5	1.1	60 h	44	(Ashengroph et al., 2011)

Table 2.3 (Continue)

Streptomyces sannanensis MTCC 6637	Ferulic acid	0.97	0.4	20 days	48	(Ghosh et al., 2007)
Paenibacillus lactis SAMS-2001	Ferulic acid	0.485	0.241	18 h	57	(Mishra et al., 2016)
Bacillus Licheniformis SHL1	Ferulic acid	1	0.494	45 h	60	(Ashengroph et al., 2012b)
Streptomyces halstedii	Ferulic acid	1	0.8	24 h	80	(Brunati et al., 2004)
Halomonas elongata strain Mar	Ferulic acid	0.97	0.722	6 h	86	(Abdelkafi et al., 2006)
Ochrobactrum anthropi	Vanillin	2	2.2	7 h	95	(Girawale et al., 2022)
Recombinant *P. fluorescens* KT2440 knocking out *van*AB gene encoding vanillate-O-demethylase	Ferulic acid	2	1.9	22 h	95	(Upadhyay et al., 2020)
Streptomyces viridosporus T7A.	Vanillin	1.5	1.6	48 h	96	(Pometto & Crawford, 1983)
Serratia marcescens	Vanillin	2	2.16	20 h	100	(Perestelo et al., 1989)

CHAPTER III

MATERIAL AND METHOD

3.1 Laboratory equipment and chemicals

3.1.1 Laboratory equipments

Laboratory equipments	Company	Country
Autoclave, NLS-3020	Sanyo Electric Co,.LTD	Japan
Autoclave, HV-110	Hiramaya	Japan
Centrifuge, Prism R	Labnet	USA
Fermenter 5-L, MDFT-500	Marubishi	Japan
High Performance Liquid Chromotography (HPLC), LC-20	Shimazu	Japan
Hot plate stirrer	Lab Tech	Korea
Incubator shaker, innova 4000	New Brunswick scientific	USA
Incubator shaker, innova 4340	New Brunswick scientific	USA
Laminar flow cabinet	Biobase	China
Micropipette 10, 20, 200, 1000 uL	Gilson	France
pH meter	Mettler Toledo	USA
Refrigerated centrifuge, 5804R	Eppendorf	USA
Refrigerated centrifuge, combi514R	Hanil Scientific Inc.	South Korea
Synergi 4 µm Hydro-RP 80 Å, LC Column 50 x 1 mm	Phenomenax	USA
UV-visible spectrophotometer, biomate 3S	Thermo Scientific	USA
Ultra-low temperature freezer	New Brunswick scientific	USA

3.1.2 Laboratory chemicals

(1) Chemicals

Chemicals	**Supplier**	**Country**	**CAS number**
Ammonium chloride, NH_4Cl	Merck	Germany	12125-02-9
Apocynol	TCI	Japan	2480-86-6
Copper sulfate pentahydrate, $CuSO_4.5H_2O$	Scharlau	Spain	7758-99-8
trans-Ferulic acid	TCI	Japan	537-98-4
Ferrous sulfate / Iron (II) sulfate	Carlo Erba Reagent	Italy	7782-63-0
Glucose	Ajex Finechem	Australia	50-99-7
Glycerol	Univar	Australia	56-81-5
Isopropyl-ß-D-1-thiogalactopyanoside, IPTG	Thermo Fisher Scientific	USA	367-93-1
Lactose	Krungtepchemi	Thailand	5989-81-1
Magnesiun sulfate, MgSO4	Ajex Finechem	Australia	7487-88-9
Poldimethylsiloxane emulsion, antifoam SAG4701	Krungtepchemi	Thailand	75-07-0, 140-88-5
di-Potassium hydrogen phosphate, K_2HPO_4	Ajex Finechem	Australia	7758-11-4
Potassium di-hydrogen phosphate, KH_2PO_4	Ajex Finechem	Australia	7778-77-0
Sodium chloride, NaCl	Ajex Finechem	Australia	7440-23-5
Sodium hydroxide, NaOH	Ajex Finechem	Australia	1310-73-2
Tryptone	Himedia	India	91079-40-2
Vanillin	TCI	Japan	121-33-5
Vanillic acid	TCI	Japan	121-37-6

(1) Chemicals (continue)

Chemicals	Supplier	Country	CAS number
Vanillyl alcohol	TCI	Japan	498-00-0
4-Vinylguaiacol	Sigma-aldrich	USA	7786-61-0
Yeast extract	Scharlau	Spain	8013-01-2

(2) Analytical grade solvents

Solvents	Supplier	Country	CAS number
Ethanol absolute (≥ 99% purity)	Merck	Germany	64-17-5
Phosphoric acid (85% w/w)	Kemaus	Australia	7664-38-2
Hydrochloric acid (37% w/w)	Merck	Germany	7647-01-0

(3) HPLC grade organics solvents

Organics solvents	Supplier	Country	CAS number
Acetonitrile (99% purity)	Burdick & Jackson	USA	75-05-8
Methanol (99% purity)	Burdick & Jackson	USA	67-56-1

3.2 Strains, plasmid, and media

The recombinant *E. coli* strain BL21(DE3) harboring pETDuet-*padC-vdh* and pCDFDuet-(*ado* or *cypd*) used in this study was obtained from the culture collection of Biocatalyst and Environmental Biotechnology Research Unit, Department of Biochemistry, Faculty of Science, Chulalongkorn University.

Caffeic acid 3-*O*-Methyltransferase, *comt* gene from *Arabidobsis thalina* (Genbank accession numbers AY062837.1) was codon optimized for *Escherichia coli* (Rodrigues *et al*.,

2020) and synthesized in a pRSFDuet-1 plasmid (Novagen, USA) which equipped with an N-terminal 6xHis tag by IDTDNA. The pRSFDuet-*comt* plasmid was transformed into *E. coli* BL21(DE3) (Novagen, USA) by electroporation.

The recombinant *E. coli* BL21(DE3) were grown in LB medium agar (Appendix A) supplemented with antibiotics depend on the plasmid(s) present in the strains, 50 mg/mL of streptomycin for pCDFDuet-1, 100 mg/mL of ampicillin for pETDuet-1 and/or 50 mg/mL of kanamycin for pRSFDuet-1 (the optimum of antibiotic concentration for stability of plasmid) at 37 °C for 16 h and then maintained at 4 °C. The strain was sub-cultured at monthly interval.

3.3 The production of vanillic acid using resting cells in shake flask scale

3.3.1 Batch bioconversion

3.3.1.1 Effect of initial substrate concentration and pH on vanillic acid production

(1) Preparation of whole cells

An overnight culture (1%, v/v) of the recombinant strain *E. coli* BL21(DE3) harboring plasmid pETDuet-*padC-vdh* and pCDFDuet-*ado* was inoculated into 250 mL of LB medium supplemented with 50 µg/mL streptomycin and 100 µg/mL ampicillin in 1 L-baffled Erlenmeyer flask. Cells were cultured at 37 °C and shaking condition 200 rpm. After OD600 of cultures reached 0.6, 0.2 mM of IPTG and 1 mM of $FeSO_4$ were added to the culture. The cultures were further incubated at 16 °C, 125 rpm for 16 h. Then, the cells were centrifuged at 6000 rpm for 10 min, and washed twice with sterilized 0.85% (w/v) NaCl and resuspended in Tris-HCl (100 mM, pH 8.0), Glycine-NaOH (100 mM, pH 9.0), or Glycine-NaOH buffer (100 mM, pH 10.0) to obtain OD_{600} = 40 (equivalent to 17.6 g/L or 10^{12} CFU/mL). These cells were used in batch and fed-batch bioconversion.

(2) Production of vanillic acid by *E. coli* resting cells

The influence of initial ferulic acid concentrations and pH were performed in batch bioconversion. Various initial concentrations of ferulic acid ranging from 1.0, 5.0, 10.0 to 20.0 mM were added into the cell suspension with OD_{600} = 40 in buffer pH 8.0, 9.0, or 10.0. The bioconversion was conducted at 37 °C, 200 rpm. At 0, 1, 6, 12, 24, and 48 h, the sample was collected to investigate vanillic acid production by using HPLC and the viability of the recombinant *E. coli* cells using plate count technique. The culture without substrate inoculation were used as control.

3.3.2 Fed-batch bioconversion

3.3.2.1 Effect of pH on vanillic acid production

The effect of initial ferulic acid concentrations was studied in fed-batch bioconversion**.** The initial concentration of 1.0 mM ferulic acid were added every 12 h into the cell suspension with OD_{600} = 40 in buffer pH 8.0, 9.0, or 10.0. The bioconversion was conducted at 37 °C, 200 rpm. At 0, 1, 6, 12, 24, and 48 h, the sample was collected to investigate vanillic acid production by using HPLC and the viability of the recombinant *E. coli* cells using plate count technique. The culture without substrate inoculation were used as control.

3.4 Vanillic acid production by a two-step bioprocess

3.4.1 First stage: bioconversion of caffeic acid to ferulic acid by *E. coli* resting cells

(1) Preparation of whole cells

An overnight culture (1%, v/v) of the recombinant strain *E. coli* BL21(DE3) harboring plasmid pRSFDuet-*comt* was inoculated into 250 mL of LB medium supplemented with 50 μg/mL kanamycin in 1 L-baffled Erlenmeyer flask. Cells were cultured at 37 °C and shaking condition 200 rpm. After OD_{600} of cultures reached 0.8, 0.1 mM of IPTG was added

to the culture. The cultures were further incubated at 25 °C, 125 rpm for 20 h. Then, the cells culture were centrifuged at 6000 rpm for 10 min, and washed twice with sterilized 0.85% NaCl and resuspended in M9 medium (Appendix B) pH 7.5 to obtain OD_{600} = 40 (equivalent to 18 g CDW/L).

(2) Production of ferulic acid from caffeic acid

Bioconversion of caffeic acid to ferulic acid was performed in batch and fed-batch bioconversion. Two mM of ferulic acid and L-methionine were added into the cell suspension with OD_{600} = 40 in M9 medium pH 7.5. The bioconversion was conducted at 26 °C, 200 rpm. At 0, 3, 6, 12, 24, and 36 h, the sample was collected to investigate vanillic acid production by using HPLC. Then, the cells culture were centrifuged at 20 °C, 5000 rpm for 10 min. The supernatant was filtered through a 0.22-μm filter and maintained on 4 °C for the second stage reaction.

3.4.2 Second stage: bioconversion of ferulic acid to vanillic acid by *E. coli* resting cells

(1) Preparation of whole cells

An overnight culture (1%, v/v) of the recombinant strain *E. coli* BL21(DE3) harboring plasmid pETDuet-*padC-vdh* and pCDFDuet-*(ado or cypd)* was inoculated into 250 mL of LB medium supplemented with 50 μg/mL streptomycin and 100 μg/mL ampicillin in 1 L-baffled Erlenmeyer flask. Cells were cultured at 37 °C and shaking condition 200 rpm. After OD600 of cultures reached 0.6 (*ado* gene) and 0.8 (*cypd* gene), 0.2 mM of IPTG, 1 mM of $FeSO_4$ (cofactor for *ado* gene), and 0.5 mM 5-aminolevunilic acid (cofactor for *cypD* gene) were added to the culture. The cultures were further incubated at 25 °C, 125 rpm for 20 h. Then, the cells culture were centrifuged at 6000 rpm for 10 min, and washed twice with sterilized 0.85% NaCl and resuspended in Tris-HCl buffer (100 mM, pH 8.0) to obtain OD_{600} = 40 (equivalent to 17 g CDW/L).

(2) Production of vanillic acid from ferulic acid prepared by the first-stage reaction.

Bioconversion of ferulic acid to vanillic acid was performed in batch bioconversion. One mM of ferulic acid prepared by the first-stage reaction was added into the cell suspension with OD_{600} = 40 in Tris-HCl buffer (100 mM, pH 8.0). The bioconversion was conducted at 37 °C, 200 rpm. At 0, 1, 6, 12, 24, and 36 h, the sample was collected to investigate vanillic acid production by using HPLC.

3.5 The production of vanillic acid using growing cells in shake flask scale

3.5.1 Batch bioconversion

3.5.1.1 Effect of IPTG concentrations, carbon source, medium, and type of inducer on vanillic acid production

(1) Preparation of bacteria inoculums

An overnight culture (1%, v/v) of the recombinant strain *E. coli* BL21(DE3) harboring plasmid pETDuet-*padC-vdh* and pCDFDuet-*ado* was inoculated into 50 mL of fermentation medium supplemented with 50 μg/mL streptomycin and 100 μg/mL ampicillin in 250 mL-baffled Erlenmeyer flask. Cells were cultured at 37 °C and shaking condition 200 rpm. After OD600 of cultures reached 0.6, 0.2 mM of IPTG and 1 mM of $FeSO_4$ were added to the culture. The cultures were further incubated at 16 °C, 125 rpm for 16 h.

(2) Production of vanillic acid by *E. coli* growing cells

To investigate the influence of IPTG concentrations, carbon source, medium, and type of inducer on the bioconversion of ferulic acid to vanillic acid by the recombinant *E. coli* BL21 (DE3), different conditions of IPTG (0.2, 0.4, and 1.2 mM), glucose or glycerol (1, 5, 10, and 20 g/L), medium (LB, 2XLB, and formulated medium), and inducer (0.2 mM IPTG; 5, 10 g/L lactose; 5,10 g/L galactose) were evaluated in this study. The 1 mM ferulic acid was added to culture. The bioconversion was conducted at 37 °C, 200 rpm. At 0, 1, 6, 12, 24, and 48 h, the sample was collected to investigate vanillic acid production by using HPLC.

The cell growth (OD_{600}) was determined by using an UV-Visible Spectrophotometer (BioMate™ 3S, USA).

3.6 Vanillic acid production by *E. coli* growing cells in 5-L bioreactor

3.6.1 Effect of inducer type, inoculum percentage on vanillic acid production

The overnight culture (1, 5, and 10 % (v/v)) of the recombinant strain *E. coli* BL21(DE3) harboring plasmid pETDuet-*padC-vdh* and pCDFDuet-*ado* was inoculated into of LB medium supplemented with 50 μg/mL streptomycin and 100 μg/mL ampicillin in 250 mL-baffled Erlenmeyer flask. Cells were cultured at 37 °C and shaking condition 200 rpm for 16 h. The influences of various inoculum volumes (1, 5, and 10% (v/v)) were evaluated. The cultures were inoculated into the 5-L fermenter containing 3-L of formulated medium pH 8.0 supplemented with 1 g/L glycerol. The bioconversion was conducted at 37 °C and 200 rpm with 1 L/min aeration rate. After OD_{600} of cultures reached 2.5 (mid log phase), different types of inducers (0.2 mM IPTG, 5, 10 g/L glucose and galactose) and 1 mM of $FeSO_4$ were added to the culture. 1 mM ferulic acid (0.2 g/L) was added after 16 h of induction. The culture sample was collected to investigate vanillic acid production by using HPLC, cell growth, glucose consumption. All experiment set-ups were performed in triplicates, and the average values were reported.

3.7 Analytical procedure

3.7.1 Cell growth determination

The cell growth was determined by an UV-Visible Spectrophotometer at wavelength 600 nm and determination of the cell dry weight (CDW). One mL of cultivation broth was collected and centrifuged in pre-dried and pre-weighted 1.5-mL microtubes at 10,000 rpm for 10 min. The cell was washed with 0.8 mL sterilized 0.85% NaCl, then centrifuged at 10,000 rpm for 10 min. The saline was removed, and the pellet was dried for 24 h at 60 °C and weighed with a precision balance.

3.7.2 Substrate and product analysis

High performance liquid chromatography (HPLC) was used to analyze the amount of substrate, product, and by-products present in the medium broth. The samples from medium broth were centrifuged at 10,000 rpm for 10 min and the supernatant was filtered using a 0.2 μm syringe filter. Production of vanillic acid and by-products were analyzed by using HPLC (Shimazu SPD-20A, Japan) with Synergi Hydro-RP 80A column (4.6 mm×250 mm) and UV-VIS detector (SPD-20A). The mobile phase consisted of mobile phase A (0.1% (v/v) phosphoric acid in UP water) and mobile phase B (acetonitrile). The column temperature was maintained at 40 °C and a 0.6 mL/min flow rate. The gradient elution programs were as follows: (i) equilibrate the column with 75% mobile phase A and 25% mobile phase B for 10 min, (ii) run gradient from 25 to 75% mobile phase B for 5 min, (iii) run gradient from 75 to 95% mobile phase B for 5 min, (iv) run gradient from 95 to 75% mobile phase B for 5 min, and (v) wash with 25% mobile phase B for 15 min. Production of vanillic acid and other aromatics were monitored by measuring the absorbance at 200 and 310 nm.

3.7.3 The viability of *E. coli* cells determination

Colony Forming Unit (CFU) was used for determining the viability of *E. coli* cells on toxicity of ferulic acid. Twelve-fold serial dilution of bacteria was prepared by diluting the samples from the culture medium with sterilized 0.85% NaCl. The serial dilution of cell suspension 1 mL was dropped on LB agar plates. The plates were incubated at 37°C. Number of bacterial colonies on the agar plate was accounted for the viability of *E. coli* cell on toxicity of ferulic acid.

3.7.4 Statistical analysis

All statistical analyses were performed in Microsoft Excel. Experimental data were expressed as mean ± standard deviation (SD). Statistical significance was set at $p < 0.05$.

CHAPTER IV

RESULTS AND DISCUSSION

Conceptual framework of results and discussion

The results and discussion of this thesis are presented in order as shown in the following flow chart.

4.1 Construction of the recombinant *E. coli* strain BL21(DE3) for vanillic acid production using caffeic acid as substrate

4.2 Optimization of vanillic acid production using the recombinant *E. coli* strain BL21(DE3) in shake flask scale

4.2.1 Growth-independent production (resting cell)

- Batch bioconversion

4.2.1.1 Effect of ferulic acid (FA) concentrations and pH

4.2.1.2 Toxicity test of FA concentrations and pH

Fed-batch bioconversion

4.2.1.3 Effect of pH

Toxicity test of pH

4.2.1.4 A two-step bioprocess

4.2.2 Growth-dependent production (growing cell)

- Batch bioconversion

4.2.2.1 Effect of IPTG concentrations

4.2.2.2 Effect of carbon source

4.2.2.3 Effect of medium

4.2.2.4 Effect of inducer

4.3 Optimization of vanillic acid production using the recombinant *E. coli* strain BL21(DE3) in 5-L bioreactor

4.3.1 Growth-dependent production (growing cell)

4.3.1.1 Effect of inducer

4.3.1.2 Effect of inoculum size

4.1 Construction of the recombinant *E. coli* strain BL21(DE3) for vanillic acid production using caffeic acid as substrate

The *comt* gene encoding caffeic acid 3-*O*-methyltransferase from *Arabidobsis thaliana* (Genbank accession numbers AY062837.1) was codon-optimized for *E. coli* by Rodrigues et *al*. (2020). The synthesized gene was incorporated into the pRSFDuet-1 plasmid (Novagen, USA), resulting in pRSFDuet-*comt* (Figure 4.1). The pRSFDuet-*comt* plasmid was transformed into *E. coli* BL21(DE3) (Novagen, USA) by electroporation.

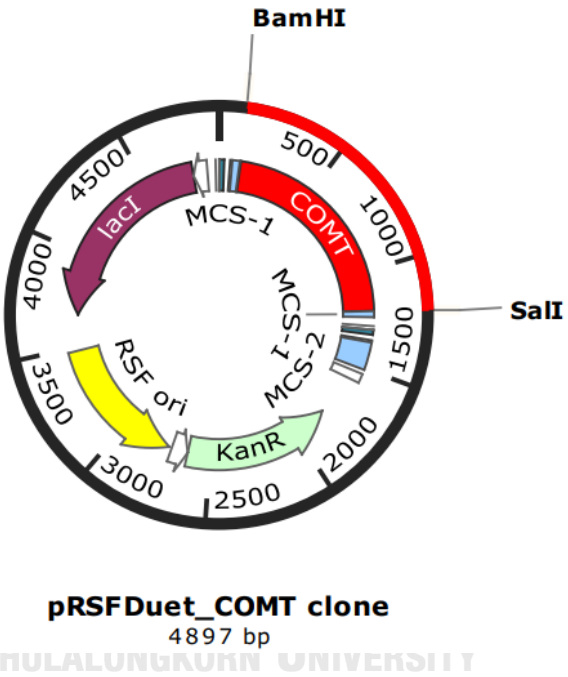


Figure 4.1 pRSFDuet plasmid containing comt gene

4.2 Optimization of vanillic acid production using the recombinant *E. coli* BL21(DE3) in shake flask scale

4.2.1 The production of vanillic acid using resting cells

4.2.1.1 Effect of initial substrate concentration and pH on batch vanillic acid production

In this part, the effects of two important parameters i.e. initial substrate concentration and pH on the growth-independent bioconversion of ferulic acid to vanillic acid were investigated using the recombinant *E. coli* strain BL21(DE3) co-harboring pETDuet-*padc*-*vdh* and pCDFDuet-*ado*. The tested initial concentrations of ferulic acid were 1, 5, 10, and 20 mM, whereas the pH of the bioconversion medium was tested at pH 8, 9, and 10.

The synthesis pathway of vanillic production showed as follows: firstly, the decarboxylation of ferulic acid leads to the formation of 4-vinylguaiacol, which catalyzed by phenolic acid decarboxylase (PadC). 4-Vinylguaiacol is converted to vanillin by aromatic dioxygenase (Ado). Vanillin is further oxidized to vanillic acid by vanillin dehydrogenase (Vdh). Upon the addition of ferulic acid, it was rapidly converted to 4-vinylguaiacol due to the high activity of the overexpressed phenolic acid decarboxylase. At the initial concentration of 1 and 5 mM, ferulic acid was completely converted to 4-vinylguaiacol within 1 hour (Figure 4.2A, 4.2B). As shown in Table 4.1 and Figure 4.2, the vanillic acid yields depend on the initial concentration of ferulic acid. Regardless of pH, the increasing ferulic acid concentration resulted in the decreasing vanillic acid yield. At 1 mM ferulic acid, 4-vinylguaiacol was almost completely consumed within 6 hours and the obtained vanillic acid yields were 62% - 96%. At the higher ferulic acid concentration of 10 and 20 mM, 4-vinylguaiacol accumulated before being gradually converted to vanillic acid within 24 – 48 hours (Figure 4.2C and 4.2D). The vanillic acid yields decreased to 9% - 16% when the ferulic acid concentration increased to 20 mM. Due to the high cytoxicity of 4-vinylguaicol (L. Li et al., 2019), the accumulation of 4-vinylguaiacol in *E. coli* affected to cell death during

the bioconversion (Figure 4.3). The addition of 10 mM 4-vinylguaiacol decreased the activity of phenolic acid decarboxylase and bacterial cells by 80% and 60%, respectively (Li et al., 2019). The property of 4-vinylguaiacol is a hydrophobic compound, it has strong affinity to cell membrane and readily transport into the cells, these cause *E. coli* cells to be easily lysed and making the cells unrecyclable (Jung et al., 2013; Luo et al., 2021).

Apocynol is a vanilla-like aromatic compound and an unwanted by-product of the abiotic oxidation/hydration of 4-vinylguaiacol (Vanbeneden et al., 2008). Accordingly, Kotchaplai et al. (2022) detected the high formation of apocynol at pH 7 during the production of vanillin, the production of apocynol affected the decrease of vanillin production (Kotchaplai et al., 2022). Therefore, pH 8, 9, and 10 of bioconversion medium be used to investigate for reduce the formation of apocynol. At the initial ferulic acid of 1 mM, the production of vanillic acid decreased from 96% at pH 8 to 83% and 62% at pH 9 and 10, respectively (Table 4.1). The reported optimum pH for the activity of PadC, Ado and Vdh were 5, 9 and 8, respectively (Cavin et al., 1998; Mitsui et al., 2010; Ni et al., 2018). The activity of PadC decreased by more than 90% at pH 9 (Cavin et al., 1998). The activity of Ado decreased by approximately 40% at pH 10 (Ni et al., 2018). At pH 10, the activity and stability of vanillin dehydrogenase from *Burkholderia cepacia* decreased by approximately 20% and 40%, respectively (Mitsui et al., 2010). Similar results were observed by Yan et al. (2016), pH higher than 8.5 adversely affected the production of vanillin which is a precursor for producing vanillic acid by reducing the activity of enzymes involved in bioconversion (Yan et al., 2016). The pH-dependent vanillic acid yield was less observed at the high initial concentration of ferulic acid (Figure 4.2D).

Table 4.1 Vanillic acid production in presence of various pH and ferulic acid concentrations in batch bioconversion.

Medium	pH	Ferulic acid concentration (mM)	Production of vanillic acid (mM)	Productivity (mg/L.h)	%Molar of vanillic acid yield
100 mM Tris-HCl	8.0	1	0.92 ± 0.02	3.22 ± 0.07	95.8 ± 2.10
		5	3.15 ± 0.02	11.0 ± 0.10	65.1 ± 0.40
		10	4.92 ± 0.01	17.2 ± 0.50	57.7 ± 0.10
		20	1.87 ± 0.02	6.55 ± 0.70	10.9 ± 0.10
100 mM Glycine-NaOH	9.0	1	0.79 ± 0.02	2.77 ± 0.07	83.2 ± 2.10
		5	3.40 ± 0.01	11.9 ± 0.00	71.7 ± 0.20
		10	2.01 ± 0.01	7.04 ± 0.04	23.2 ± 0.20
		20	1.68 ± 0.01	5.88 ± 0.04	9.49 ± 0.06
100 mM Glycine-NaOH	10.0	1	0.58 ± 0.02	2.03 ± 0.07	61.7 ± 1.90
		5	2.53 ± 0.04	8.86 ± 0.04	57.9 ± 0.90
		10	3.24 ± 0.01	11.4 ± 0.00	42.4 ± 0.10
		20	2.79 ± 0.01	9.77 ± 0.04	16.3 ± 0.00

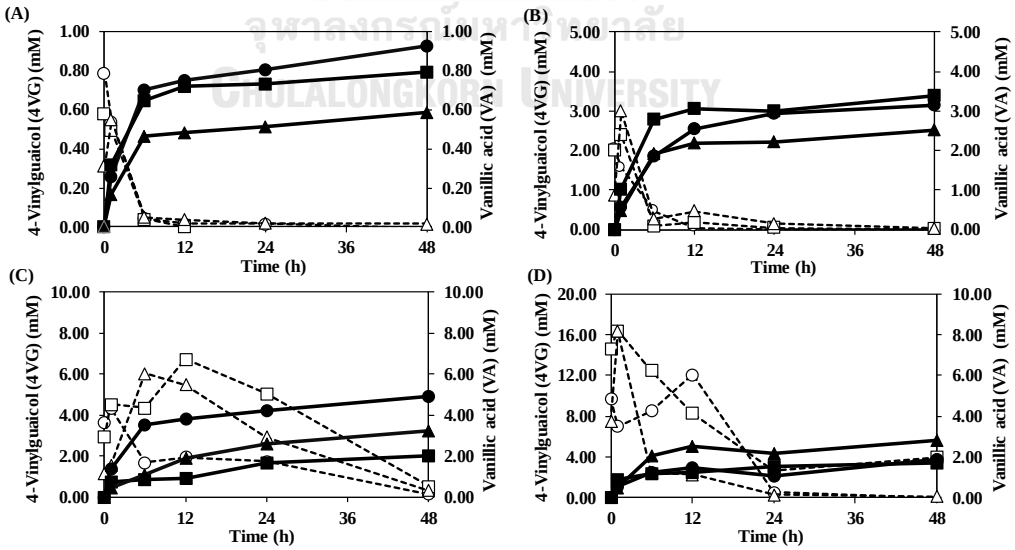


Figure 4.2 The effect of pH and ferulic acid concentration on vanillic acid production. Initial ferulic acid concentration of (A) 1 mM, (B) 5 mM, (C) 10 mM, and (D) 20 mM was used.

pH of (●) 8, (■) 9, and (▲) 10 was studied. The production was conducted using an initial OD_{600} of 40 at 37 °C, 200 rpm. The close symbol represents vanillic acid production and the open symbol represents 4-vinylguaiacol concentration. Due to the rapid conversion of ferulic acid to 4-vinylguaiacol within 1 hour, no ferulic acid was shown in the graphs.

4.2.1.2 Toxicity of ferulic acid concentration on the recombinant *E. coli* strain BL21(DE3)

As shown in Figure 4.2C and 4.2D, when the initial concentration of ferulic acid increased, vanillic acid production decreased. This is possibly due to the toxicity of 4-vinylguaicol remaining in the medium (Li et al., 2019). The concentration- and pH-dependent toxicity of ferulic acid to cells was shown in Figure 4.3. Exposure to 20 mM of ferulic acid completely inactivated the recombinant *E. coli* strain BL21(DE3) within 6 hours, regardless of pH. According to Chen et al. 2016, the higher ferulic acid concentration (more than 4 mM, 1.5 g/L) adversely affected *B. subtilis* cells, resulting in lower product achievement. (Chen et al., 2016). Considering its toxicity, 1 mM ferulic acid and pH 8 appears to be the most suitable condition for vanillic acid production by using the recombinant *E. coli* harboring pETDuet-*padC-vdh* and pCDFDuet-*ado*.

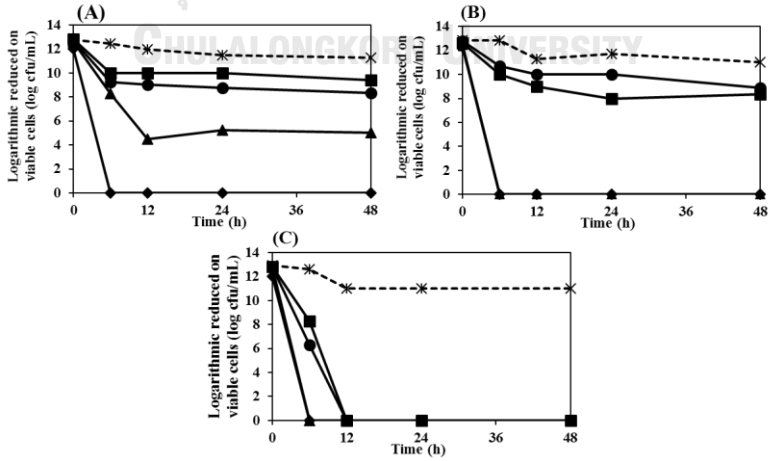


Figure 4.3 Toxicity of ferulic acid concentration on the recombinant E. coli strain BL21(DE3) at (A) pH 8, (B) pH 9 and (C) pH 10 in batch bioconversion. Cells with an initial concentration of OD_{600} = 40 (10^{12} CFU/mL) were exposed to (●) 1 mM, (■) 5 mM, (▲) 10

mM, and (◆) 20 mM of ferulic acid. The culture without supplemented ferulic acid (control) was indicated by (✳).

4.2.1.3 The effect of different pH and toxicity of ferulic acid on fed-batch vanillic acid production

Fed-batch bioconversion was started by adding 1 mM ferulic acid 1 mM to the conversion medium at the 0, 12, 24, and 36 hours of conversion. Similar to batch conversion, the increasing pH from 8.0 to 10.0 resulted in decreased vanillic acid production (Figure 4.4). The highest conversion molar yield of ferulic acid to vanillic acid was 77% (2.67 ± 0.02 mM) after 48 hours of conversion under pH 8 (Figure 4.4A). The vanillic acid molar yield at pH 9 and 10 declined to 76 and 26%, respectively (Figure 4.4B, 4.4C). Moreover, fed-batch bioconversion with increasing pH affected bacterial cells (Figure 4.5). As shown in Figure 4.5C, under pH 10, the viable bacterial cells decreased from 12 to 8 log CFU/mL within 6 hours after adding 1 mM of ferulic acid to the medium. The accumulation of 4-vinylguaiacol in the medium led to decreased vanillic acid production (Figure 4.4C) due to the high cytotoxicity of 4-vinylguaiacol to *E. coli*, consequently, cell death during the process (Li et al., 2019). Accordingly, pH 8 was chosen as the optimum pH for further growth-dependent vanillic acid production.

Table 4.2 Fed-batch vanillic acid production at various pH and concentrations of ferulic acid

Medium	pH	Ferulic acid concentration (mM)	Production of vanillic acid (mM)	Productivity of vanillic acid (mg/L.h)	% Molar yield of vanillic acid
100 mM Tris-HCl	8.0	4	2.67 ± 0.02	9.35 ± 0.07	77 ± 2
100 mM Glycine-NaOH	9.0	4	2.45 ± 0.05	8.58 ± 0.17	76 ± 5
100 mM Glycine-NaOH	10.0	4	0.85 ± 0.03	2.97 ± 0.11	26 ± 3

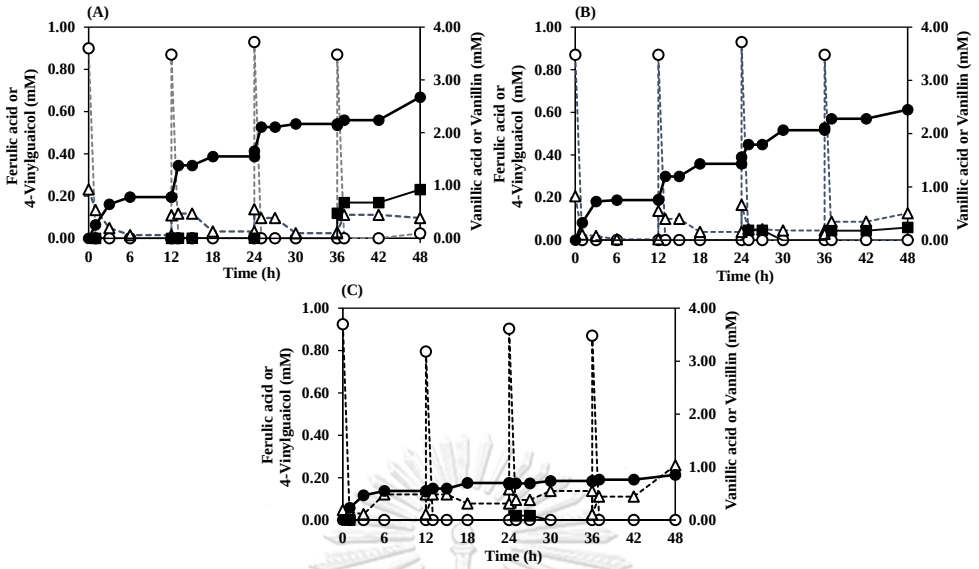


Figure 4.4 The influence of ferulic acid concentrations and pH on fed-batch vanillic acid production. pHs of (A) 8, (B) 9, and (C) 10 were used. The concentrations of ferulic acid (○), 4-vinylguaiacol (△), vanillin (■) and vanillic acid (●) are represented throughout the run time. The production was conducted using an initial OD_{600} of 40 at 37 °C, 200 rpm

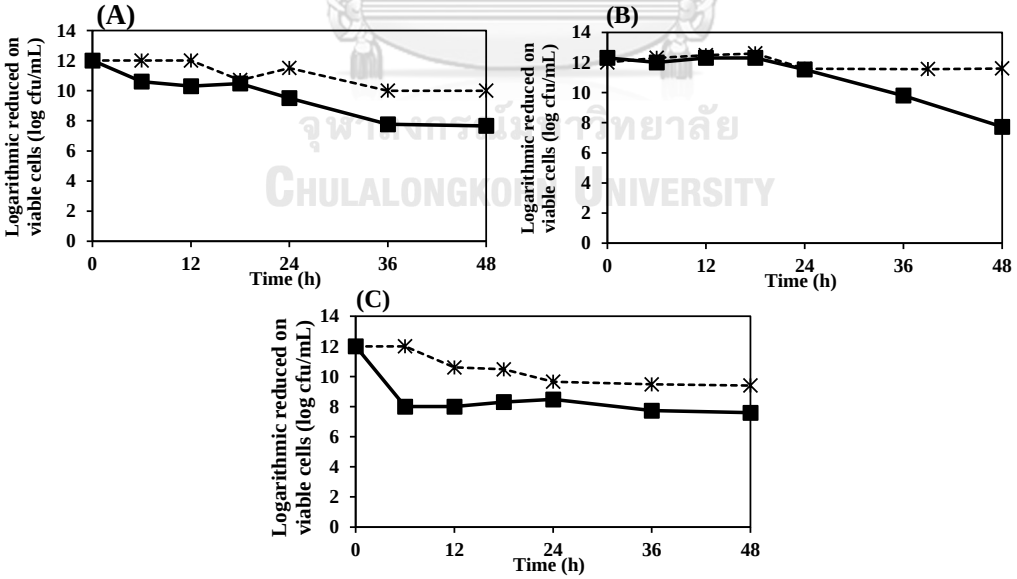


Figure 4.5 Toxicity of pH on the recombinant E. coli strain BL21(DE3) at (A) pH 8, (B) pH 9 and (C) pH 10 in fed-batch bioconversion. Cells with an initial concentration of OD_{600} = 40

(10^{12} CFU/ml) were exposed to (■) 1 mM of ferulic acid at 0, 12, 24, and 36 h. The culture without supplemented ferulic acid (control) was indicated by (✳).

4.2.1.4 Vanillic acid production by a two-step bioprocess using the recombinant *E. coli* BL21(DE3) resting cell in shake flask scale

For the conversion of caffeic acid to vanillic acid, a two-step bioconversion was conducted. In the first step reaction, caffeic acid was converted to ferulic acid using *E. coli* BL21(DE3) cells carrying pRSFDuet-*comt*. The obtained ferulic acid was then further converted to vanillic acid using *E. coli* BL21(DE3) cells carrying pETduet-*padC-vdh* and pCDFDuet-*ado* in the second step.

In the first step, both batch and fed-batch production of ferulic acid from caffeic acid were conducted at 25 °C, 200 rpm. The conversion medium was supplemented with 2 mM of DL-methionine as methyl group donor, and 2 mM of caffeic acid was used as substrate. Both batch and fed-batch ferulic acid production resulted in comparable ferulic acid production (Figure 4.6A, B). The recombinant *E. coli* BL21(DE3) expressing *comt* completely converted 2 mM of caffeic acid (360 mg/L) to 2 mM ferulic acid (388 mg/L) within 36 hours of conversion. This result is similar to the previous study, the recombinant *E. coli* BL21(DE3) carrying pRSFDuet-*comt* showed high activity to convert caffeic acid to ferulic acid (Rodrigues et al., 2020).

In the second step, the recombinant *E. coli* BL21(DE3) cells harboring pRSFDuet-*comt* were removed from the conversion medium. The recombinant *E. coli* BL21(DE3) co-harboring pETDuet-*padC-vdh*/pCDFDuet-*ado* cells were then added to the previously obtained conversion medium containing 1 mM of ferulic acid. In the second step, 1 mM vanillic acid (168 mg/L, 100% molar yield) was obtained within 48 hours of conversion (Figure 4.7).

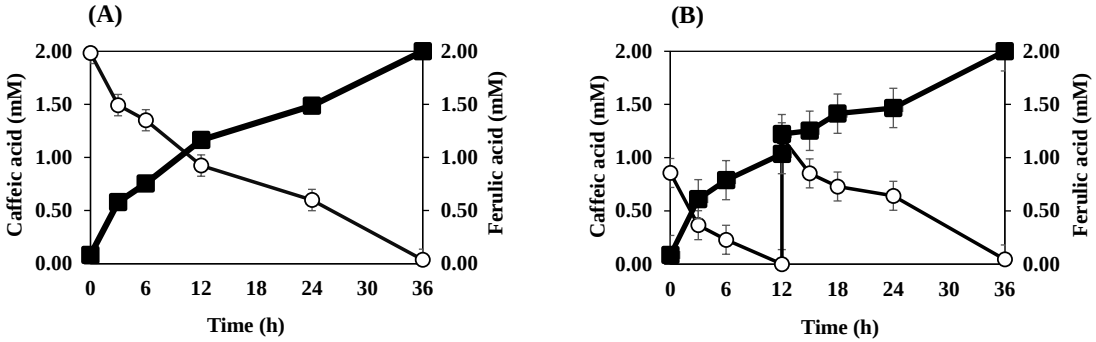


Figure 4.6 Bioconversion profile of E. coli BL21(DE3).pRSFDuet-comt in the first stage of vanillic production by a two-step bioprocess. (A) batch and (B) fed-batch bioconversion were studied. The concentrations of caffeic acid (○) and ferulic acid (■) are represented throughout the run time. The production was conducted using an initial OD_{600} of 40 (18 mg/ml CDW) at 25 °C, 200 rpm, 2 mM of caffeic acid was used as substrate.

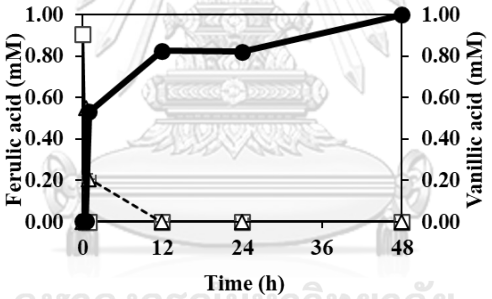


Figure 4.7 Bioconversion profile of E. coli BL21(DE3) co-harboring pETduet-padc-vdh/pCDFDuet-ado in the second stage. One mM of ferulic acid prepared by the first-stage reaction was used as substrate. The concentrations of ferulic acid (□), 4-vinylguaiacol (△), and vanillic acid (●) are represented throughout the run time. The production was conducted using an initial OD_{600} of 40 (17 mg/ml CDW) at 37 °C, 200 rpm.

4.2.2 Optimization of vanillic acid production using growing cells in shake flask scale

4.2.2.1 The effect of IPTG concentration on vanillic acid production

The optimum IPTG concentration is an important factor for gene expression and thus vanillic acid production. In this part, the effect of IPTG at different concentrations (0.2, 0.4, and 1.2 mM) on vanillic acid production was investigated. IPTG was initially added into LB medium at OD_{600} = 0.6 and cell cultivation was further carried out under 125 rpm, at 25 °C for 16 h.

Figure 4.8 showed the effect of IPTG concentrations on the production of 4-vinylguaiacol and vanillic acid, in comparison to the control (no IPTG added). Without IPTG addition, only approximately 50% of ferulic acid was converted to 4-vinylguaiacol, and that no vanillin or vanillic acid was detected (Figure 4.8D). The highest vanillic acid production of 0.57 ± 0.03 mM (95 ± 5 mg/L, 59% molar yield) was obtained with the addition of 0.2 mM of IPTG. However, vanillic acid production was slightly decreased to 0.53 ± 0.03 mM (90 ± 4 mg/L, 56% molar yield) when 1.2 mM IPTG was used (Figure. 4.8C). The higher IPTG concentrations did not result in higher vanillic acid production in this study. This is probably due to the metabolic burden on the cells which can further affect productivity (Malakar & Venkatesh, 2012). High expression of heterologous proteins could lead to reduced cell growth (Couto et al., 2017), while low expression levels of proteins might decrease the encounter between the enzymes and the substrates (Moon et al., 2010).

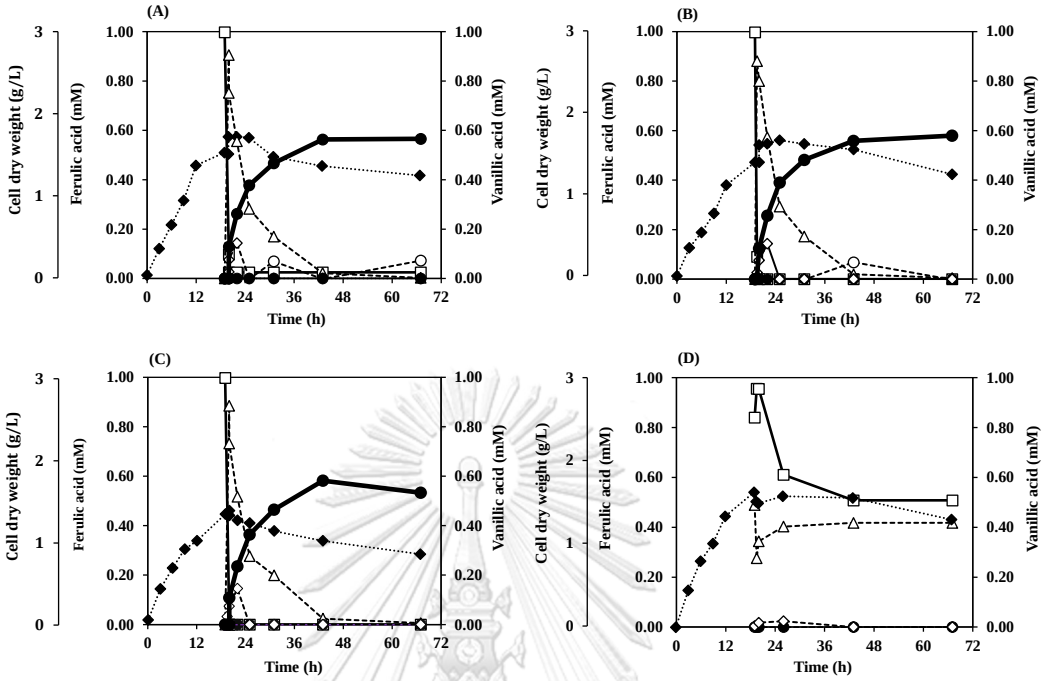


Figure 4.8 The effect of different IPTG concentrations on shake flask production of vanillic acid. The concentrations of IPTG: (A) 0.2 mM, (B) 0.4 mM, (C) 1.2 mM, and (D) no IPTG were studied. Cell dry weight (◆), the concentrations of ferulic acid (□), 4-vinylguaiacol (△), apocynol (◇), vanillin (○), and vanillic acid (●) are represented throughout the run time. In the growth phase, 1% (v/v) of inoculum was inoculated to 50 mL of Luria-Bertani medium containing in 250-mL baffled flasks, cultivated at 37°C and 200 rpm. When OD_{600} reached 0.6**,** the different concentrations of IPTG and 1 mM $FeSO_4$ were added to the culture**,** incubated at 20°C and 200 rpm for 16 hours. The production phase was started with addition of 1 mM (194 mg/L) of ferulic acid, incubated at 37°C, and 200 rpm.

Table 4.3 Comparison of shake flask production of vanillic acid with different concentrations of IPTG (0, 0.2, 0.4, and 1.2 mM) using growing cells of E. coli BL21(DE3) harboring pETDuet-padC-vdh and pCDFDuet-ado.

Concentrations of IPTG (mM)	Titer of vanillic acid (mM)	Titer of vanillic acid (mg/L)	Productivity of vanillic acid (mg/L.h)	% Molar yield of vanillic acid
0.2	0.57 ± 0.03	95 ± 5	1.97 ± 0.13	59 ± 3
0.4	0.56 ± 0.02	94 ± 3	1.95 ± 0.06	58 ± 2
1.2	0.53 ± 0.03	90 ± 4	1.88 ± 0.08	56 ± 2
No IPTG (control)	n.d.	n.d.	n.d.	n.d.

n.d. = not detected.

4.2.2.2 The effect of carbon source on vanillic acid production

Carbon sources are important for cell growth and metabolism (Wang & Tang, 2017). In this part, the effects of carbon sources i.e. glucose or glycerol at the concentration of 1, 5 and 10 g/L on vanillic acid production were investigated. Interestingly, while the increasing concentration of glucose adversely affected vanillic acid production, the vanillic acid molar yield remained 71 – 83% when the concentrations of glycerol were 1 - 10 g/L (Figure 4.9 and Table 4.4). As shown in Figure 4.9E, the highest production of vanillic acid was 0.83 mM (140 mg/L, 83.0% molar yield), obtained from the conversion medium containing 1 mM (194 mg/L) of ferulic acid and 5 g/L glycerol. With the addition of 1 g/L glycerol in LB medium, the produced vanillic acid was 0.80 ± 0.03 mM (135 ± 5 mg/L, 80% molar yield), while LB medium supplemented with 10 g/L of glycerol yielded 0.71 ± 0.02 mM vanillic acid (119 ± 3 mg/L, 71% molar yield) (Figure 4.9D, F). The addition of 1 g/L of glucose resulted in 0.73 ± 0.02 mM vanillic acid production (122 ± 3 mg/L, 73% molar yield) within 67 hours of conversion, which is slightly lower than that of the medium supplemented with 1 g/L glycerol (Figure. 4.9A). The supplementation of 10 g/L glucose exhibited the

inhibitory effect on vanillic acid production (0.15 ± 0.02 mM, 26 ± 3 mg/L, 15% molar yield) (Figure. 4.9D).

Glucose is very effective carbon source in industrial fermentations of *E. coli* because it supports fast growth of bacteria, relatively inexpensive, and the initial substrate and precursor in many pathways (Gosset, 2005). However, growth of *E. coli* on the presence of excess glucose under aerobic conditions causes the formation of acidic-by-products, which is acetate (Luli & Strohl, 1990; El-Mansi & Holms, 1989; Kleman & Strohl, 1994). As shown in Figure 4.9D, the *E. coli* grown in the presence of 10 g/L glucose might produce acetate, resulting a decreased of pH of conversion medium from pH 8 to 7. For *E. coli*, the decreasing of pH in the culture medium affected to conversion and production of metabolic compounds by overflow metabolites (Philip et al., 2018). Acetate transported out of cells caused a decrease of pH in the culture medium (Philip et al., 2019). This case, the demand of the carbon flux supply exceeds for energy generation in central metabolism (Varma & Palsson, 1994). This occurred because of the accumulation of acetate, due to overloading of the tricarboxylic acid cycle and/or the electron transport chain (Han et al., 1992). When *E. coli* grown in batch cultures with 20 g/L glucose, the production of acetate was reached a range of 0.88–5.12 g/L (Luli & Strohl, 1990). High concentrations of acetate exceeds 5 g/L, inhibited cell growth, biomass yields, and recombinant protein production (Han et al., 1992).

Glycerol can be used as a carbon source for compare the production of vanillic acid with glucose. Glycerol is a by-product from the biodiesel industry (Martínez-Gómez et al., 2012), which has shown promising for *E. coli* cultivations (Bisen et al., 2010; Ukkonen et al., 2013). When *E. coli* is cultured on glycerol, low levels or no acetate production have been detected (Oh & Liao, 2000; Peng & Shimizu, 2003). These results were similar to the study by Ni and colleagues in 2015, when compared between glucose and glycerol as carbon source for vanillin production, the vanillin titer was higher with glycerol than glucose. They concluded glycerol was a more suitable carbon source than glucose to produce vanillin that as

vanillic acid substrate (Ni et al., 2015; Ahn et al., 2008). Therefore, 1 g/L of glycerol was used as carbon source in next experiment for flask scale production of vanillic acid.

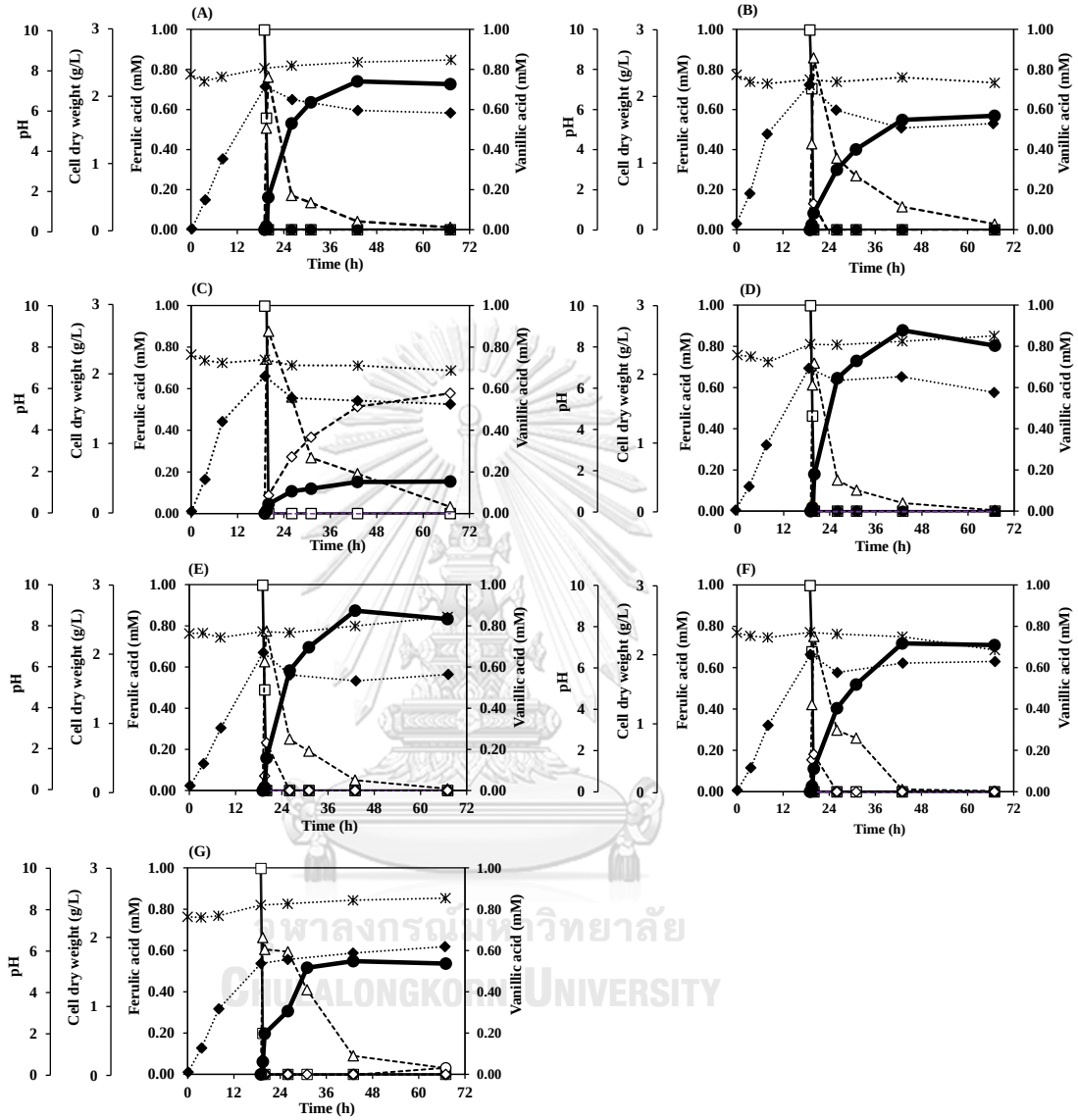


Figure 4.9 The effect of different concentrations of carbon source on shake flask production of vanillic acid. The concentrations of glucose: (A) 1 g/L, (B) 5 g/L, (C) 10 g/L, the concentrations of glycerol: (D) 1 g/L, (E) 5 g/L, (F) 10 g/L, and (G) No carbon source were studied. Cell dry weight (◆), pH (✳), the concentrations of ferulic acid (□), 4-vinylguaiacol (△), apocynol (◇), vanillin (○), and vanillic acid (●) are represented throughout the run time. In the growth phase, 1% (v/v) of inoculum was inoculated to 50 mL of LB medium supplemented with different concentrations of carbon source, cultivated at 37°C and 200 rpm.

When OD_{600} reached 0.6**,** 0.2 mM IPTG and 1 mM $FeSO_4$ were added to the culture, and incubated at 20°C and 200 rpm for 16 hours. The production phase was started with the addition of 1 mM (194 mg/L) of ferulic acid, incubated at 37°C, and 200 rpm.

Table 4.4 Comparison of vanillic acid production with different concentrations of carbon source using E. coli BL21(DE3)/pETDuet-padC-vdh/ pCDFDuet-ado growing cells. The production was conducted using LB medium of 50 mL, ferulic acid of 1 mM (194 mg/L) used as the substrate, incubated at 37 °C, and 200 rpm.

Concentrations of carbon source (g/L)	Titer of vanillic acid (mM)	Titer of vanillic acid (mg/L)	Productivity of vanillic acid (mg/L.h)	% Molar yield of vanillic acid
1 g/L glucose	0.73 ± 0.02	122 ± 3	2.54 ± 0.06	73 ± 2
5 g/L glucose	0.57 ± 0.03	96 ± 5	2.00 ± 0.10	57 ± 3
10 g/L glucose	0.15 ± 0.02	26 ± 3	0.54 ± 0.06	15 ± 2
1 g/L glycerol	0.80 ± 0.03	135 ± 5	2.81 ± 0.10	80 ± 3
5 g/L glycerol	0.83 ± 0.03	140 ± 5	2.92 ± 0.10	83 ± 3
10 g/L glycerol	0.71 ± 0.02	119 ± 3	2.47 ± 0.07	71 ± 2
No carbon source (control)	0.54 ± 0.03	90 ± 5	1.87 ± 0.10	54 ± 3

4.2.2.3 The effect of medium on vanillic acid production

To decrease the costs of the production of vanillic acid, alternative cultivation medium should be considered. The effects of three different cultivation media (LB, 2xLB, Fermentation medium) on vanillic acid production were evaluated. The composition of each medium were shown in Table 4.6. As shown in Figure. 4.10B, the recombinant *E. coli* BL21(DE) cultivated in 2xLB medium produced the highest concentration of vanillic acid (0.97 ± 0.01 mM, 97% molar yield), while the LB and fermentation medium resulted in approximately 80% and 86% vanillic acid molar yield, respectively (Figure 4.10A, 4.10C). High cell density cultivation (HCDC) of *E. coli* was carried out to improve productivity,

reduced culture volume, lower operation costs, and reduced investment in equipment (Lee, 1996). Increase *E. coli* cells to a high density can be achieved by optimization of cultivation medium for supporting cell growth (Lee, 1996). The 2xLB medium was rich cultivation media, developed for provide high *E. coli* cell yields (Brandis et al., 1989). A fermentation medium is a semi-defined minimal medium adapted from (Ng, 2018). Ng (2018) reported a new semi-defined formulated medium which shown to be useful for high cell density cultivation of *E. coli* DH5α strain ATCC 53868. By comparing between 2xLB medium and the fermentation medium, the production of vanillic acid in the fermentation medium (0.86 mM) was lower than the production in 2xLB medium; this may be due to the lower cell density (Figure 4.10C). Yeast extract of 12 g/L was added into the fermentation medium, it importantly promotes *E. coli* cell growth and biomass production. Yeast extract contains components (% w/w) of carbohydrates and derivatives (~10–35%), proteins (~45–50%) and free amino acids (~8–15%), vitamin derivatives (~5–10%), minerals and trace elements (~5–10%), nucleotides (~5–15%), and fat (~3–10%), which are necessary for microbial cell growth (Diederichs et al., 2014). Cell biomass obtained from the fermentation medium was 3.16 g CDW/L, which was slightly lower than 3.56 g CDW/L of 2xLB medium (Figure 4.8B, 4.8C). Although 2xLB medium given the highest vanillic acid production, the cost of producing vanillic acid by using 2xLB medium was 1.85-fold higher than the cost of the fermentation medium (Table 4.6). Therefore, the fermentation medium is simpler to be produced and cheaper to be used in fermentation processes. This medium will be further used in the production of vanillic acid in fermenter scale.

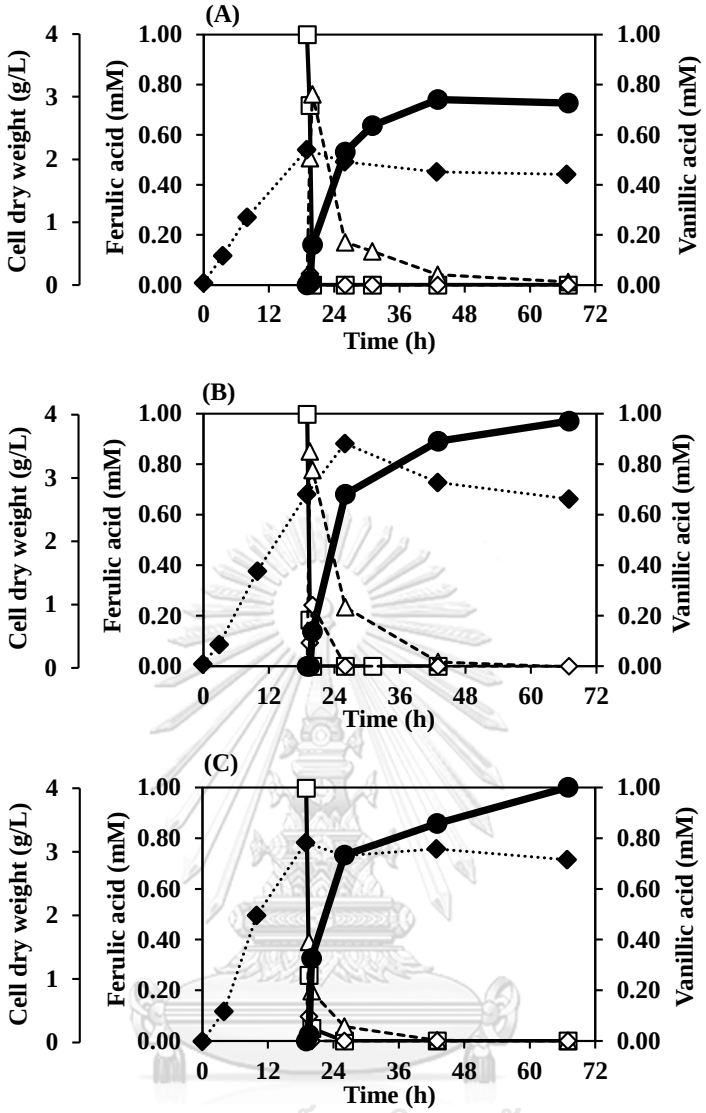


Figure 4.10 The effect of medium on shake flask production of vanillic acid. Luria-Bertani (LB) medium (A), 2x Luria-Bertani (2x LB) medium (B), Fermentation medium (C) was studied. Cell dry weight (◆), the concentrations of ferulic acid (□), 4-vinylguaiacol (△), apocynol (◇), vanillin (○), and vanillic acid (●) are represented throughout the run time. In the growth phase, 1% (v/v) of inoculum was inoculated to 50 mL of Luria-Bertani medium supplemented with different concentrations of carbon source in 250-mL baffled flasks, cultivated at 37°C and 200 rpm. When OD_{600} reached 0.6, 0.2 mM IPTG and 1 mM $FeSO_4$ were added to the culture, and incubated at 20°C and 200 rpm for 16 h. The production phase was started with the addition of 1 mM (194 mg/L) of ferulic acid, incubated at 37°C, and 200 rpm.

Table 4.5 Comparison of vanillic acid production with different medium using E. coli BL21(DE3)/pETDuet-padC-vdh/pCDFDuet-ado growing cells. The production was conducted using Luria-Bertani, 2x-Luria-Bertani, and fermentation medium volume of 50 mL supplemented with glycerol of 1 g/L, ferulic acid of 1 mM (194 mg/L) as substrate, incubated at 37 °C, and 200 rpm.

Medium type	Titer of vanillic acid (mM)	Titer of vanillic acid (mg/L)	Productivity of vanillic acid (mg/L.h)	% Molar yield of vanillic acid
LB	0.80 ± 0.03	135 ± 5	2.81 ± 0.10	80 ± 3
2xLB	0.97 ± 0.01	163 ± 1	3.40 ± 0.01	97 ± 1
Fermentation	0.86 ± 0.04	145 ± 7	3.01 ± 0.15	86 ± 4

Table 4.6 Composition and cost of medium for vanillic acid production used in this study.

Compositions	**Unit cost (Bath/ unit)**	**Consumption of medium (unit/ liter)**			**Cost of medium (Bath/ unit)**		
		LB	**2x LB**	**Fermentation**	**LB**	**2x LB**	**Fermentation**
Tryptone	3430/ 0.5 kg	10 g	20 g		68.60	137.20	
Yeast extract	3640/ 0.5 kg	5 g	10 g	12 g	36.40	72.80	87.36
NaCl	260/ 1 kg	10 g	20 g	5 g	2.60	5.20	1.30
K_2HPO_4	970/ 0.5 kg			16.282 g			23.77
KH_2PO_4	730/ 0.5 kg			0.888 g			1.40
NH_4Cl	670/ 0.5 kg			1 g			1.34
$MgSO_4.7H_2O$	1584/ 0.5 kg			0.24 g			0.38
Glycerol	2152/ 2.5 liter			0.79 mL			0.69
Total cost (Bath/ liter)					107.6	215.20	116.24

4.2.2.4 The effect of different inducers on vanillic acid production

Genes encoding phenolic acid decarboxylase (padC), aromatic dioxygenase (ado), and vanillin dehydrogenase (vdh) were expressed in E. coli BL21(DE3) under control of the T7 promoter and the lac operator of plasmid pETDuet and pCDFDuet. IPTG, which is the inducer for the T7 promoter is an essential component for protein expression and vanillic acid production. However, the high cost of IPTG affects the cost of conversion medium. In this part, to develop a more cost-effective conversion medium, IPTG was replaced by several natural sugars i.e. lactose and galactose, and the vanillic acid production was investigated. The induction of gene expression with IPTG was used as a control. Lactose and galactose have previously been reported to induce gene expression in the recombinant E. coli cells (Menzella et al., 2003; Yildirim & Mackey, 2003; Xu et al., 2012). Lactose is a natural inducer of the lac operon and a substrate of β-galactosidase (encoded by lacZ) for protein expression (Dvorak et al., 2015). Concentrations of lactose up to 10 g/L (30 mM) are used to induce the protein expression at levels that can be achieved with ≤1 mM IPTG (Neubauer et al., 1992). Lactose can be transported to E. coli BL21(DE3) cell by lactose permease (LacY), then converted into allolactose by β-galactosidase before binding to the lac repressor, thereby depressing expression of the genes for lac permease, transacetylase, and β-galactosidase (Wong et al., 1997). The remain of lactose and allolactose can be hydrolyzed to galactose and glucose, which then metabolized as carbon source in E. coli (Wong et al., 1997). The advantage of galactose over IPTG is a much weaker inducer of the lac operon than IPTG without metabolic burden to *E. coli* and increased the recombinant protein production in wild-type *E. coli* strains (Mattanovich et al., 1998; Barkley et al., 1975). As shown in Figure 4.11, using 0.2 mM IPTG as an inducer resulted in the highest vanillic acid (0.86 ± 0.01 mM, 86% molar yield). In comparison to galactose, using lactose as an inducer resulted in a slightly higher vanillic acid molar yield. Vanillic acid 78% molar yield was obtained from the induction with 5 g/L (15 mM) lactose (Figure 4.11B), whereas 75% vanillic acid molar yield

was produced from 5 g/L (29 mM) galactose (Figure 4.11D). The high cost and toxicity of IPTG make it unfeasible as an inducer in microbial fermentation at industrial scales (Dvorak et al., 2015; Briand et al., 2016). *E. coli* BL21(DE3) lacks the galactose-metabolism enzyme galactokinase, making it unable to metabolize galactose and allowing enough galactose level for targeted protein overexpression (Studier et al., 2009). Compared with IPTG and galactose, lactose is cheaper, not toxic to cell, and increase the solubility of the target protein (Tian et al., 2011; Lim et al., 2013).

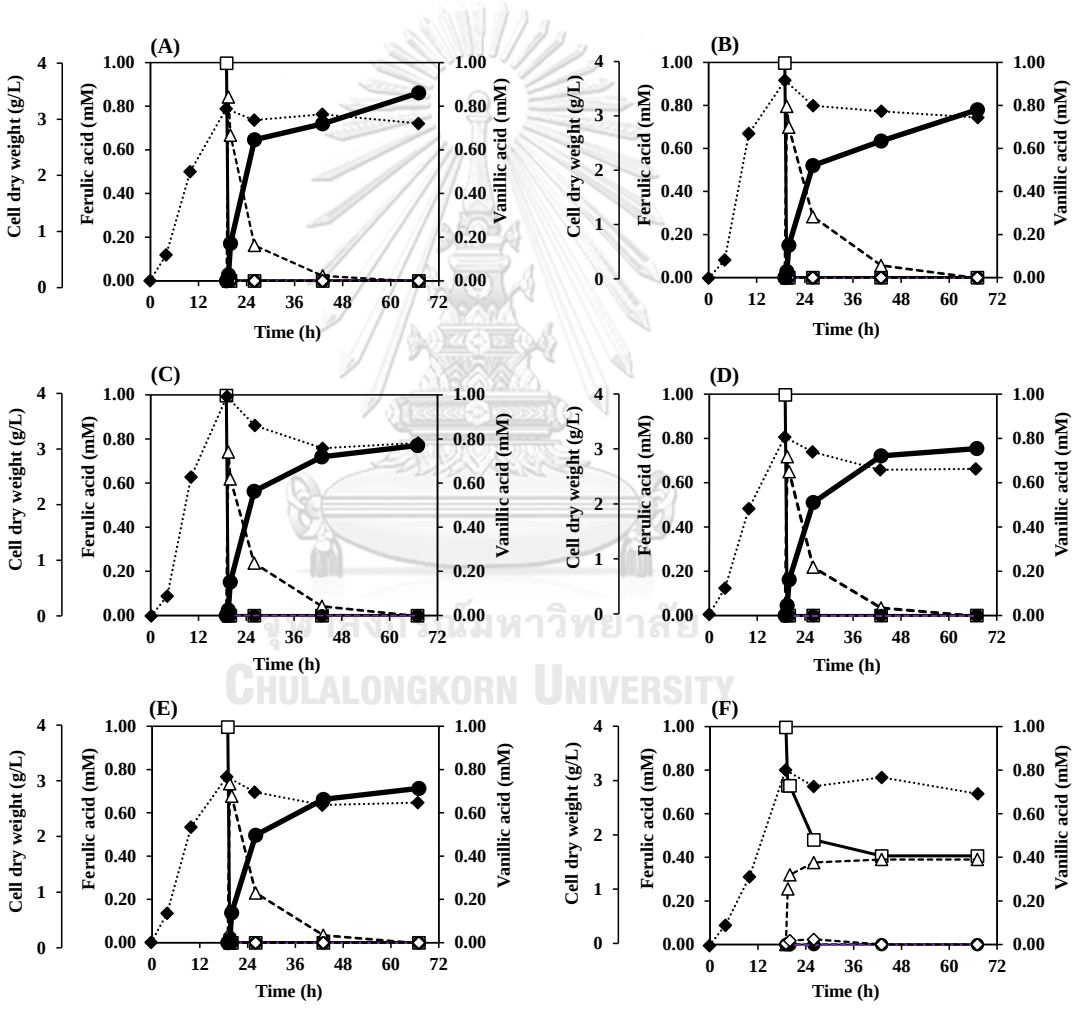


Figure 4.11 The effect of different inducers on shake flask production of vanillic acid. The concentration of 0.2 mM IPTG (A), 5 g/L lactose (B), 10 g/L lactose (C), 5 g/L galactose (D), 10 g/L galactose, and no inducer (F) were studied. Cell dry weight (◆), the concentrations of ferulic acid (□), 4-vinylguaiacol (△), apocynol (◇), vanillin (○), and

vanillic acid (●) are represented throughout the run time. In the growth phase, 1% (v/v) of inoculum was inoculated to 50 mL of fermentation medium supplemented with 1 g/L glycerol containing in 250-mL baffled flasks, cultivated at 37°C and 200 rpm. When OD_{600} reached 0.6**,** the different inducer and 1 mM $FeSO_4$ were added to the culture and incubated at 20°C and 200 rpm for 16 hours. The production phase was started with the addition of 1 mM (194 mg/L) of ferulic acid, incubated at 37°C, and 200 rpm.

Table 4.7 Comparison of vanillic acid production with different inducer type using E. coli BL21(DE3)/pETDuet-padC-vdh/pCDFDuet-ado growing cells. The production was conducted using fermentation medium of 50 mL supplemented with glycerol of 1 g/L, ferulic acid of 1 mM (194 mg/L) as substrate, incubated at 37 °C, and 200 rpm.

Inducer type	Titer of vanillic acid (mM)	Titer of vanillic acid (mg/L)	Productivity of vanillic acid (mg/L.h)	% Molar yield of vanillic acid
0.2 mM IPTG	0.86 ± 0.01	145 ± 1	3.02 ± 0.02	86 ± 1
5 g/L Lactose	0.78 ± 0.03	131 ± 5	2.73 ± 0.10	78 ± 3
10 g/L Lactose	0.77 ± 0.02	130 ± 3	2.70 ± 0.07	77 ± 2
5 g/L Galactose	0.75 ± 0.02	127 ± 3	2.64 ± 0.06	75 ± 2
10 g/L Galactose	0.71 ± 0.03	120 ± 5	2.50 ± 0.10	71 ± 3
No inducer (control)	n.d.	n.d.	n.d.	n.d.

n.d. = not detected.

4.3 Vanillic acid production by the recombinant *E. coli* growing cells in 5-L bioreactor

4.3.1 Effect of inducer type, inoculum percentage on vanillic acid production using *E. coli* BL21(DE3)/pETDuet-*padc-vdh*/pCDFDuet-*ado*

4.3.1.1 Effect of inducer type on vanillic acid production

The effects of inducers on the 5-L bioreactor production of vanillic acid were investigated by using 0.2 mM IPTG and different concentrations of lactose and galactose (5 g/L, 10 g/L). In the process of fermentation, obtaining higher the cell growth of bacteria

and increasing the expression of target protein were essential for decreasing the cost (Wong et al. 1998). Lactose and galactose, natural inducer of lac operon, they have proved to be non-toxic to bacterial cell and metabolized by bacteria as a carbon source (Wong et al., 1997). Due to toxicity of IPTG, made it unsuitable for large-scale production of recombinant proteins in *E. coli* (Donovan et al. 1996). Contrary to flask-scale vanillic acid production, the molar yield of vanillic acid production using 5 g/L of lactose as an inducer was 59% (0.58 ± 0.03 mM), which was higher than that using 0.2 mM IPTG as an inducer (0.43 mM, 45% molar yield) (Figure 4.12A, B). The high rate of proteins expression in large-scale fermentation were much easier to form inclusion body than the low rate of proteins expression (Choi et al., 2006). IPTG induced protein expression at high rate, while lactose could decrease the expression rate of target protein, which was benefit for soluble expression (Tian et al., 2011). The higher concentration of either lactose or galactose adversely affected vanillic acid production. The molar yields of vanillic acid were 17% and 10% when using 10 g/L of lactose and galactose as an inducer (Figure 4.12C, E). Lactose and galactose were not only inducer, but also could be utilized as a carbon source by *E. coli* (Deng et al., 2014; Qiu et al., 2014). Lactose could be converted into glucose and galactose *via* lactose metabolism in *E. coli* (Nath et al., 2014). The use of excessive carbon sources (lactose or galactose) might increase excess glucose which produce acetate as acidic fermentation by-product in the medium (Eiteman & Altman, 2006), this leads to reduce the culture pH and enzyme activity for vanillic acid production (Deng et al., 2014; Qiu et al., 2014). Thus, the induction with 5 g/L lactose was chosen for further optimization of vanillic acid production.

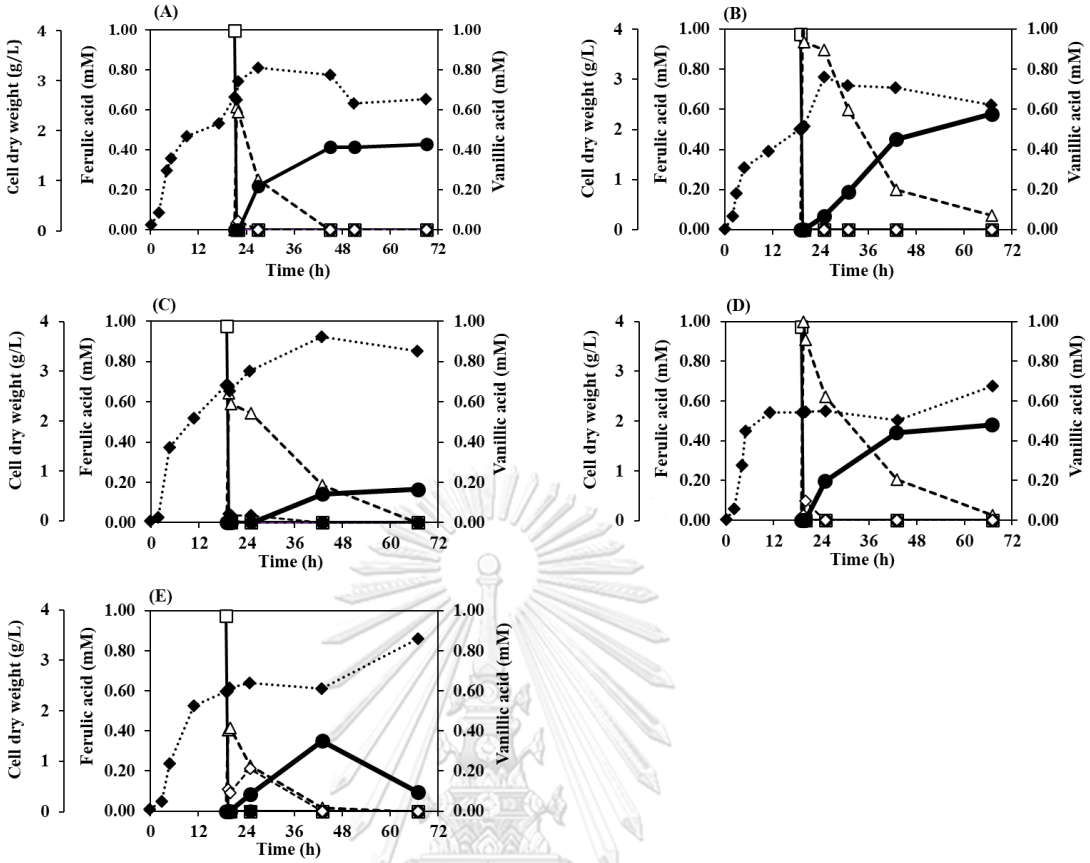


Figure 4.12 The effect of different inducer on vanillic acid production in 5-L bioreactor. The concentration of IPTG 0.2 mM (A), lactose of 5 g/L (B), 10 g/L (C), galactose of 5 g/L (D), and 10 g/L (E) were studied. Cell dry weight (◆), the concentrations of ferulic acid (□), 4-vinylguaiacol (△), apocynol (◇), vanillin (○), and vanillic acid (●) are represented throughout the run time. The production was conducted using 3 Liters of fermentation medium, ferulic acid of 1 mM (194 mg/L) as substrate, incubated at 37 °C, and 200 rpm. The pH of the culture medium was maintained at 8.0 ± 0.1 with 2 M HCl and 2 M NaOH. The aeration rate was controlled at 1 vvm.

Table 4.8 Comparison of vanillic acid production with different inducer type using E. coli BL21(DE3)/pETDuet-padC-vdh/pCDFDuet-ado growing cells in 5-L bioreactor. The production was conducted using fermentation medium of 3 L supplemented with glycerol of 1 g/L, ferulic acid of 1 mM (194 mg/L) as substrate, incubated at 37 °C, and 200 rpm. The pH of culture medium was maintained at 8.0 ± 0.1 with 2 M HCl and 2 M NaOH. The aeration rate was controlled at 1 vvm.

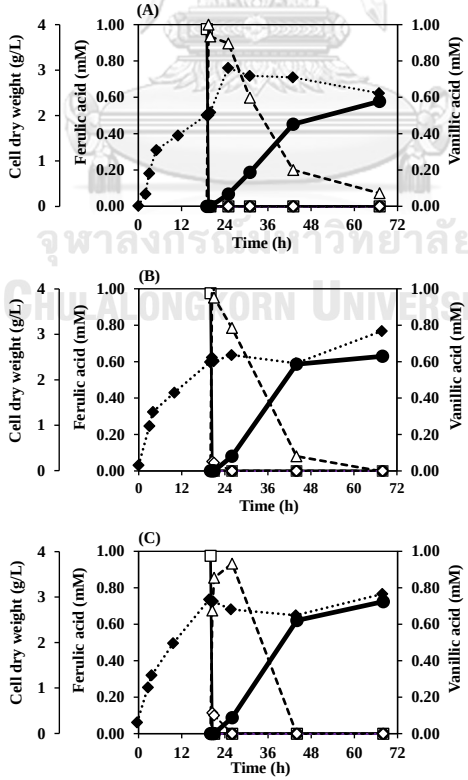
Inducer type	Titer of vanillic acid (mM)	Titer of vanillic acid (mg/L)	Productivity of vanillic acid (mg/L.h)	% Molar yield of vanillic acid
0.2 mM IPTG	0.43 ± 0.05	72 ± 8	1.51 ± 0.16	45 ± 5
5 g/L Lactose	0.58 ± 0.03	98 ± 5	2.03 ± 0.12	59 ± 3
10 g/L Lactose	0.17 ± 0.04	29 ± 7	0.60 ± 0.15	17 ± 4
5 g/L Galactose	0.48 ± 0.02	81 ± 3	1.68 ± 0.07	49 ± 2
10 g/L Galactose	0.09 ± 0.04	15 ± 6	0.32 ± 0.10	10 ± 4

4.3.1.2 Effect of inoculum percentage on vanillic acid production

The effects of different inoculum sizes (1, 5 and 10% (v/v)) on vanillic acid production were investigated by using 3 Liters of fermentation medium containing 1 mM ferulic acid as substrate. The fermentation condition was 37 °C, and agitation of 200 rpm. As shown in Figure 4.13, the increased inoculum sizes resulted in the higher molar yield of vanillic acid. The highest vanillic acid of 74% molar yield (0.72 ± 0.04 mM, 121 ± 6 mg/L) was obtained using the inoculum size of 10% (v/v) (Figure. 4.13C). The conversion rate of 4-vinylguaiacol in vanillic acid production with the inoculum size of 10% (v/v) is higher than the lower inoculum size, 4-vinylguaiacol was completely converted to vanillic acid after added ferulic acid for 24 h (Figure. 4.13C). The higher inoculum size led to faster bacterial consumption and nutrient depletion as compared to the lower inoculum size (Mehmood et al.,

2022). The use of 10% (v/v) of the inoculum size might be a critical cellular density, hampering to reach a high vanillic acid yield after 67 h. As shown in Figure 4.13, the decrease of vanillic acid production rate after added ferulic acid for 24 h was due perhaps to the increasing limitation of key nutrients, and accumulation of greater amounts of growth inhibitory metabolites. However, the results disagreed with the research conducted by Guo and colleagues in 2022 which showed that the production of vanillin, which is a substrate for producing vanillic acid, decreased when using the higher inoculum 7% (v/v), due to the inadequate of basic nutrient for bacterial growth and metabolism, thus decreasing the efficiency of strains to convert ferulic acid to produce vanillin (Gou et al., 2022). According to the result, 10% was the optimum inoculum for large-scale vanillic acid production in 5-L bioreactor.

Figure 4.13 The effect of different inoculum percentage on vanillic acid production in 5-L



bioreactor. The inoculum percentage of 1% (A), 5% (B), and 10% (v/v) were studied. Cell dry weight (◆), the concentrations of ferulic acid (□), 4-vinylguaiacol (△), apocynol (◇),

vanillin (○), and vanillic acid (●) are represented throughout the run time. The production was conducted using fermentation medium of 3 L, ferulic acid of 1 mM (194 mg/L) as substrate, incubated at 37 °C, and 200 rpm. The pH of culture medium was maintained at 8.0 ± 0.1 with 2 M HCl and 2 M NaOH. The aeration rate was controlled at 1 vvm.

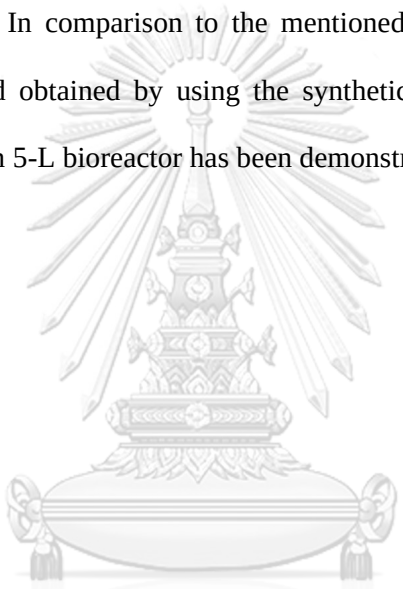
Table 4.9 Comparison of vanillic acid production with different inoculum percentage using E. coli BL21(DE3)/pETDuet-padC-vdh/pCDFDuet-ado growing cells in 5-L bioreactor. The production was conducted using fermentation medium of 3 L supplemented with glycerol of 1 g/L, ferulic acid of 1 mM (194 mg/L) as substrate, incubated at 37 °C, and 200 rpm. The pH of culture medium was maintained at 8.0 ± 0.1 with 2 M HCl and 2 M NaOH. The aeration rate was controlled at 1 vvm.

Inoculum percentage (%, v/v)	Titer of vanillic acid (mM)	Titer of vanillic acid (mg/L)	Productivity of vanillic acid (mg/L.h)	% Molar yield of vanillic acid
1	0.58 ± 0.03	98 ± 5	2.03 ± 0.12	59 ± 3
5	0.63 ± 0.05	106 ± 8	2.21 ± 0.17	64 ± 5
10	0.72 ± 0.04	121 ± 6	2.52 ± 2.65	74 ± 4

In the current study, the recombinant *E. coli* BL21(DE3) harboring pETduet-*padC-vdh* and pCDFDuet-*ado* could produce the maximum production of vanillic acid 0.83 g/L (4.92 mM, 58% molar yield) from 1.94 g/L of ferulic acid within 48 hours in shake flask scale (Table 4.1). The highest of vanillic acid molar yield 100% was obtained from 0.19 g/L of ferulic acid in two-step bioprocess in shake flask scale (Figure 4.7). With the production of vanillic acid in 5-L fermenter, the maximum vanillic acid 121 mg/L (0.72 mM, 74% molar yield) was achieved from 194 mg/L of ferulic acid within 67 hours. (Table 4.9)

The engineered *Pseudomonas putida* KT2440 could produce vanillic acid with 95 ± 1.4% molar yield from 2 g/L of ferulic acid in 22 hours (Upadhyay et al., 2020). The vanillic acid of 2.2 g/L or 95% yield was obtained from 2 g/L of vanillin when *Ochrobactrum*

anthropic was used (Girawale et al., 2022). By using *Streptomyces sannanensis* MTCC 6637 which possesses CoA-dependent vanillic acid production, the vanillic acid yield of 48% (0.49 g/L) was obtained from 1 g/L (5 mM) of ferulic acid in 20 days (Ghosh et al., 2007). With *P. resinovorans* SPR1 strain, vanillic acid was produced at 1.1 g/L (44% molar yield) from 2.5 g/L of eugenol as the sole carbon source after 60 hours (Ashengroph et al., 2011). The resting cells of *Halomonas elongata* DSM 2581, under hypersaline conditions, produced 0.7 g/L (3.95 mM) or 40% yield of vanillic acid from 1.94 g/L of ferulic acid within 10 hours (Abdelkafi et al., 2008). In comparison to the mentioned studies, this study reported the higher vanillic acid yield obtained by using the synthetic pathway and that the upscaled vanillic acid production in 5-L bioreactor has been demonstrated.



CHAPTER V

CONCLUSION

Owing to its properties, vanillic acid production is important compound for various industries, resulting in the growing demand. The bioproduction of vanillic acid, particularly from the abundant lignin-derived compounds, is considered as a sustainable alternative. Recently, the Coenzyme A-independent production of vanillin from ferulic acid has been reported. To further develop this pathway for vanillic acid production, herein, the synthetic pathway consisting of four genes which are *comt*, *padC*, *vdh*, and *ado* was constructed and expressed in the recombinant *E. coli* strain BL21(DE3). Such strain showed an effective vanillic acid production with 96 ± 2 % vanillic acid yield when the growth-independent conversion was conducted with an initial OD_{600} of 40 in 100 mM Tris-HCl buffer pH 8, and ferulic acid 0.194 g/L (1 mM) as the substrate. The maximum production of vanillic acid was 0.95 ± 0.002 g/L (4.92 ± 0.01 mM, 58 ± 1 % molar yield) from 1.94 g/L (10 mM) of ferulic acid within 48 hours in shake flask scale. A two-step bioconversion process was established to produce vanillic acid from caffeic acid. The production of vanillic acid reached 0.168 ± 0.003 g/L (1.00 ± 0.02 mM, 100 ± 2 % molar yield) from 0.180 g/L of caffeic acid (1 mM) within 48 hours. For the production of vanillic acid in 5-L fermenter, the maximum vanillic acid of 0.121 ± 0.006 g/L (0.72 ± 0.04 mM, 74 ± 4 % molar yield) was achieved from 0.194 g/L of ferulic acid within 67 hours. This study suggests this recombinant *E. coli* might be a practical strain for scaling up vanillic acid production and further development for industrial application.

REFERENCES

Abdelkafi, S., Labat, M., Gam, Z. B. A., Lorquin, J., Casalot, L., & Sayadi, S. (2008). Optimized conditions for the synthesis of vanillic acid under hypersaline conditions by *Halomonas elongata* DSM 2581 T resting cells. *World Journal of Microbiology and Biotechnology*, *24*(5), 675-680.

Abdelkafi, S., Sayadi, S., Ali Gam, Z. B., Casalot, L., & Labat, M. (2006). Bioconversion of ferulic acid to vanillic acid by *Halomonas elongata* isolated from table-olive fermentation. *FEMS microbiology letters*, *262*(1), 115-120.

Achterholt, S., Priefert, H., & Steinbüchel, A. (2000). Identification of *Amycolatopsis sp.* strain HR167 genes, involved in the bioconversion of ferulic acid to vanillin. *Applied microbiology and biotechnology*, *54*(6), 799-807.

Ahn, J.-O., Lee, H.-W., Saha, R., Park, M.-S., Jung, J.-K., & Lee, D.-Y. (2008). Exploring the effects of carbon sources on the metabolic capacity for shikimic acid production in *Escherichia coli* using in silico metabolic predictions. *Journal of microbiology and biotechnology*, *18*(11), 1773-1784.

Ashengroph, M., & Amini, J. (2017). Bioconversion of isoeugenol to vanillin and vanillic acid using the resting cells of *Trichosporon asahii*. *3 Biotech*, *7*(6), 1-9.

Ashengroph, M., Nahvi, I., Zarkesh-Esfahani, H., & Momenbeik, F. (2011). *Pseudomonas resinovorans* SPR1, a newly isolated strain with potential of transforming eugenol to vanillin and vanillic acid. *New biotechnology*, *28*(6), 656-664.

Ashengroph, M., Nahvi, I., Zarkesh-Esfahani, H., & Momenbeik, F. (2012a). Conversion of isoeugenol to vanillin by *Psychrobacter sp.* strain CSW4. *Applied biochemistry and biotechnology*, *166*(1), 1-12.

Ashengroph, M., Nahvi, I., Zarkesh-Esfahani, H., & Momenbeik, F. (2012b). Novel strain of *Bacillus licheniformis* SHL1 with potential converting ferulic acid into vanillic acid. *Annals of microbiology*, *62*(2), 553-558.

Barghini, P., Montebove, F., Ruzzi, M., & Schiesser, A. (1998). Optimal conditions for bioconversion of ferulic acid into vanillic acid by *Pseudomonas fluorescens* BF13 cells. *Applied microbiology and biotechnology*, *49*(3), 309-314.

Barkley, M. D., Riggs, A. D., Jobe, A., & Bourgeois, S. (1975). Interaction of effecting ligands with lac repressor and repressor-operator complex. *Biochemistry*, *14*(8), 1700-1712.

Bayer, I. S., Guzman-Puyol, S., Heredia-Guerrero, J. A., Ceseracciu, L., Pignatelli, F., Ruffilli, R., Cingolani, R., & Athanassiou, A. (2014). Direct transformation of edible vegetable waste into bioplastics. *Macromolecules*, *47*(15), 5135-5143.

Beckham, G. T., Johnson, C. W., Karp, E. M., Salvachúa, D., & Vardon, D. R. (2016). Opportunities and challenges in biological lignin valorization. *Current opinion in biotechnology*, *42*, 40-53.

Bisen, P. S., Sanodiya, B. S., Thakur, G. S., Baghel, R. K., & Prasad, G. (2010). Biodiesel production with special emphasis on lipase-catalyzed transesterification. *Biotechnology letters*, *32*(8), 1019-1030.

Bisson, C., Salmon, R. C., West, L., Rafferty, J. B., Hitchcock, A., Thomas, G. H., & Kelly, D. J. (2022). The structural basis for high-affinity uptake of lignin-derived aromatic compounds by proteobacterial TRAP transporters. *The FEBS Journal*, *289*(2), 436-456.

Biswas, S., Mohammad, M. M., Movileanu, L., & van den Berg, B. (2008). Crystal structure of the outer membrane protein OpdK from *Pseudomonas aeruginosa*. *Structure*, *16*(7), 1027-1035.

Borchert, A. J., Henson, W. R., & Beckham, G. T. (2022). Challenges and opportunities in biological funneling of heterogeneous and toxic substrates beyond lignin. *Current opinion in biotechnology*, *73*, 1-13.

Brandis, J., Ditullio, D., Lee, J., & Armiger, W. (1989). Process controlled temperature induction during batch fermentations for recombinant DNA products. In *Computer Applications in Fermentation Technology: Modelling and Control of Biotechnological Processes* (pp. 235-251). Springer.

Brar, S. K., Sarma, S. J., & Pakshirajan, K. (2016). *Platform chemical biorefinery: future green chemistry*. Elsevier.

Briand, L., Marcion, G., Kriznik, A., Heydel, J.-M., Artur, Y., Garrido, C., Seigneuric, R., & Neiers, F. (2016). A self-inducible heterologous protein expression system in *Escherichia coli*. *Scientific reports*, *6*(1), 1-11.

Brunati, M., Marinelli, F., Bertolini, C., Gandolfi, R., Daffonchio, D., & Molinari, F. (2004). Biotransformations of cinnamic and ferulic acid with actinomycetes. *Enzyme and microbial technology*, *34*(1), 3-9.

Cai, C., Xu, Z., Zhou, H., Chen, S., & Jin, M. (2021). Valorization of lignin components into gallate by integrated biological hydroxylation, O-demethylation, and aryl side-chain oxidation. *Science advances*, *7*(36), eabg4585.

Cavin, J. F., Dartois, V., & Diviès, C. (1998). Gene cloning, transcriptional analysis, purification, and characterization of phenolic acid decarboxylase from *Bacillus subtilis*. *Appl Environ Microbiol*, *64*(4), 1466-1471.

Chaudhry, M. T., Huang, Y., Shen, X.-H., Poetsch, A., Jiang, C.-Y., & Liu, S.-J. (2007). Genome-wide investigation of aromatic acid transporters in *Corynebacterium glutamicum*. *Microbiology*, *153*(3), 857-865.

Chen, P., Yan, L., Wu, Z., Li, S., Bai, Z., Yan, X., Wang, N., Liang, N., & Li, H. (2016). A microbial transformation using *Bacillus subtilis* B7-S to produce natural vanillin from ferulic acid. *Scientific reports*, *6*(1), 1-10.

Choi, J. H., Keum, K. C., & Lee, S. Y. (2006). Production of recombinant proteins by high cell density culture of *Escherichia coli*. *Chemical engineering science*, *61*(3), 876-885.

Civolani, C., Barghini, P., Roncetti, A. R., Ruzzi, M., & Schiesser, A. (2000). Bioconversion of ferulic acid into vanillic acid by means of a vanillate-negative mutant of *Pseudomonas fluorescens* strain BF13. *Applied and Environmental Microbiology*, *66*(6), 2311-2317.

Couto, M. R., Rodrigues, J. L., & Rodrigues, L. R. (2017). Optimization of fermentation conditions for the production of curcumin by engineered *Escherichia coli*. *Journal of the Royal Society Interface*, *14*(133), 20170470.

D'Arrigo, I., Cardoso, J. G., Rennig, M., Sonnenschein, N., Herrgård, M. J., & Long, K. S. (2019). Analysis of *Pseudomonas putida* growth on non-trivial carbon sources using transcriptomics and genome-scale modelling. *Environmental microbiology reports*, *11*(2), 87-97.

Delisi, R., Ciriminna, R., Parrino, F., Palmisano, L., Xu, Y. J., & Pagliaro, M. (2016). One-Pot, Clean Synthesis of Vanillic Acid from Ferulic Acid. *ChemistrySelect*, *1*(3), 626-629.

Deng, S., Su, E., Ma, X., Yang, S., & Wei, D. (2014). High-level soluble and functional expression of Trigonopsis variabilis D-amino acid oxidase in *Escherichia coli*. *Bioprocess and biosystems engineering*, *37*(8), 1517-1526.

Diederichs, S., Korona, A., Staaden, A., Kroutil, W., Honda, K., Ohtake, H., & Büchs, J. (2014). Phenotyping the quality of complex medium components by simple online-monitored shake flask experiments. *Microbial cell factories*, *13*(1), 1-14.

Dvorak, P., Chrast, L., Nikel, P. I., Fedr, R., Soucek, K., Sedlackova, M., Chaloupkova, R., de Lorenzo, V., Prokop, Z., & Damborsky, J. (2015). Exacerbation of substrate toxicity

by IPTG in *Escherichia coli* BL21 (DE3) carrying a synthetic metabolic pathway. *Microbial cell factories*, *14*(1), 1-15.

Eiteman, M. A., & Altman, E. (2006). Overcoming acetate in *Escherichia coli* recombinant protein fermentations. *Trends in biotechnology*, *24*(11), 530-536.

El-Mansi, E., & Holms, W. (1989). Control of carbon flux to acetate excretion during growth of *Escherichia coli* in batch and continuous cultures. *Microbiology*, *135*(11), 2875-2883.

Fiormarkets report. (2022, December 14). Global vanillic acid market growth. In fiormarkets.com. Retrieved from http://www.fiormarkets.com/report/global-vanillic-acid-cas-121-34-6-market-growth-2019-2024-373272.html.

Fu, B., Xiao, G., Zhang, Y., & Yuan, J. (2021). One-pot bioconversion of lignin-derived substrates into gallic acid. *Journal of Agricultural and Food Chemistry*, *69*(38), 11336-11341.

Furukawa, H., Morita, H., Yoshida, T., & Nagasawa, T. (2003). Conversion of isoeugenol into vanillic acid by *Pseudomonas putida* I58 cells exhibiting high isoeugenol-degrading activity. *Journal of bioscience and bioengineering*, *96*(4), 401-403.

Ghosh, S., Sachan, A., Sen, S. K., & Mitra, A. (2007). Microbial transformation of ferulic acid to vanillic acid by *Streptomyces sannanensis* MTCC 6637. *Journal of Industrial Microbiology and Biotechnology*, *34*(2), 131-138.

Gioia, C., Banella, M., Marchese, P., Vannini, M., Colonna, M., & Celli, A. (2016). Advances in the synthesis of bio-based aromatic polyesters: novel copolymers derived from vanillic acid and ε-caprolactone. *Polymer Chemistry*, *7*(34), 5396-5406.

Girawale, S. D., Meena, S. N., Nandre, V. S., Waghmode, S. B., & Kodam, K. M. (2022). Biosynthesis of vanillic acid by *Ochrobactrum anthropi* and its applications. *Bioorganic & Medicinal Chemistry*, *72*, 117000.

Gitzinger, M., Kemmer, C., Fluri, D. A., El-Baba, M. D., Weber, W., & Fussenegger, M. (2012). The food additive vanillic acid controls transgene expression in mammalian cells and mice. *Nucleic Acids Res*, *40*(5), e37.

Gosset, G. (2005). Improvement of Escherichia coli production strains by modification of the phosphoenolpyruvate: sugar phosphotransferase system. *Microbial cell factories*, *4*(1), 1-11.

Gou, J., Guo, Y., Liu, H., Zhao, Y., Zhu, R., Dang, Y., Liu, N., Chen, M., & Chen, X. (2022). Process optimization of vanillin production by conversion of ferulic acid by *Bacillus megaterium*. *Journal of the Science of Food and Agriculture*.

Graf, N., & Altenbuchner, J. (2014). Genetic engineering of *Pseudomonas putida* KT2440 for rapid and high-yield production of vanillin from ferulic acid. *Applied microbiology and biotechnology*, *98*(1), 137-149.

Han, K., Lim, H. C., & Hong, J. (1992). Acetic acid formation in *Escherichia coli* fermentation. *Biotechnology and bioengineering*, *39*(6), 663-671.

Harwood, C. S., Nichols, N. N., Kim, M.-K., Ditty, J. L., & Parales, R. E. (1994). Identification of the pcaRKF gene cluster from *Pseudomonas putida*: involvement in chemotaxis, biodegradation, and transport of 4-hydroxybenzoate. *Journal of Bacteriology*, *176*(21), 6479-6488.

Horn, S. J., Vaaje-Kolstad, G., Westereng, B., & Eijsink, V. (2012). Novel enzymes for the degradation of cellulose. *Biotechnology for biofuels*, *5*(1), 45.

Isikgor, F. H., & Becer, C. R. (2015). Lignocellulosic biomass: a sustainable platform for the production of bio-based chemicals and polymers. *Polymer Chemistry*, *6*(25), 4497-4559.

Jung, D.-H., Choi, W., Choi, K.-Y., Jung, E., Yun, H., Kazlauskas, R. J., & Kim, B.-G. (2013). Bioconversion of p-coumaric acid to p-hydroxystyrene using phenolic acid

decarboxylase from *B. amyloliquefaciens* in biphasic reaction system. *Applied microbiology and biotechnology*, *97*(4), 1501-1511.

Jung, D. H., Kim, E. J., Jung, E., Kazlauskas, R. J., Choi, K. Y., & Kim, B. G. (2016). Production of p-hydroxybenzoic acid from p-coumaric acid by *Burkholderia glumae* BGR1. *Biotechnology and bioengineering*, *113*(7), 1493-1503.

Kleman, G. L., & Strohl, W. R. (1994). Acetate metabolism by *Escherichia coli* in high-cell-density fermentation. *Applied and Environmental Microbiology*, *60*(11), 3952-3958.

Kotchaplai, P., Ninrat, J., Mahipant, G., & Vangnai, A. S. (2022). Involvement of Cytochrome P450 in Organic-Solvent Tolerant *Bacillus subtilis* GRSW1-B1 in Vanillin Production *via* Ferulic Acid Metabolism. *Fermentation*, *8*(10), 508.

Kumar, A., Kumar, J., & Bhaskar, T. (2020). Utilization of lignin: A sustainable and eco-friendly approach. *Journal of the Energy Institute*, *93*(1), 235-271.

Lee, S. Y. (1996). High cell-density culture of *Escherichia coli*. *Trends in biotechnology*, *14*(3), 98-105.

Li, L., Long, L., & Ding, S. (2019). Bioproduction of high-concentration 4-vinylguaiacol using whole-cell catalysis harboring an organic solvent-tolerant phenolic acid decarboxylase from *Bacillus atrophaeus*. *Frontiers in microbiology*, *10*, 1798.

Lim, H. G., Seo, S. W., & Jung, G. Y. (2013). Engineered *Escherichia coli* for simultaneous utilization of galactose and glucose. *Bioresource technology*, *135*, 564-567.

Linger, J. G., Vardon, D. R., Guarnieri, M. T., Karp, E. M., Hunsinger, G. B., Franden, M. A., Johnson, C. W., Chupka, G., Strathmann, T. J., & Pienkos, P. T. (2014). Lignin valorization through integrated biological funneling and chemical catalysis. *Proceedings of the National Academy of Sciences*, *111*(33), 12013-12018.

Liu, H., Liu, Z.-H., Zhang, R.-K., Yuan, J. S., Li, B.-Z., & Yuan, Y.-J. (2022). Bacterial conversion routes for lignin valorization. *Biotechnology Advances*, 108000.

Luli, G. W., & Strohl, W. R. (1990). Comparison of growth, acetate production, and acetate inhibition of *Escherichia coli* strains in batch and fed-batch fermentations. *Applied and Environmental Microbiology*, *56*(4), 1004-1011.

Luo, Y., Wang, C.-Z., Sawadogo, R., Yuan, J., Zeng, J., Xu, M., Tan, T., & Yuan, C.-S. (2021). 4-Vinylguaiacol, an active metabolite of ferulic acid by enteric microbiota and probiotics, possesses significant activities against drug-resistant human colorectal cancer cells. *ACS omega*, *6*(7), 4551-4561.

Malakar, P., & Venkatesh, K. (2012). Effect of substrate and IPTG concentrations on the burden to growth of *Escherichia coli* on glycerol due to the expression of Lac proteins. *Applied microbiology and biotechnology*, *93*(6), 2543-2549.

Martínez-Gómez, K., Flores, N., Castañeda, H. M., Martínez-Batallar, G., Hernández-Chávez, G., Ramírez, O. T., Gosset, G., Encarnación, S., & Bolivar, F. (2012). New insights into *Escherichia coli* metabolism: carbon scavenging, acetate metabolism and carbon recycling responses during growth on glycerol. *Microbial cell factories*, *11*(1), 1-21.

Mattanovich, D., Kramer, W., Lüttich, C., Weik, R., Bayer, K., & Katinger, H. (1998). Rational design of an improved induction scheme for recombinant *Escherichia coli*. *Biotechnology and bioengineering*, *58*(2-3), 296-298.

Mehmood, T., Saeed, S., Hussain, N., & Waseem, R. (2022). Biotransformation of wheat straw into biovanillin by solid-state fermentation and optimization of conditions parameters through response surface methodology. *Biomass Conversion and Biorefinery*, 1-10.

Menafn report. (2022, December 14). Vanillic acid market in UK manufacturing and consumption outlook and forecast 2020-2026. In memafn.com. Retrieved from https://menafn.com/1100897739/Vanillic-Acid-Market-in-UK-Manufacturing-and-Consumption-Outlook-and-Forecast-2020-2026.

Menzella, H. G., Ceccarelli, E. A., & Gramajo, H. C. (2003). Novel *Escherichia coli* strain allows efficient recombinant protein production using lactose as inducer. *Biotechnology and bioengineering*, *82*(7), 809-817.

Mishra, S., Kullu, M., Sachan, A., Vidyarthi, A. S., & Sachan, S. G. (2016). Bioconversion of ferulic acid to vanillic acid by *Paenibacillus lactis* SAMS-2001. *Annals of microbiology*, *66*(2), 875-882.

Mitsui, R., Hirota, M., Tsuno, T., & Tanaka, M. (2010). Purification and characterization of vanillin dehydrogenases from alkaliphile *Micrococcus sp.* TA1 and neutrophile *Burkholderia cepacia* TM1. *FEMS Microbiology Letters*, *303*(1), 41-47.

Moon, T. S., Dueber, J. E., Shiue, E., & Prather, K. L. J. (2010). Use of modular, synthetic scaffolds for improved production of glucaric acid in engineered *E. coli*. *Metabolic engineering*, *12*(3), 298-305.

Mori, K., Kamimura, N., & Masai, E. (2018). Identification of the protocatechuate transporter gene in *Sphingobium sp.* strain SYK-6 and effects of overexpression on production of a value-added metabolite. *Applied microbiology and biotechnology*, *102*(11), 4807-4816.

Motedayen, N., Ismail, M. B., & Nazarpour, F. (2013). Bioconversion of ferulic acid to vanillin by combined action of *Aspergillus niger* K8 and *Phanerochaete crysosporium* ATCC 24725. *African Journal of biotechnology*, *12*(47), 6618-6624.

Muheim, A., & Lerch, K. (1999). Towards a high-yield bioconversion of ferulic acid to vanillin. *Applied microbiology and biotechnology*, *51*(4), 456-461.

Murga, R., Sanz, M. T., Beltrán, S., & Cabezas, J. L. (2004). Solubility of syringic and vanillic acids in supercritical carbon dioxide. *Journal of Chemical & Engineering Data*, *49*(4), 779-782.

Nanda, S., Mohammad, J., Reddy, S. N., Kozinski, J. A., & Dalai, A. K. (2014). Pathways of lignocellulosic biomass conversion to renewable fuels. *Biomass Conversion and Biorefinery*, *4*(2), 157-191.

Nanda, S., Mohanty, P., Pant, K. K., Naik, S., Kozinski, J. A., & Dalai, A. K. (2013). Characterization of North American lignocellulosic biomass and biochars in terms of their candidacy for alternate renewable fuels. *Bioenergy Research*, *6*(2), 663-677.

Nath, A., Mondal, S., Chakraborty, S., Bhattacharjee, C., & Chowdhury, R. (2014). Production, purification, characterization, immobilization, and application of β-galactosidase: a review. *Asia-Pacific Journal of Chemical Engineering*, *9*(3), 330-348.

Neubauer, P., Hofmann, K., Holst, O., Mattiasson, B., & Kruschke, P. (1992). Maximizing the expression of a recombinant gene in *Escherichia coli* by manipulation of induction time using lactose as inducer. *Applied microbiology and biotechnology*, *36*(6), 739-744.

Ng, W. (2018). High cell density cultivation of *Escherichia coli* DH5α in shake flasks with a new formulated medium (2167-9843).

Nguyen, L. T., Tran, M. H., & Lee, E. Y. (2021). Co-upgrading of ethanol-assisted depolymerized lignin: A new biological lignin valorization approach for the production of protocatechuic acid and polyhydroxyalkanoic acid. *Bioresource technology*, *338*, 125563.

Ni, J., Tao, F., Du, H., & Xu, P. (2015). Mimicking a natural pathway for de novo biosynthesis: natural vanillin production from accessible carbon sources. *Scientific reports*, *5*(1), 1-12.

Ni, J., Wu, Y.-T., Tao, F., Peng, Y., & Xu, P. J. J. o. t. A. C. S. (2018). A coenzyme-free biocatalyst for the value-added utilization of lignin-derived aromatics. *140*(47), 16001-16005.

Nichols, N. N., & Harwood, C. S. (1997). PcaK, a high-affinity permease for the aromatic compounds 4-hydroxybenzoate and protocatechuate from *Pseudomonas putida*. *Journal of Bacteriology*, *179*(16), 5056-5061.

Oh, M. K., & Liao, J. C. (2000). Gene expression profiling by DNA microarrays and metabolic fluxes in *Escherichia coli*. *Biotechnology progress*, *16*(2), 278-286.

Pearl, I. A. (1946). Reactions of vanillin and its derived compounds. I. The reaction of vanillin with silver oxide1. *Journal of the American Chemical Society*, *68*(3), 429-432.

Peng, L., & Shimizu, K. (2003). Global metabolic regulation analysis for *Escherichia coli* K12 based on protein expression by 2-dimensional electrophoresis and enzyme activity measurement. *Applied microbiology and biotechnology*, *61*(2), 163-178.

Perestelo, F., Dalcón, M., & De La Fuente, G. (1989). Production of vanillic acid from vanillin by resting cells of *Serratia marcescens*. *Applied and Environmental Microbiology*, *55*(6), 1660-1662.

Pérez, J., Munoz-Dorado, J., De la Rubia, T., & Martinez, J. (2002). Biodegradation and biological treatments of cellulose, hemicellulose and lignin: an overview. *International microbiology*, *5*(2), 53-63.

Pernstich, C., Senior, L., MacInnes, K. A., Forsaith, M., & Curnow, P. (2014). Expression, purification and reconstitution of the 4-hydroxybenzoate transporter PcaK from *Acinetobacter sp.* ADP1. *Protein expression and purification*, *101*, 68-75.

Philip, P., Kern, D., Goldmanns, J., Seiler, F., Schulte, A., Habicher, T., & Büchs, J. (2018). Parallel substrate supply and pH stabilization for optimal screening of *E. coli* with the membrane-based fed-batch shake flask. *Microbial cell factories*, *17*(1), 1-17.

Pometto 3rd, A., & Crawford, D. (1983). Whole-cell bioconversion of vanillin to vanillic acid by *Streptomyces viridosporus*. *Applied and Environmental Microbiology*, *45*(5), 1582-1585.

Qiu, J., Su, E.-Z., Wang, H.-L., Cai, W.-W., Wang, W., & Wei, D.-Z. (2014). Cloning, overexpression, and characterization of a high enantioselective nitrilase from *Sphingomonas wittichii* RW1 for asymmetric synthesis of (R)-phenylglycine. *Applied biochemistry and biotechnology, 173*(2), 365-377.

Quiroz-Castañeda, R. E., & Folch-Mallol, J. L. (2013). Hydrolysis of biomass mediated by cellulases for the production of sugars. *Sustainable degradation of lignocellulosic biomass techniques, applications and commercialization. InTech*, 119-155.

Rodrigues, J. L., Gomes, D., & Rodrigues, L. R. (2020). A combinatorial approach to optimize the production of curcuminoids from tyrosine in *Escherichia coli*. *Frontiers in Bioengineering and Biotechnology, 8*, 59.

Salmon, R. C., Cliff, M. J., Rafferty, J. B., & Kelly, D. J. (2013). The CouPSTU and TarPQM transporters in *Rhodopseudomonas palustris*: redundant, promiscuous uptake systems for lignin-derived aromatic substrates. *PloS one, 8*(3), e59844.

Studier, F. W., Daegelen, P., Lenski, R. E., Maslov, S., & Kim, J. F. (2009). Understanding the differences between genome sequences of *Escherichia coli* B strains REL606 and BL21 (DE3) and comparison of the E. coli B and K-12 genomes. *Journal of molecular biology, 394*(4), 653-680.

Tian, H., Tang, L., Wang, Y., Wang, X., Guan, L., Zhang, J., Wu, X., & Li, X. (2011). Lactose induction increases production of recombinant keratinocyte growth factor-2 in *Escherichia coli. International Journal of Peptide Research and Therapeutics, 17*(2), 123-129.

Ukkonen, K., Mayer, S., Vasala, A., & Neubauer, P. (2013). Use of slow glucose feeding as supporting carbon source in lactose autoinduction medium improves the robustness of protein expression at different aeration conditions. *Protein expression and purification, 91*(2), 147-154.

Upadhyay, P., Singh, N. K., Tupe, R., Odenath, A., & Lali, A. (2020). Biotransformation of corn bran derived ferulic acid to vanillic acid using engineered *Pseudomonas putida* KT2440. *Preparative Biochemistry & Biotechnology*, *50*(4), 341-348.

Vanbeneden, N., Saison, D., Delvaux, F., & Delvaux, F. R. (2008). Decrease of 4-vinylguaiacol during beer aging and formation of apocynol and vanillin in beer. *Journal of Agricultural and Food Chemistry*, *56*(24), 11983-11988.

Varma, A., & Palsson, B. O. (1994). Stoichiometric flux balance models quantitatively predict growth and metabolic by-product secretion in wild-type *Escherichia coli* W3110. *Applied and Environmental Microbiology*, *60*(10), 3724-3731.

Wada, A., Prates, É. T., Hirano, R., Werner, A. Z., Kamimura, N., Jacobson, D. A., Beckham, G. T., & Masai, E. (2021). Characterization of aromatic acid/proton symporters in *Pseudomonas putida* KT2440 toward efficient microbial conversion of lignin-related aromatics. *Metabolic engineering*, *64*, 167-179.

Wang, X., & Tang, C. (2017). Optimal growth of microbes on mixed carbon sources. *bioRxiv*, 120667.

Wilsens, C. H. R. M., Deshmukh, Y. S., Liu, W., Noordover, B. A. J., Yao, Y., Meijer, H. E. H., & Rastogi, S. (2015). Processing and performance of aromatic-aliphatic thermotropic polyesters based on vanillic acid. *Polymer*, *60*(Supplement C), 198-206.

Wong, P., Gladney, S., & Keasling, J. D. (1997). Mathematical model of the lac operon: inducer exclusion, catabolite repression, and diauxic growth on glucose and lactose. *Biotechnology progress*, *13*(2), 132-143.

Wu, W., Liu, F., & Singh, S. (2018). Toward engineering *E. coli* with an autoregulatory system for lignin valorization. *Proceedings of the National Academy of Sciences*, *115*(12), 2970-2975.

Xu, C., Nasrollahzadeh, M., Selva, M., Issaabadi, Z., & Luque, R. (2019). Waste-to-wealth: biowaste valorization into valuable bio (nano) materials. *Chemical Society Reviews*, *48*(18), 4791-4822.

Xu, J., Banerjee, A., Pan, S.-H., & Li, Z. J. (2012). Galactose can be an inducer for production of therapeutic proteins by auto-induction using *E. coli* BL21 strains. *Protein expression and purification*, *83*(1), 30-36.

Yan, L., Chen, P., Zhang, S., Li, S., Yan, X., Wang, N., Liang, N., & Li, H. (2016). Biotransformation of ferulic acid to vanillin in the packed bed-stirred fermentors. *Scientific reports*, *6*(1), 1-12.

Yildirim, N., & Mackey, M. C. (2003). Feedback regulation in the lactose operon: a mathematical modeling study and comparison with experimental data. *Biophysical journal*, *84*(5), 2841-2851.

Zamzuri, N. A., Abd-Aziz, S., Rahim, R. A., Phang, L. Y., Alitheen, N. B., & Maeda, T. (2014). A rapid colorimetric screening method for vanillic acid and vanillin-producing bacterial strains. *116*(4), 903-910.

Zhang, R., Li, C., Wang, J., & Yan, Y. (2018). Microbial ligninolysis: toward a bottom-up approach for lignin upgrading. *Biochemistry*, *58*(11), 1501-1510.

APPENDIX A

Standard graph of phenolic acid compounds and HPLC chromatogram

Vanillic acid

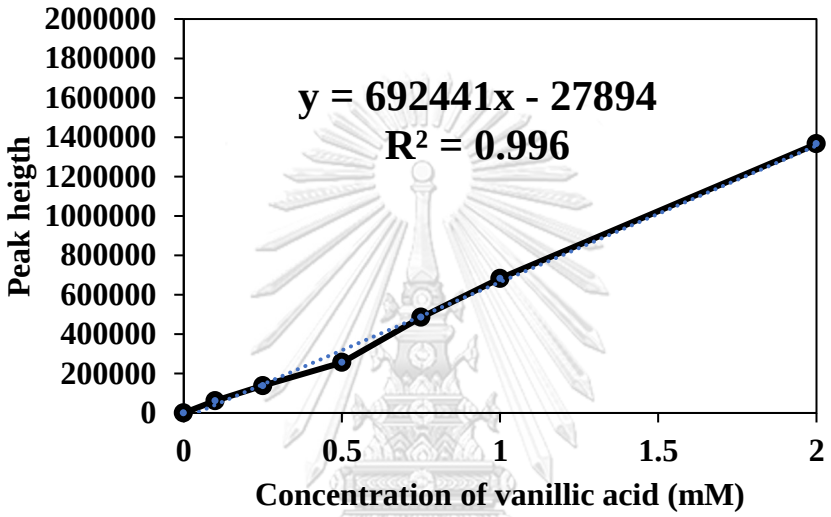


Figure A1 Standard graph of vanillic acid at 210 nm

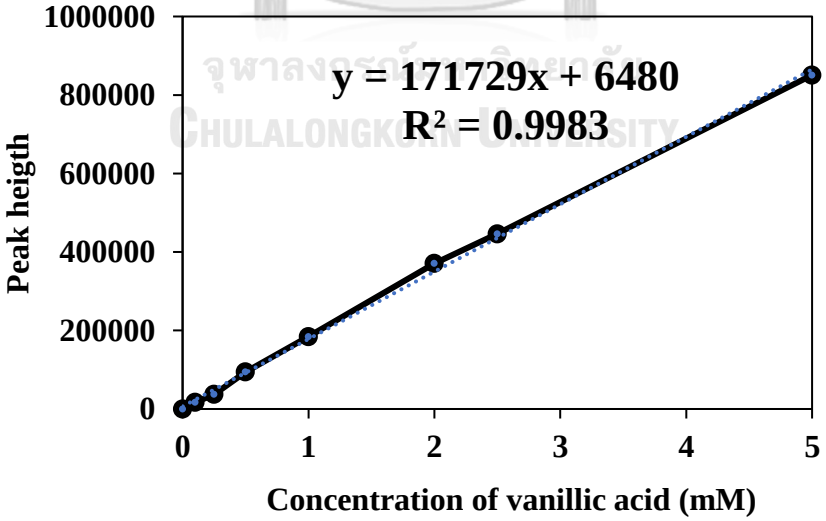


Figure A2 Standard graph of vanillic acid at 300 nm

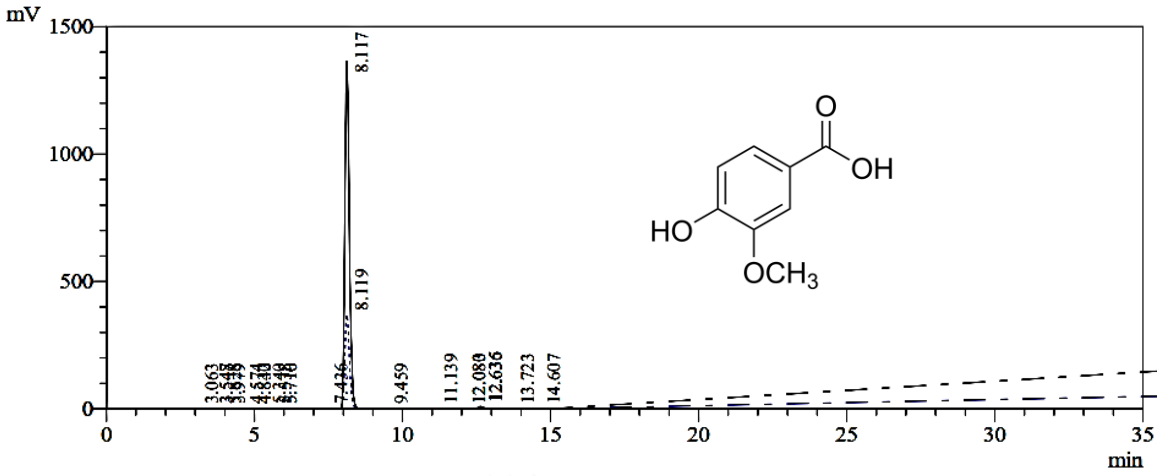


Figure A3 HPLC chromatogram of 2 mM vanillic acid (retention time = 8.11 mins)

Ferulic acid

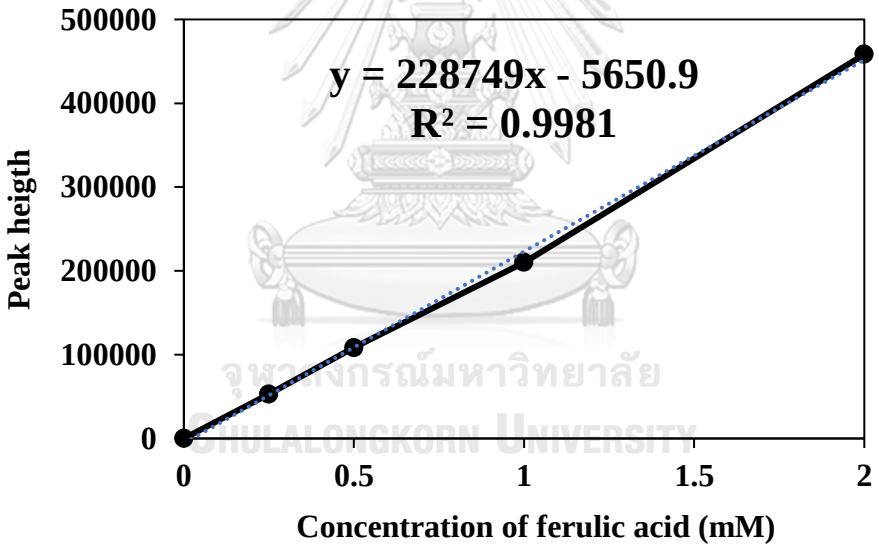


Figure A4 Standard graph of ferulic acid at 210 nm

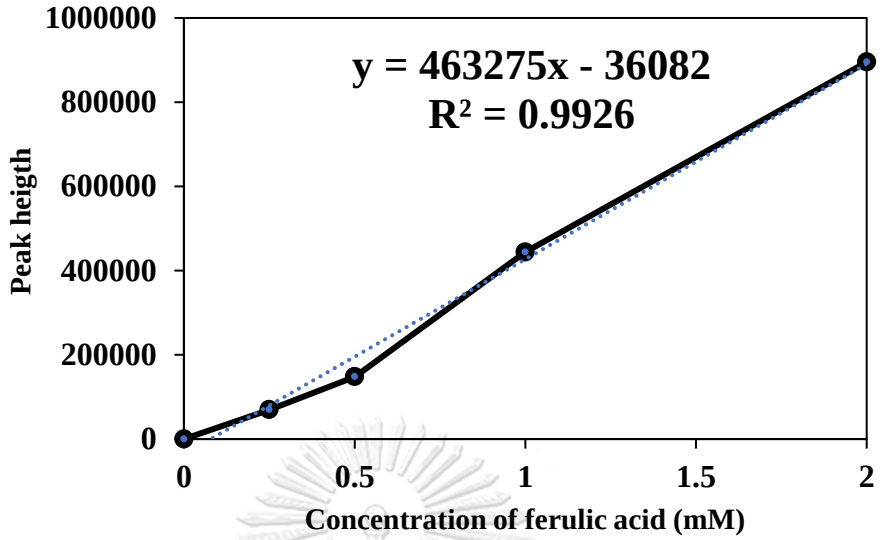


Figure A4 Standard graph of ferulic acid at 300 nm

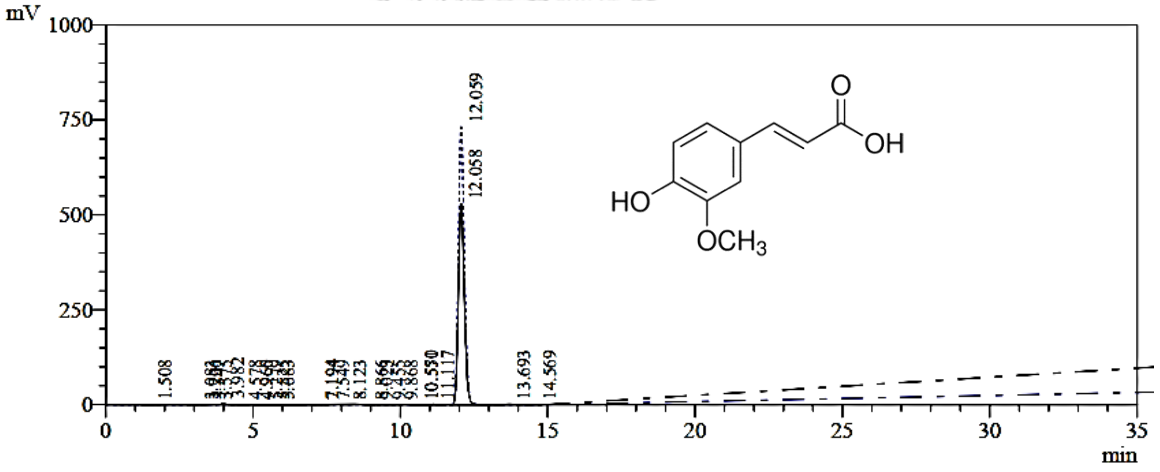


Figure A5 HPLC chromatogram of 2 mM ferulic acid (retention time = 12.05 mins)

Vanillin

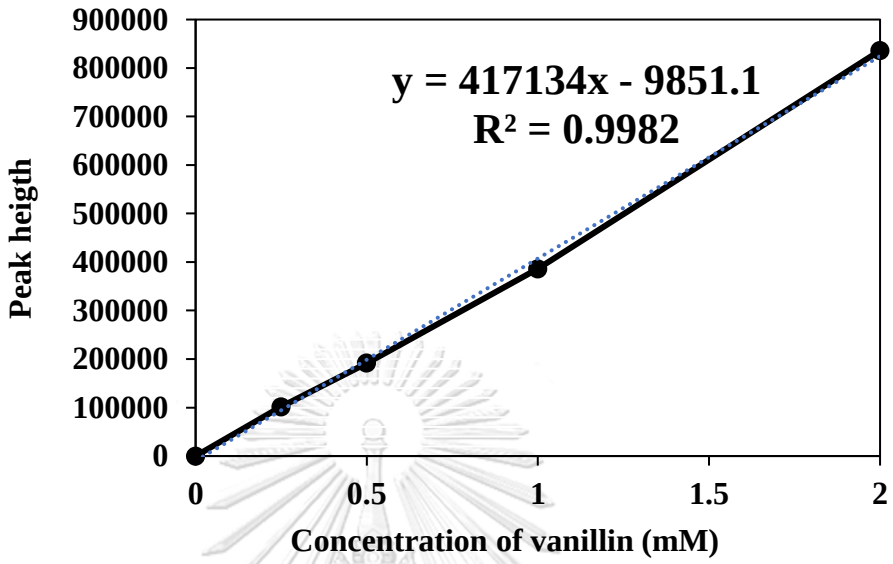


Figure A6 Standard graph of vanillin at 300 nm

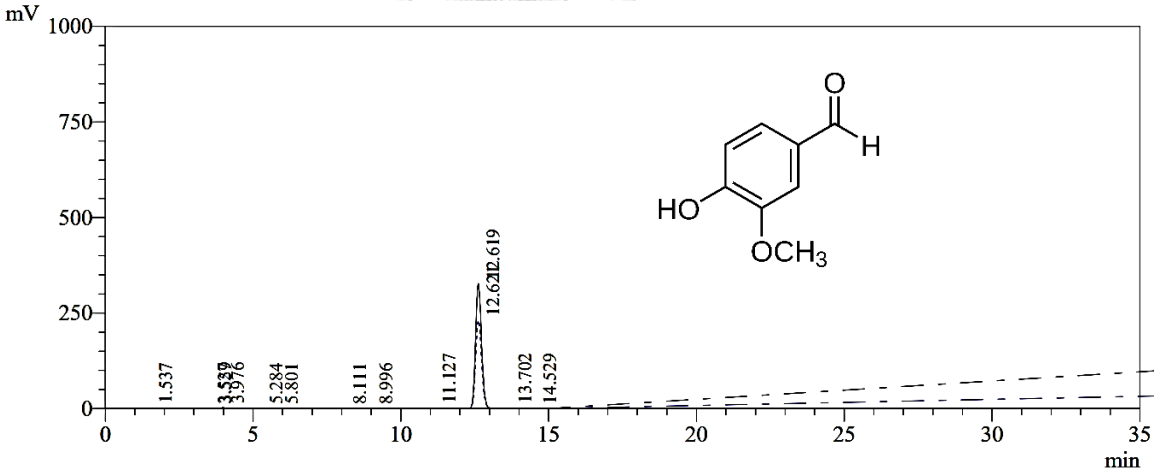


Figure A7 HPLC chromatogram of 1 mM vanillin (retention time = 12.61 mins)

Caffeic acid

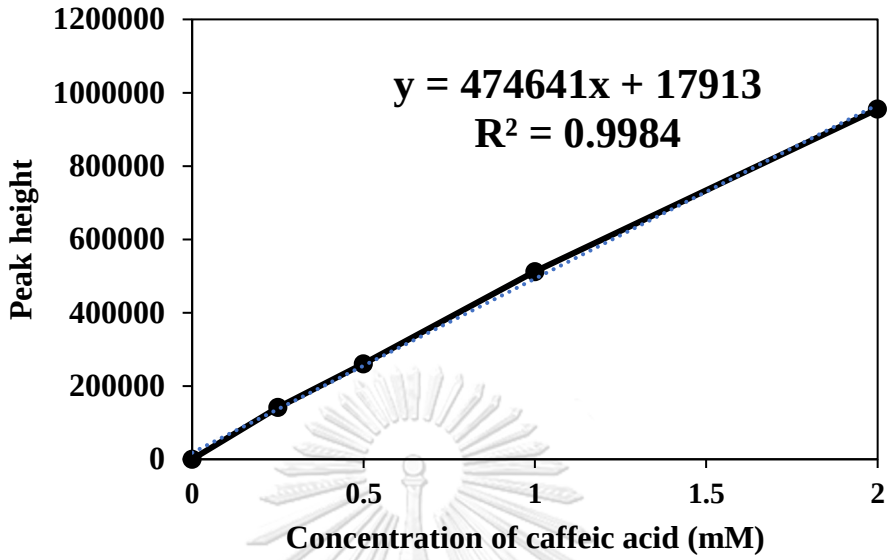


Figure A8 Standard graph of caffeic acid at 300 nm

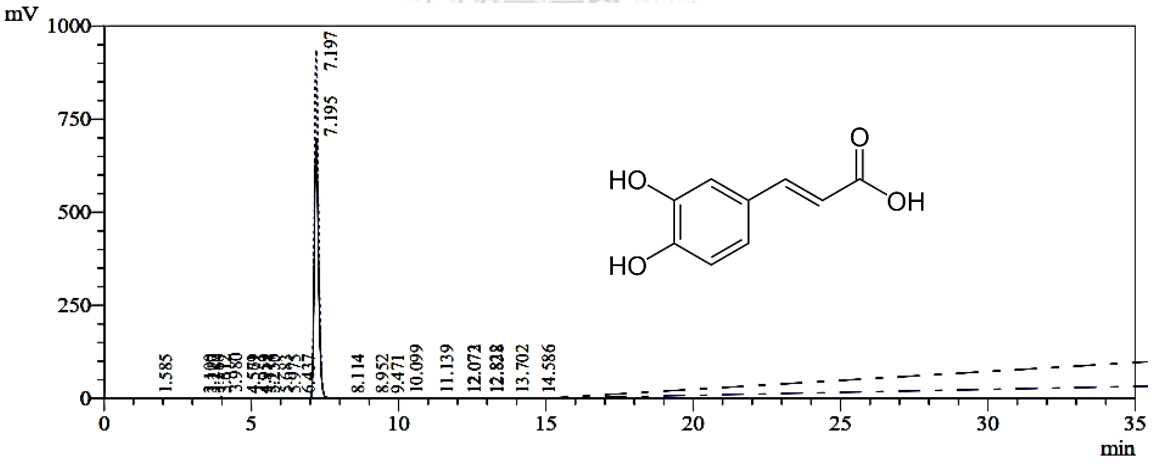


Figure A9 HPLC chromatogram of 2 mM caffeic acid (retention time = 7.19 mins)

4-Vinylguaiacol

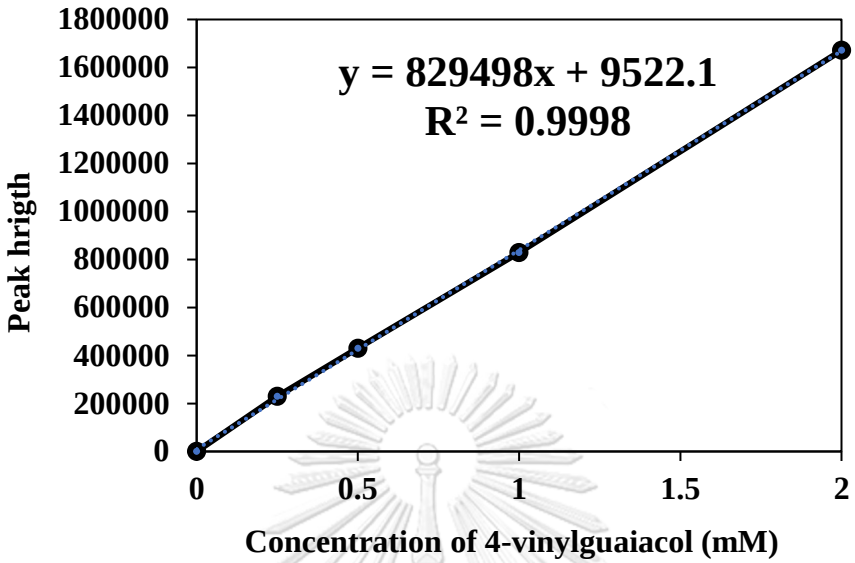


Figure A10 Standard graph of 4-vinylguaicol at 210 nm

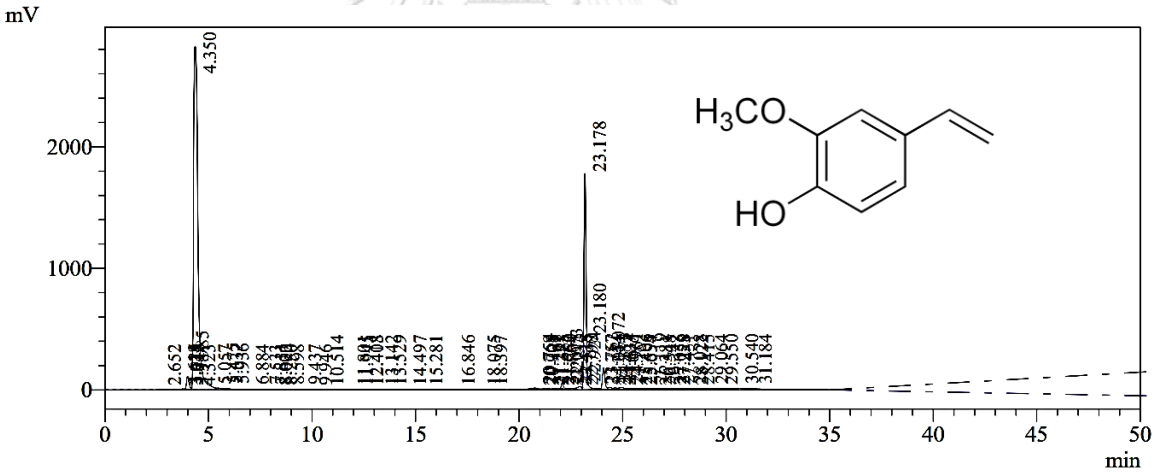


Figure A11 HPLC chromatogram of 2 mM 4-vinylguaicol (retention time = 23.17 mins)

Vanillyl alcohol

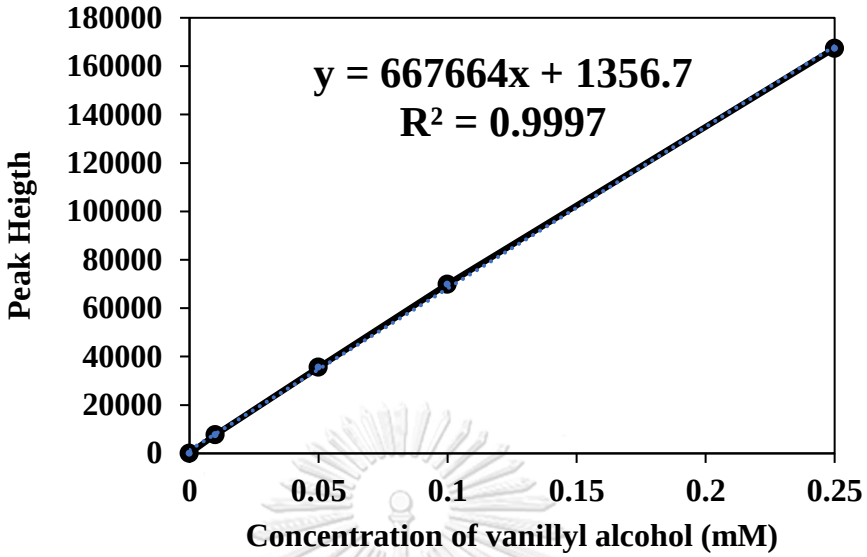


Figure A12 Standard graph of vanillyl alcohol at 210 nm

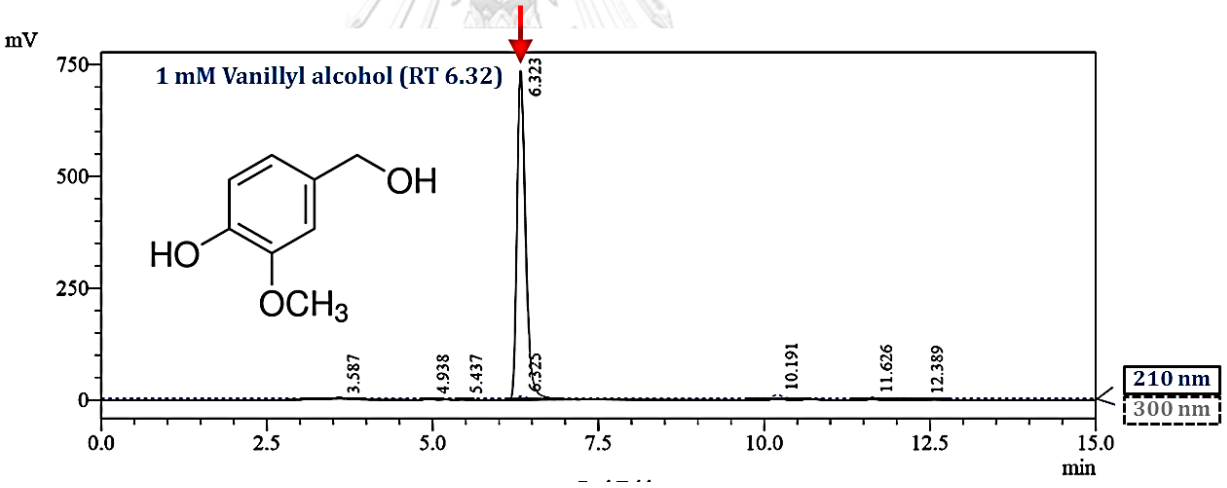


Figure A13 HPLC chromatogram of 1 mM vanillyl alcohol (retention time = 6.32 mins)

Apocynol

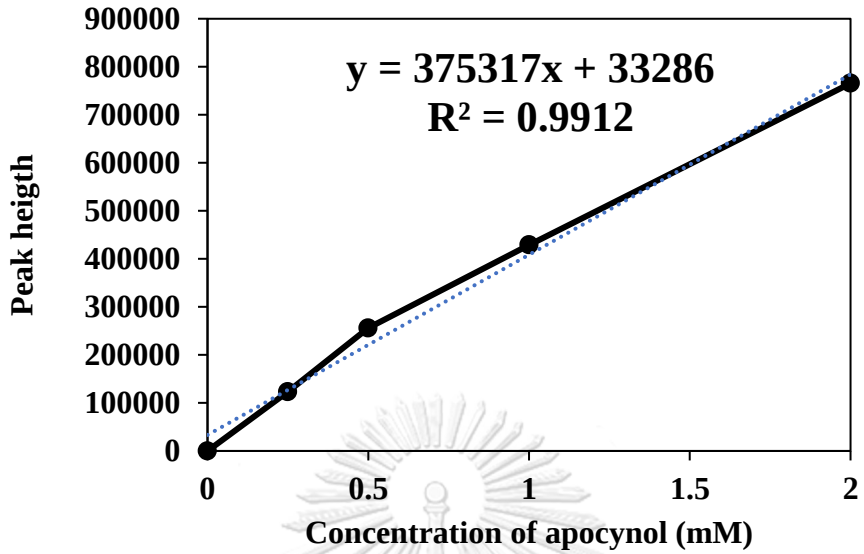


Figure A14 Standard graph of apozynol at 300 nm

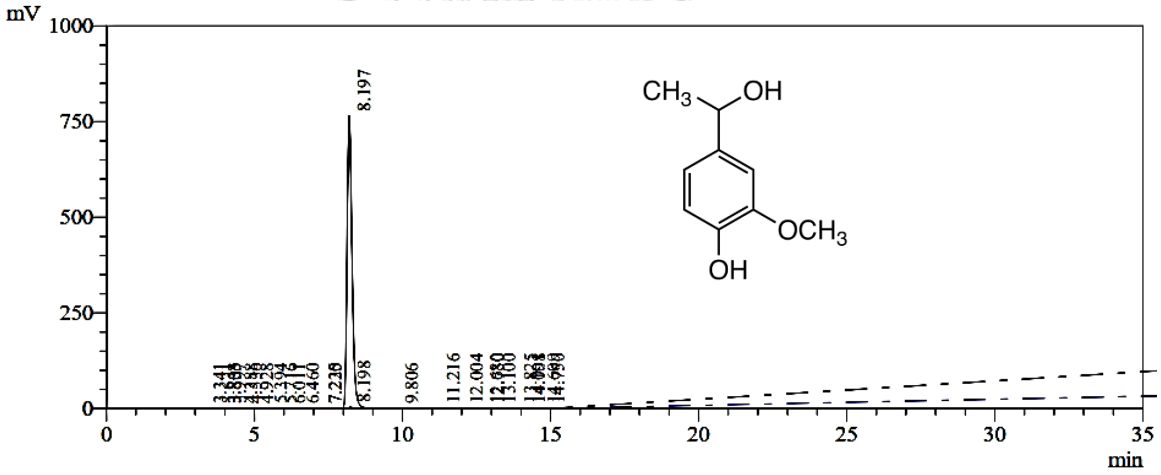


Figure A15 HPLC chromatogram of 2 mM apocynol (retention time = 8.19 mins)

HPLC chromatogram of effect of initial substrate concentration and pH on batch vanillic acid production using the resting cell of recombinant *E. coli* BL21(DE3) carrying pETDuet-*padC-vdh* and pCDFDuet-*ado*

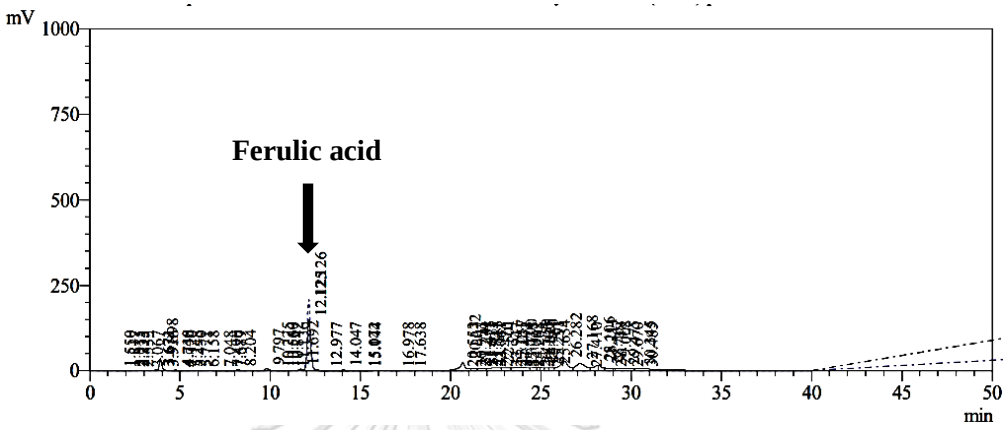


Figure A16 HPLC chromatogram obtained from batch vanillic acid production in shake flask scale using the resting cell of *E. coli* BL21(DE3) empty vector (OD_{600} = 40), conducted by Tris-HCl buffer (pH 8), 1 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 0 h.

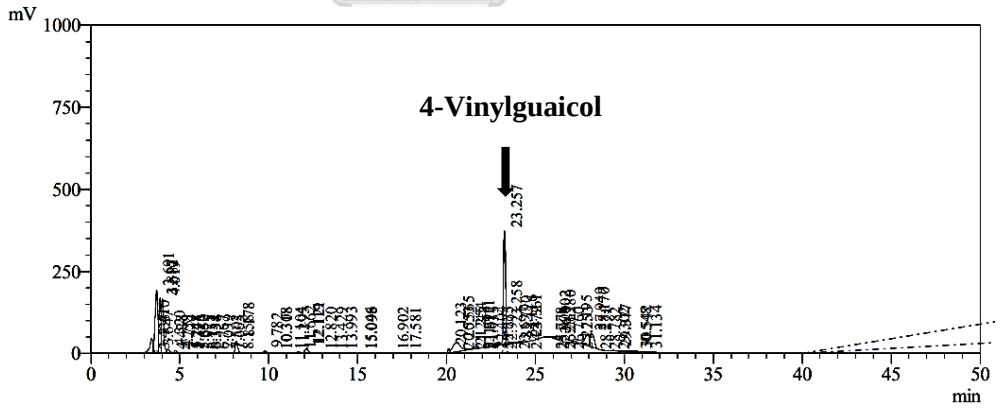


Figure A17 HPLC chromatogram obtained from batch vanillic acid production in shake flask scale using the resting cell of recombinant *E. coli* BL21(DE3)/pETDuet-*padC-vdh*/pCDFduet-*ado* (OD_{600} = 40), conducted by Tris-HCl buffer (pH 8), 1 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 0 h.

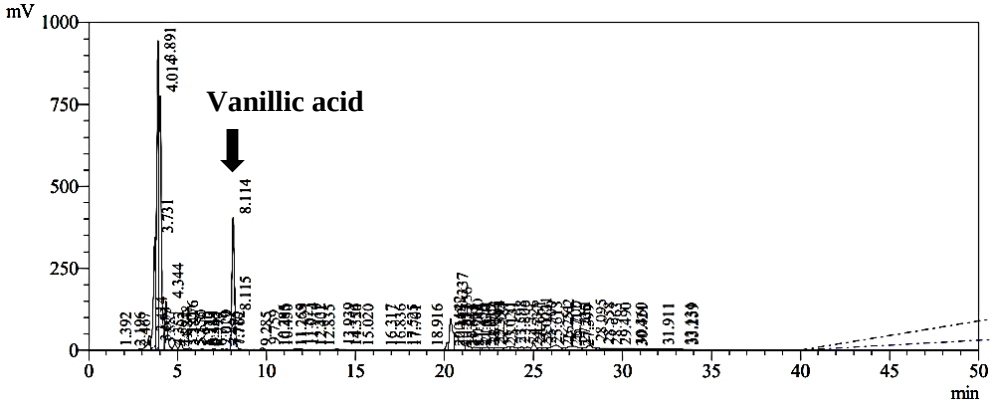


Figure A18 HPLC chromatogram obtained from batch vanillic acid production in shake flask scale using the resting cell of recombinant *E. coli* BL21(DE3)/pETDuet-*padC*-*vdh*/pCDFDuet-*ado* (OD_{600} = 40), conducted by Tris-HCl buffer (pH 8), 1 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 48 h.

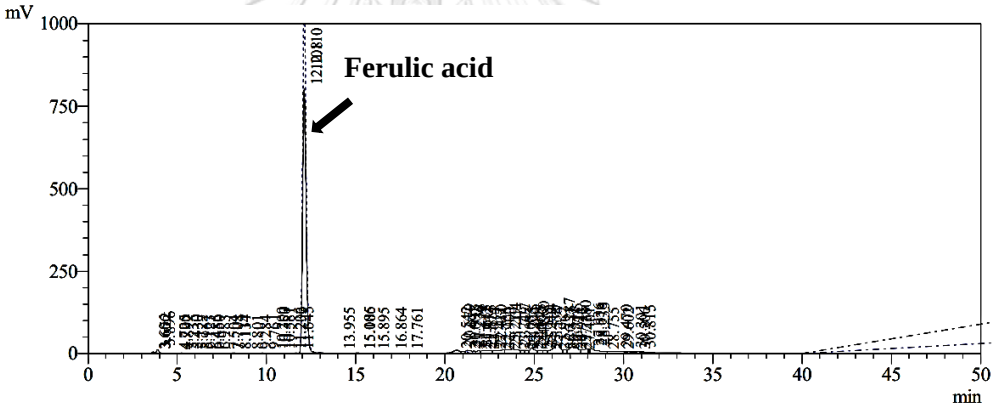


Figure A19 HPLC chromatogram obtained from batch vanillic acid production in shake flask scale using the resting cell of *E. coli* BL21(DE3) empty vector (OD_{600} = 40), conducted by Tris-HCl buffer (pH 8), 5 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 0 h.

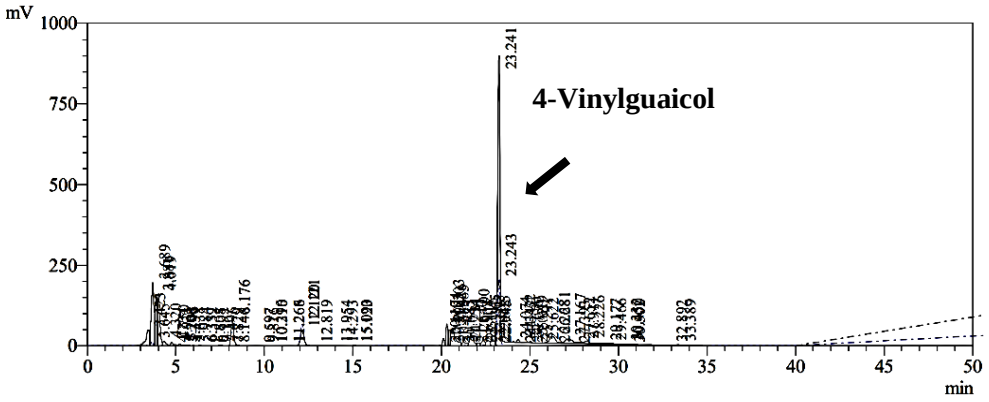


Figure A20 HPLC chromatogram obtained from batch vanillic acid production in shake flask scale using the resting cell of recombinant *E. coli* BL21(DE3)/pETDuet-*padC*-*vdh*/pCDFDuet-*ado* (OD_{600} = 40), conducted by Tris-HCl buffer (pH 8), 5 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 0 h.

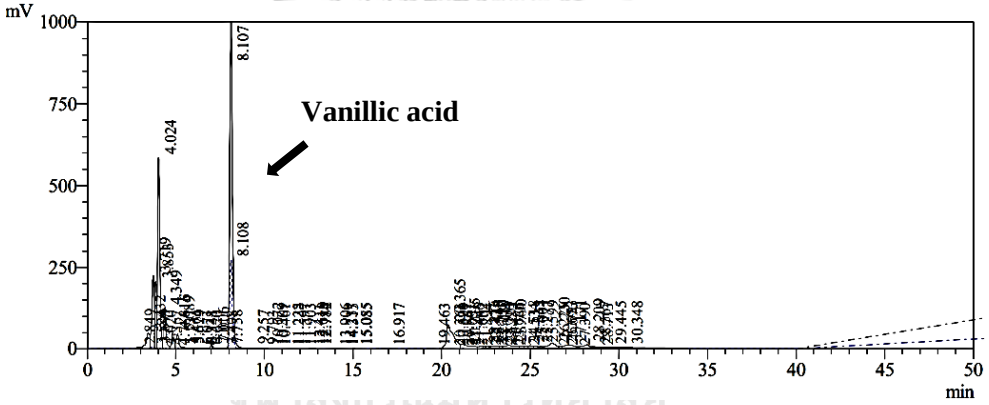


Figure A21 HPLC chromatogram obtained from batch vanillic acid production in shake flask scale using the resting cell of recombinant *E. coli* BL21(DE3)/pETDuet-*padC*-*vdh*/pCDFDuet-*ado* (OD_{600} = 40), conducted by Tris-HCl buffer (pH 8), 5 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 48 h.

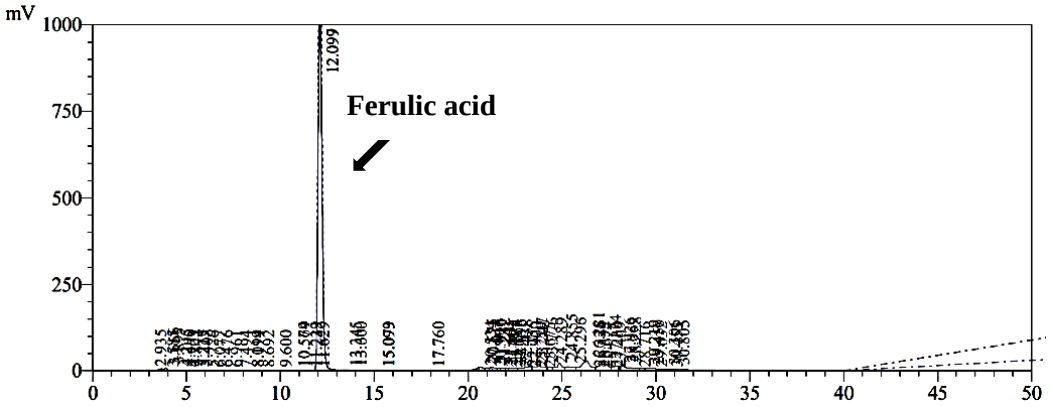


Figure A22 HPLC chromatogram obtained from batch vanillic acid production in shake flask scale using the resting cell of *E. coli* BL21(DE3) empty vector (OD_{600} = 40), conducted by Tris-HCl buffer (pH 8), 10 mM of ferulic acid as substrate, 200 rpm, and 37℃ as substrate at 0 h.

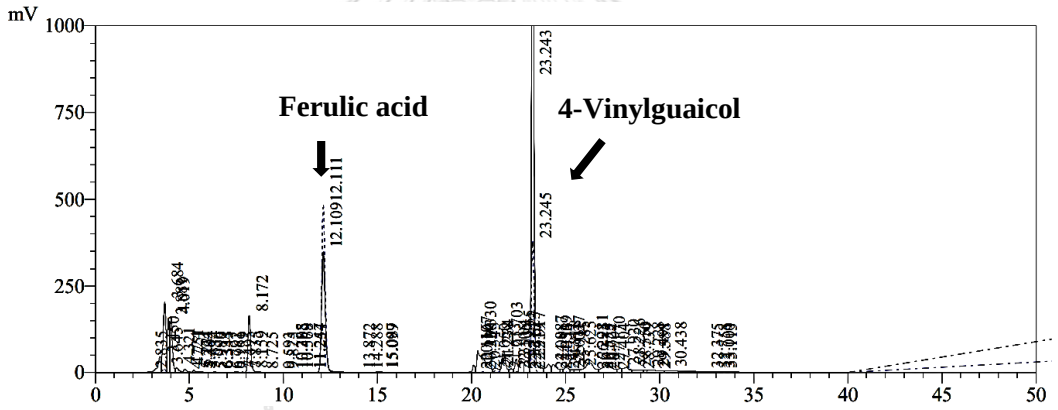


Figure A23 HPLC chromatogram obtained from batch vanillic acid production in shake flask scale using the resting cell of recombinant *E. coli* BL21(DE3)/pETDuet-*padC*-*vdh*/pCDFDuet-*ado* (OD_{600} = 40), conducted by Tris-HCl buffer (pH 8), 10 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 0 h.

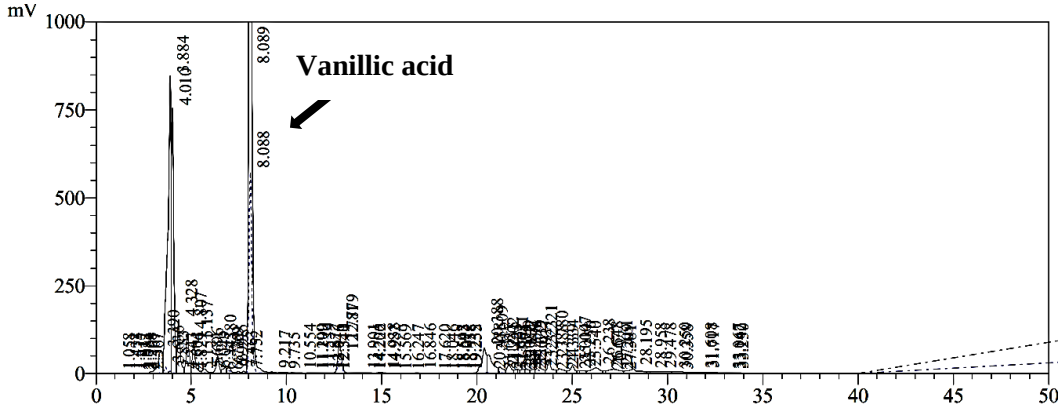


Figure A24 HPLC chromatogram obtained from batch vanillic acid production in shake flask scale using the resting cell of recombinant *E. coli* BL21(DE3)/pETDuet-*padC*-*vdh*/pCDFDuet-*ado* (OD_{600} = 40), conducted by Tris-HCl buffer (pH 8), 10 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 48 h.

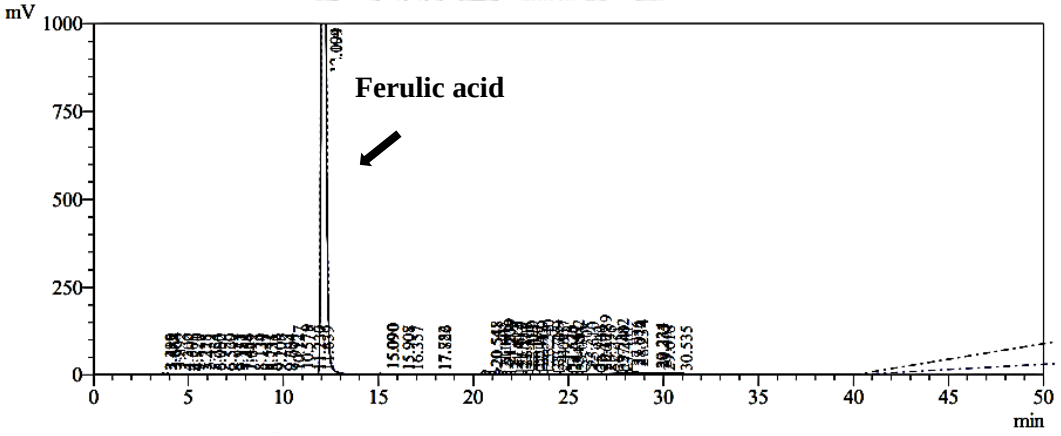


Figure A25 HPLC chromatogram obtained from batch vanillic acid production in shake flask scale using the resting cell of *E. coli* BL21(DE3) empty vector (OD_{600} = 40), conducted by Tris-HCl buffer (pH 8), 20 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 0 h.

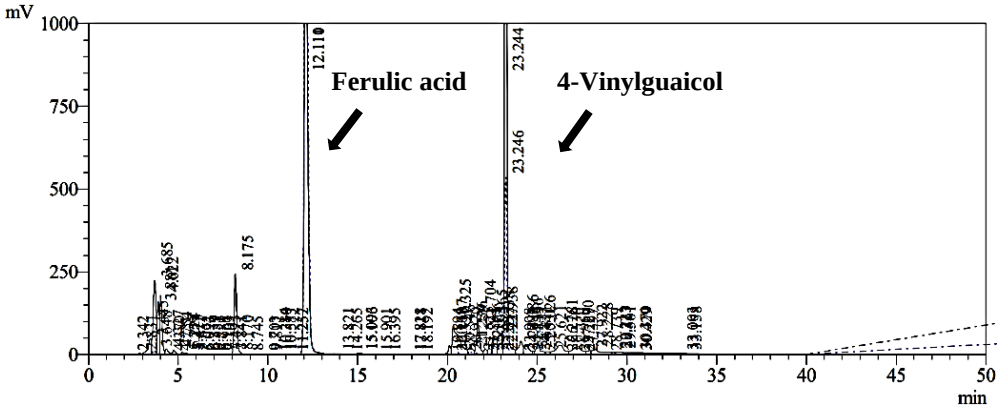


Figure A26 HPLC chromatogram obtained from batch vanillic acid production in shake flask scale using the resting cell of recombinant *E. coli* BL21(DE3)/pETDuet-*padC*-*vdh*/pCDFDuet-*ado* (OD_{600} = 40), conducted by Tris-HCl buffer (pH 8), 20 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 0 h.

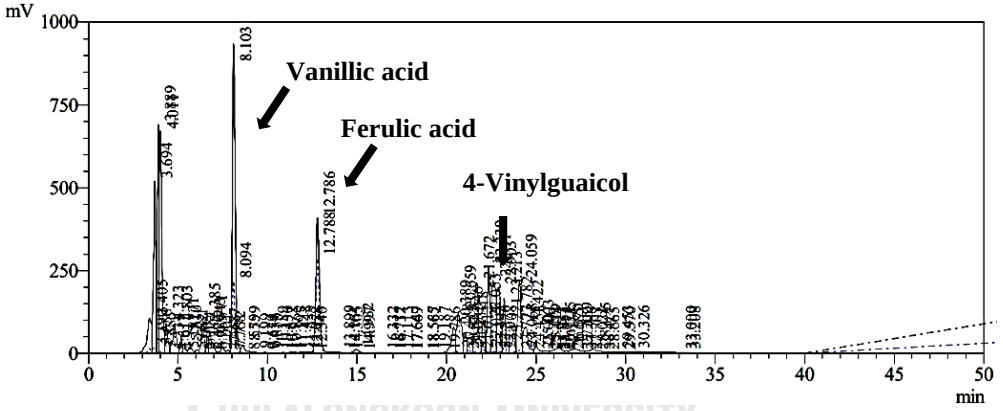


Figure A27 HPLC chromatogram obtained from batch vanillic acid production in shake flask scale using the resting cell of recombinant *E. coli* BL21(DE3)/pETDuet-*padC*-*vdh*/pCDFDuet-*ado* (OD_{600} = 40), conducted by Tris-HCl buffer (pH 8), 20 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 48 h.

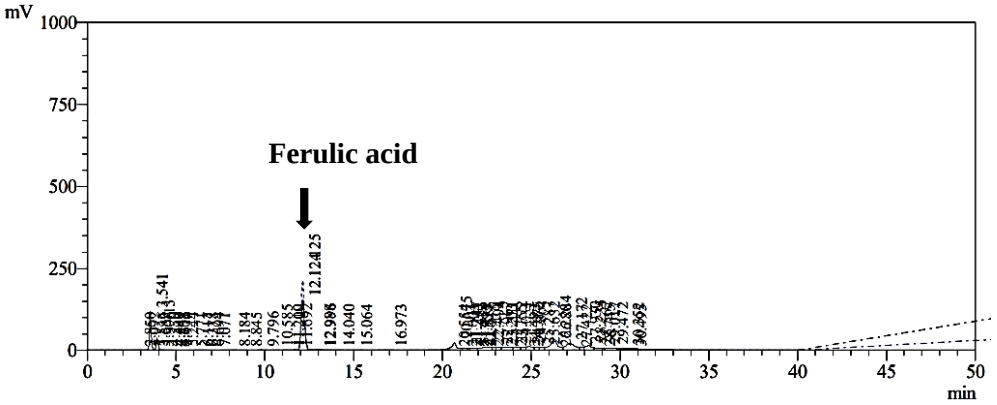


Figure A28 HPLC chromatogram obtained from batch vanillic acid production in shake flask scale using the resting cell of *E. coli* BL21(DE3) empty vector (OD_{600} = 40), conducted by glycine-NaOH buffer (pH 9), 1 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 0 h.

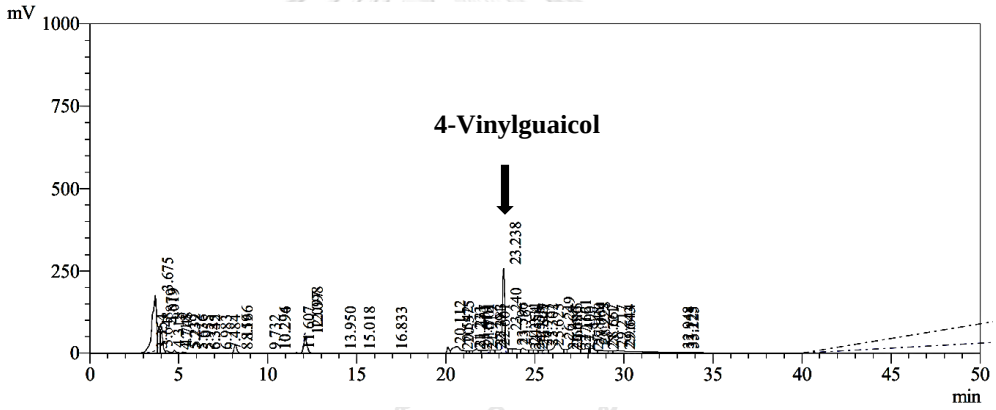


Figure A29 HPLC chromatogram obtained from batch vanillic acid production in shake flask scale using the resting cell of recombinant *E. coli* BL21(DE3)/pETDuet-*padC-vdh*/pCDFDuet-*ado* (OD_{600} = 40), conducted by glycine-NaOH buffer (pH 9), 1 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 0 h.

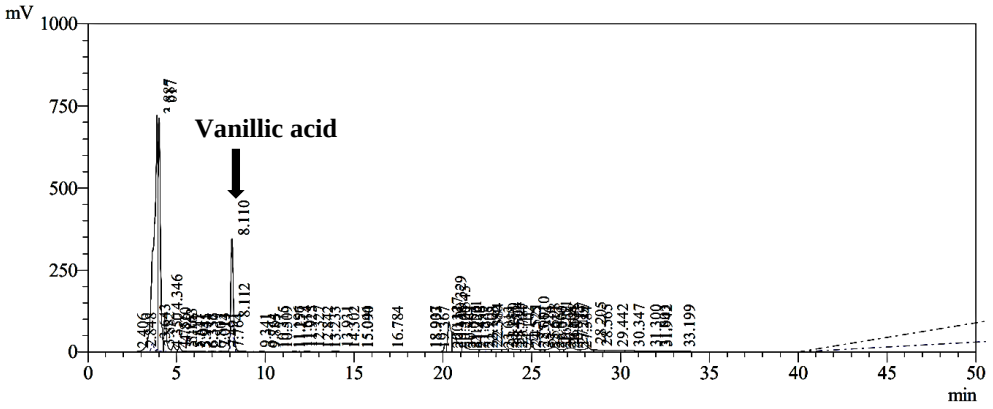


Figure A30 HPLC chromatogram obtained from batch vanillic acid production in shake flask scale using the resting cell of recombinant *E. coli* BL21(DE3)/pETDuet-*padC-vdh*/pCDFDuet-*ado* (OD_{600} = 40), conducted by glycine-NaOH buffer (pH 9), 1 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 0 h.

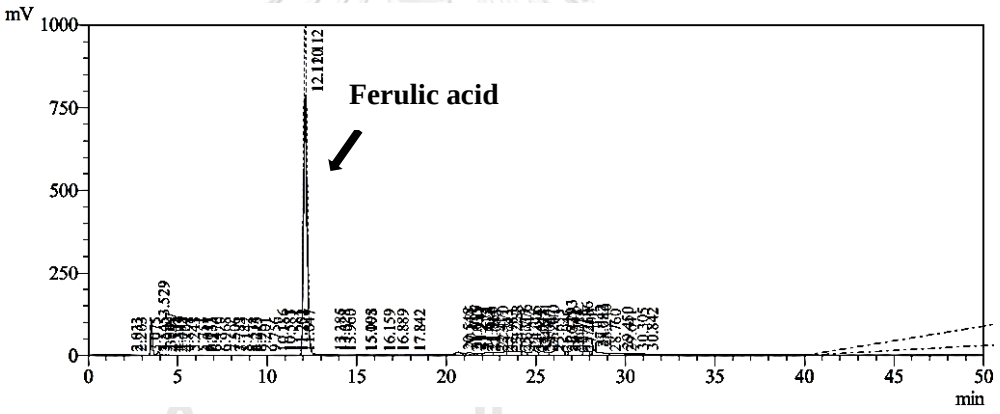


Figure A31 HPLC chromatogram obtained from batch vanillic acid production in shake flask scale using the resting cell of *E. coli* BL21(DE3) empty vector (OD_{600} = 40), conducted by glycine-NaOH buffer (pH 9), 5 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 0 h.

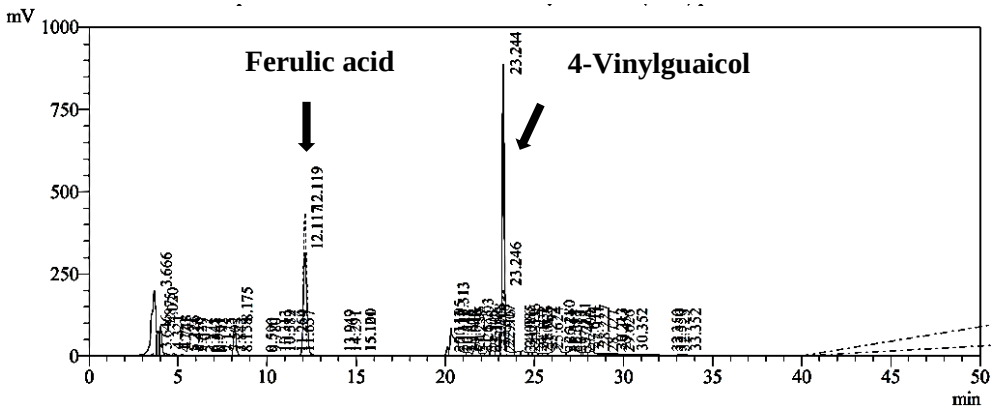


Figure A32 HPLC chromatogram obtained from batch vanillic acid production in shake flask scale using the resting cell of recombinant *E. coli* BL21(DE3)/pETDuet-*padC-vdh*/pCDFDuet-*ado* (OD_{600} = 40), conducted by glycine-NaOH buffer (pH 9), 5 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 0 h.

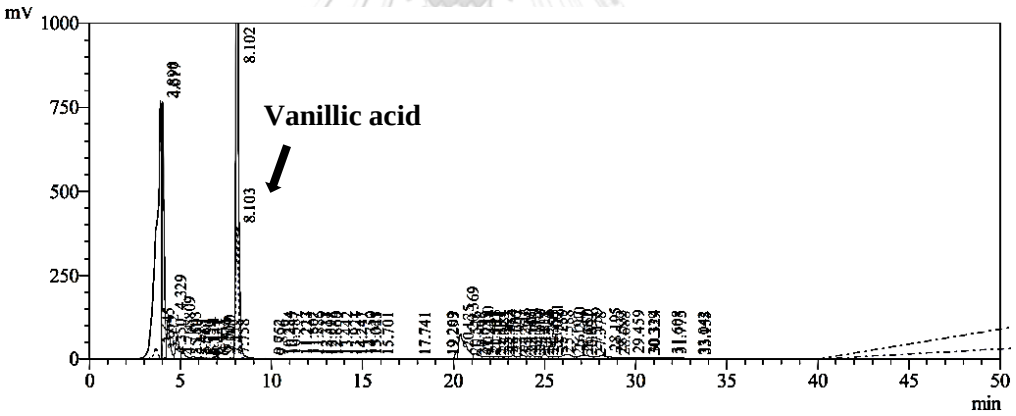


Figure A33 HPLC chromatogram obtained from batch vanillic acid production in shake flask scale using the resting cell of recombinant *E. coli* BL21(DE3)/pETDuet-*padC-vdh*/pCDFDuet-*ado* (OD_{600} = 40), conducted by glycine-NaOH buffer (pH 9), 5 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 48 h.

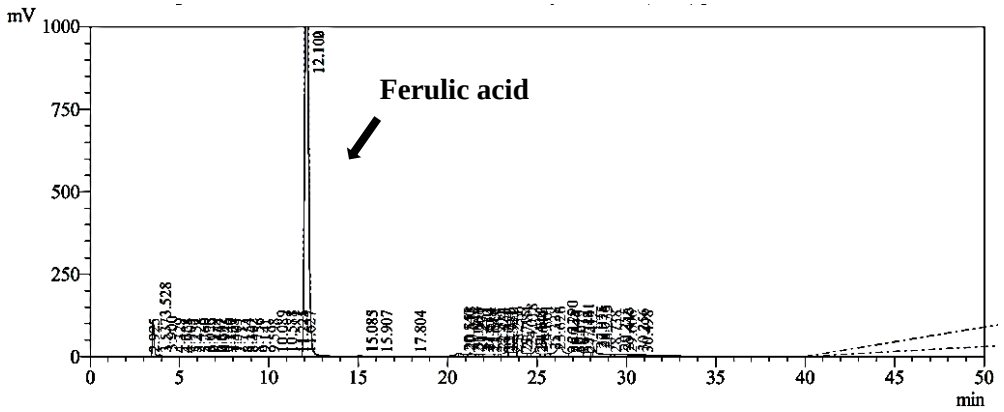


Figure A34 HPLC chromatogram obtained from batch vanillic acid production in shake flask scale using the resting cell of *E. coli* BL21(DE3) empty vector (OD_{600} = 40), conducted by glycine-NaOH buffer (pH 9), 10 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 0 h.

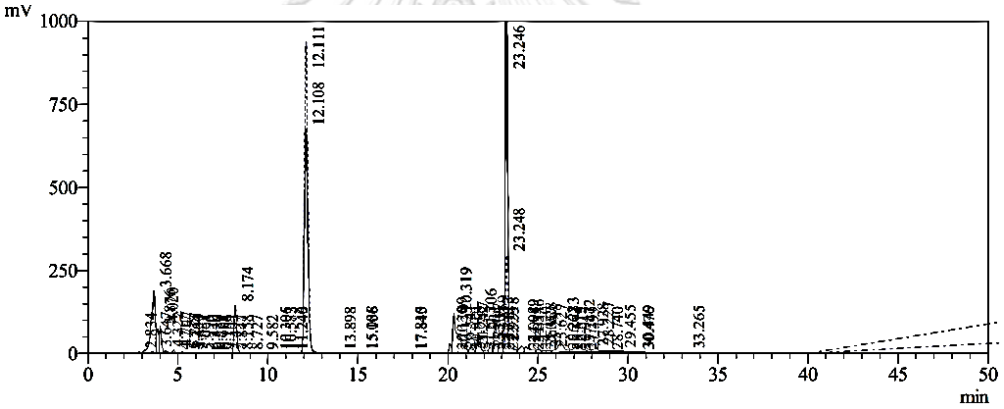


Figure A35 HPLC chromatogram obtained from batch vanillic acid production in shake flask scale using the resting cell of recombinant *E. coli* BL21(DE3)/ pETDuet-*padC*-*vdh*/pCDFDuet-*ado* (OD_{600} = 40), conducted by glycine-NaOH buffer (pH 9), 10 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 0 h.

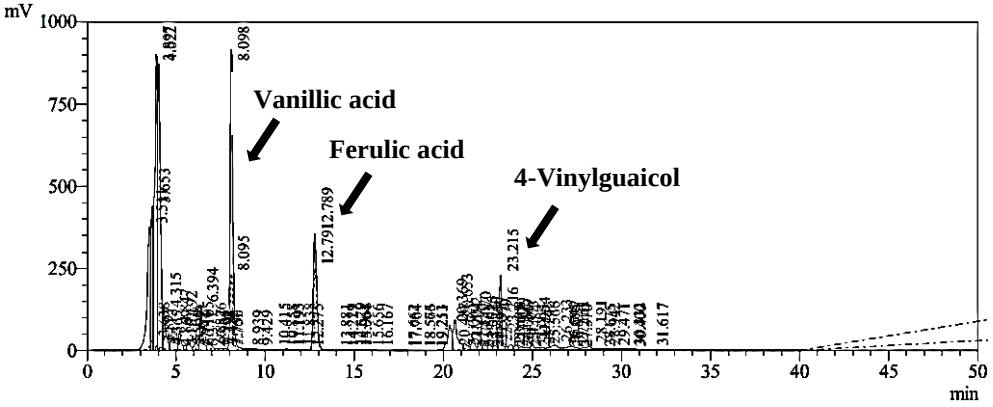


Figure A36 HPLC chromatogram obtained from batch vanillic acid production in shake flask scale using the resting cell of recombinant *E. coli* BL21(DE3)/pETDuet-*padC-vdh*/pCDFDuet-*ado* (OD_{600} = 40), conducted by glycine-NaOH buffer (pH 9), 10 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 48 h.

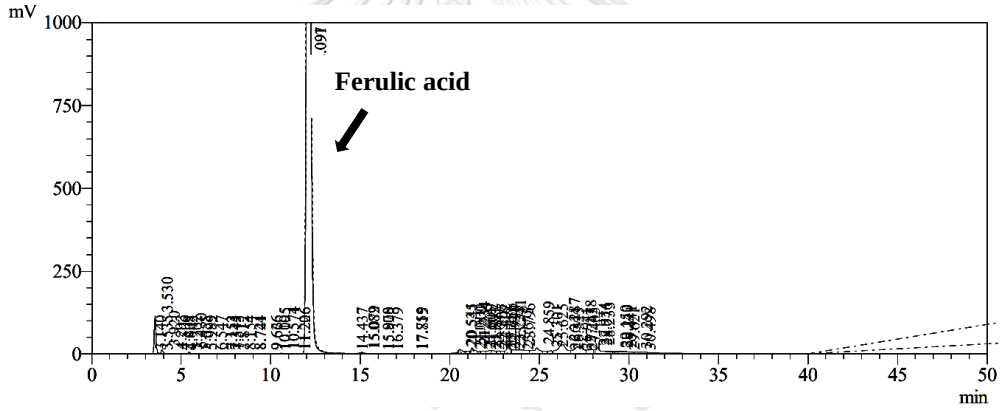


Figure A37 HPLC chromatogram obtained from batch vanillic acid production in shake flask scale using the resting cell of *E. coli* BL21(DE3) empty vector (OD_{600} = 40), conducted by glycine-NaOH buffer (pH 9), 20 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 0 h.

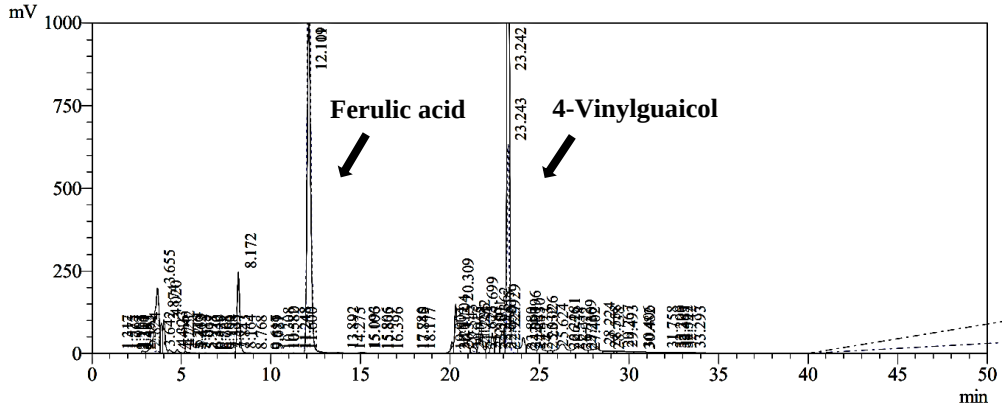


Figure A38 HPLC chromatogram obtained from batch vanillic acid production in shake flask scale using the resting cell of recombinant *E. coli* BL21(DE3)/pETDuet-*padC-vdh*/pCDFDuet-*ado* (OD_{600} = 40), conducted by glycine-NaOH buffer (pH 9), 20 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 0 h.

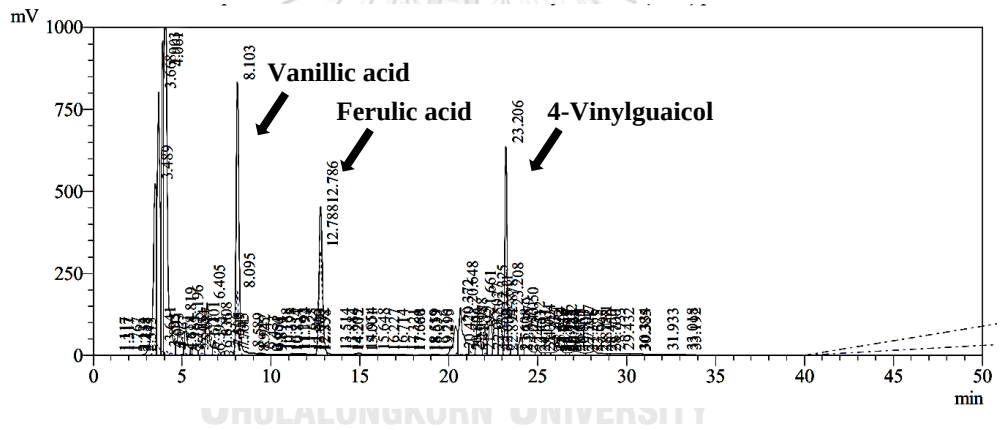


Figure A39 HPLC chromatogram obtained from batch vanillic acid production in shake flask scale using the resting cell of recombinant *E. coli* BL21(DE3)/ pETDuet-*padC-vdh*/pCDFDuet-*ado* (OD_{600} = 40), conducted by glycine-NaOH buffer (pH 9), 20 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 48 h.

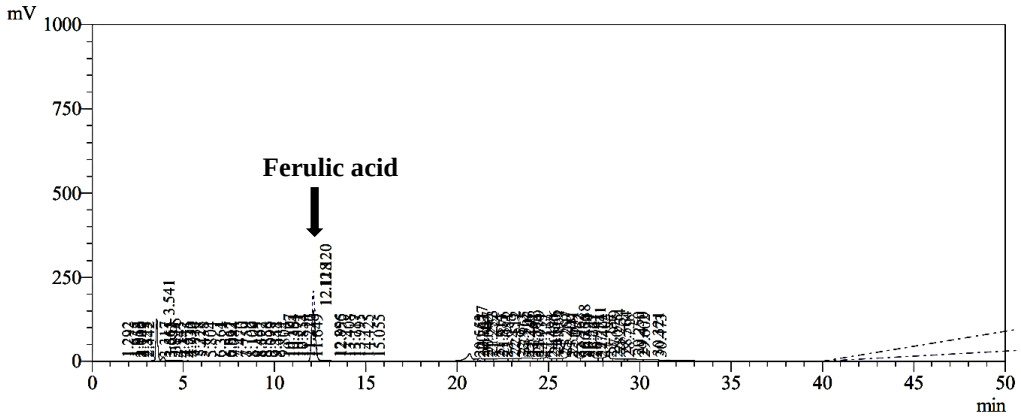


Figure A37 HPLC chromatogram obtained from batch vanillic acid production in shake flask scale using the resting cell of *E. coli* BL21(DE3) empty vector (OD_{600} = 40), conducted by glycine-NaOH buffer (pH 10), 1 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 0 h.

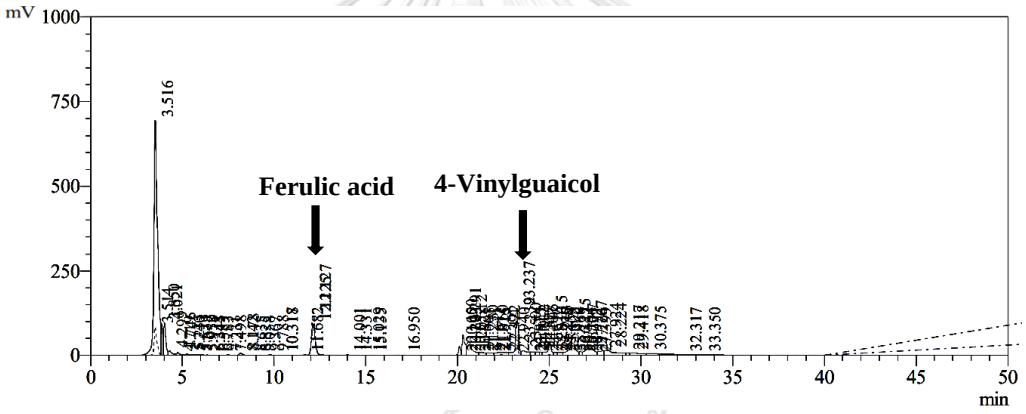


Figure A38 HPLC chromatogram obtained from batch vanillic acid production in shake flask scale using the resting cell of recombinant *E. coli* BL21(DE3)/ pETDuet-*padC-vdh*/pCDFDuet-*ado* (OD_{600} = 40), conducted by glycine-NaOH buffer (pH 10), 1 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 0 h.

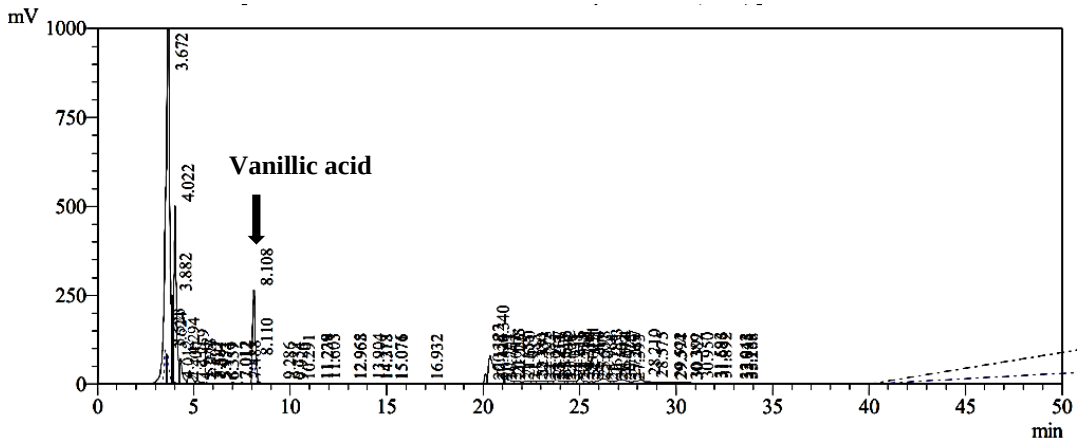


Figure A39 HPLC chromatogram obtained from batch vanillic acid production in shake flask scale using the resting cell of recombinant *E. coli* BL21(DE3)/pETDuet-*padC-vdh*/pCDFDuet-*ado* (OD_{600} = 40), conducted by glycine-NaOH buffer (pH 10), 1 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 48 h.

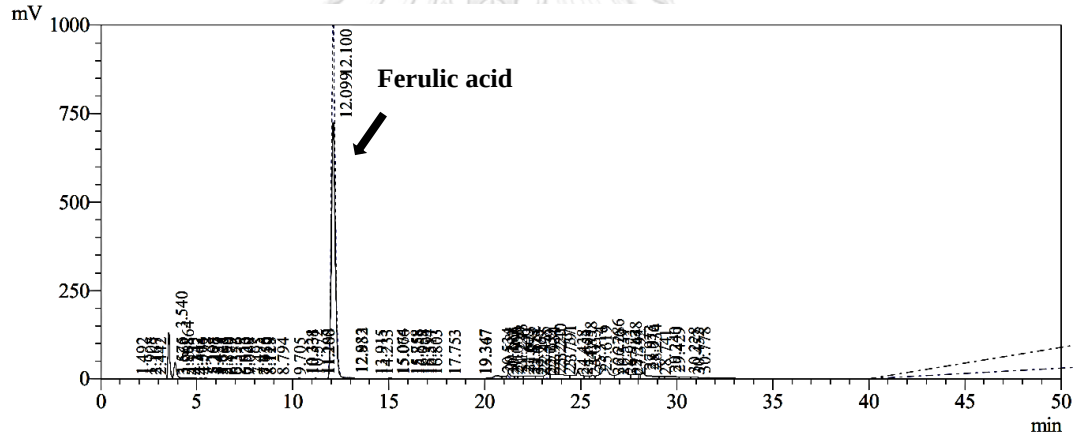


Figure A40 HPLC chromatogram obtained from batch vanillic acid production in shake flask scale using the resting cell of *E. coli* BL21(DE3) empty vector (OD_{600} = 40), conducted by glycine-NaOH buffer (pH 10), 5 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 0 h.

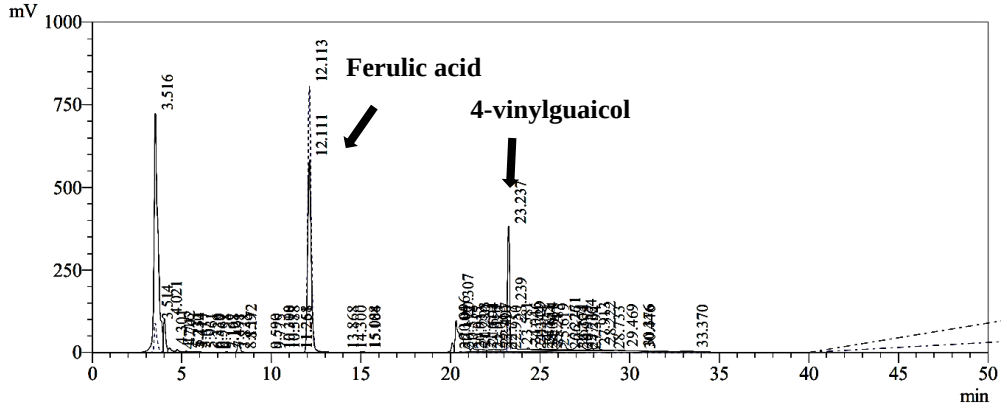


Figure A41 HPLC chromatogram obtained from batch vanillic acid production in shake flask scale using the resting cell of recombinant *E. coli* BL21(DE3)/pETDuet-*padC-vdh*/pCDFDuet-*ado* (OD_{600} = 40), conducted by glycine-NaOH buffer (pH 10), 5 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 0 h.

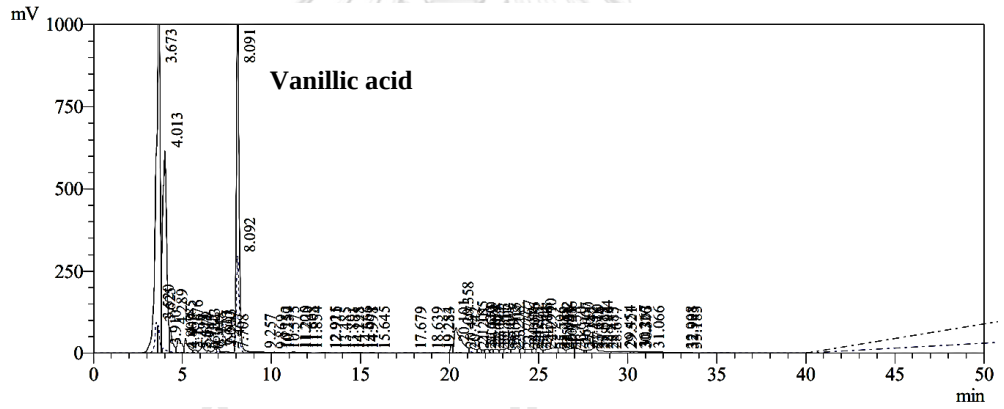


Figure A42 HPLC chromatogram obtained from batch vanillic acid production in shake flask scale using the resting cell of recombinant *E. coli* BL21(DE3)/pETDuet-*padC-vdh*/pCDFDuet-*ado* (OD_{600} = 40), conducted by glycine-NaOH buffer (pH 10), 5 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 48 h.

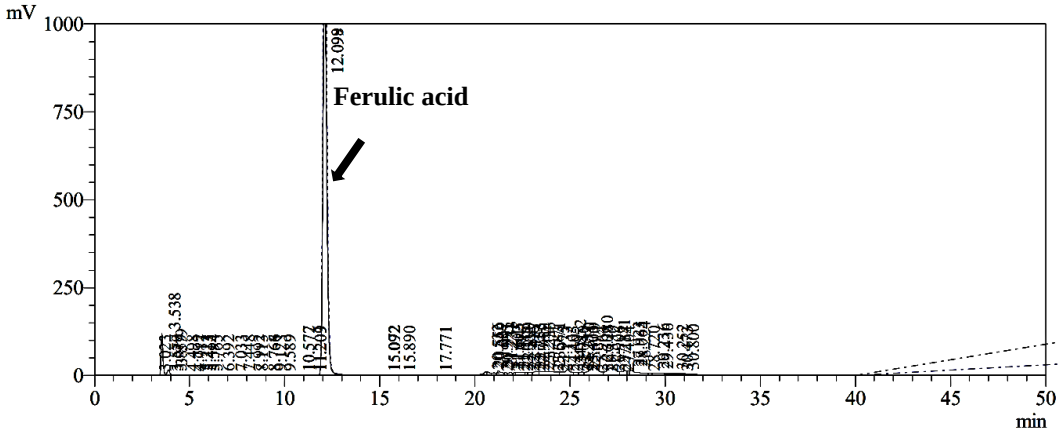


Figure A43 HPLC chromatogram obtained from batch vanillic acid production in shake flask scale using the resting cell of *E. coli* BL21(DE3) empty vector (OD_{600} = 40), conducted by glycine-NaOH buffer (pH 10), 10 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 0 h.

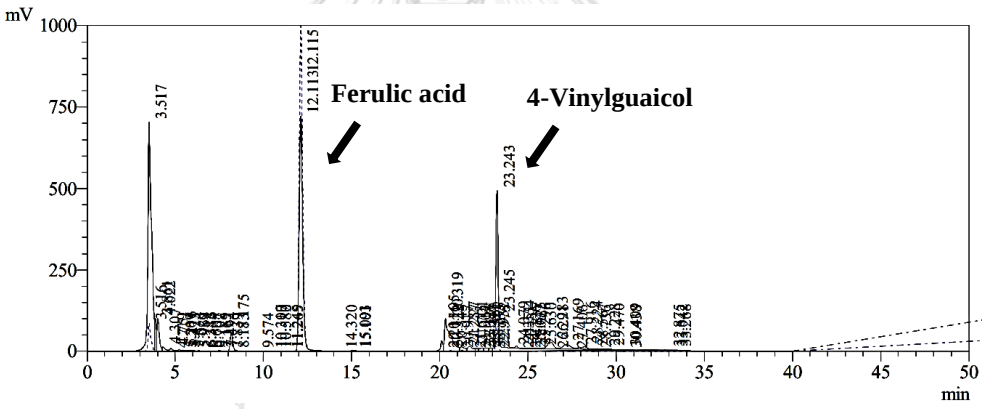


Figure A44 HPLC chromatogram obtained from batch vanillic acid production in shake flask scale using the resting cell of recombinant *E. coli* BL21(DE3)/pETDuet-*padC-vdh*/pCDFDuet-*ado* (OD_{600} = 40), conducted by glycine-NaOH buffer (pH 10), 10 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 0 h.

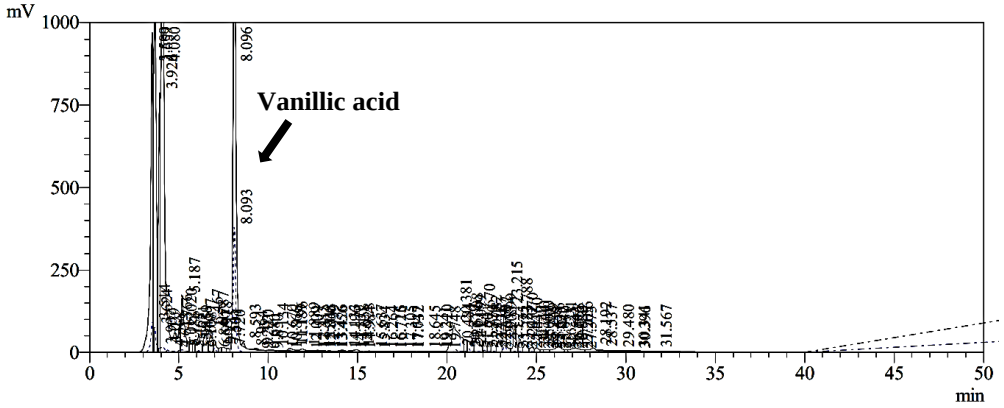


Figure A45 HPLC chromatogram obtained from batch vanillic acid production in shake flask scale using the resting cell of recombinant *E. coli* BL21(DE3)/pETDuet-*padC-vdh*/pCDFDuet-*ado* (OD_{600} = 40), conducted by glycine-NaOH buffer (pH 10), 10 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 48 h.

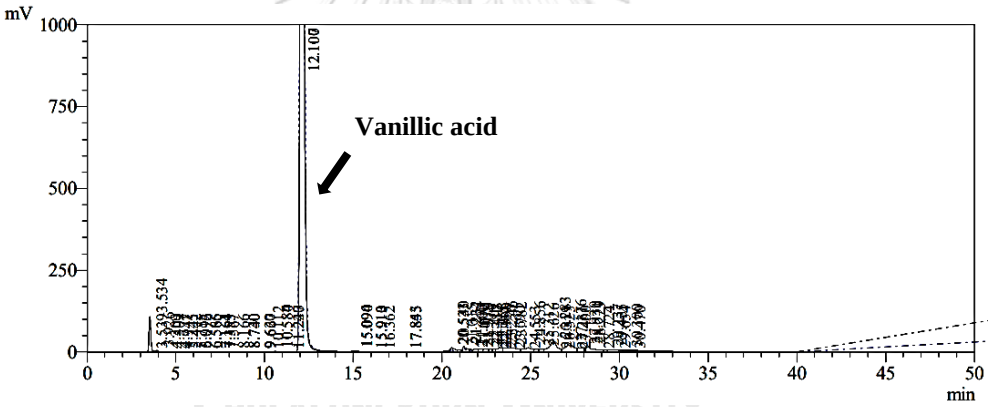


Figure A46 HPLC chromatogram obtained from batch vanillic acid production in shake flask scale using the resting cell of *E. coli* BL21(DE3) empty vector (OD_{600} = 40), conducted by glycine-NaOH buffer (pH 10), 20 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 0 h.

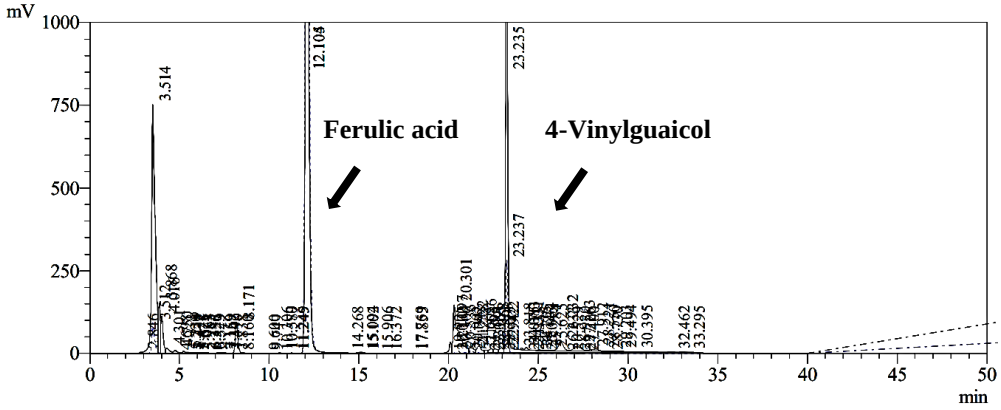


Figure A47 HPLC chromatogram obtained from batch vanillic acid production in shake flask scale using the resting cell of recombinant *E. coli* BL21(DE3)/pETDuet-*padC-vdh*/pCDFDuet-*ado* (OD_{600} = 40), conducted by glycine-NaOH buffer (pH 10), 20 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 0 h.

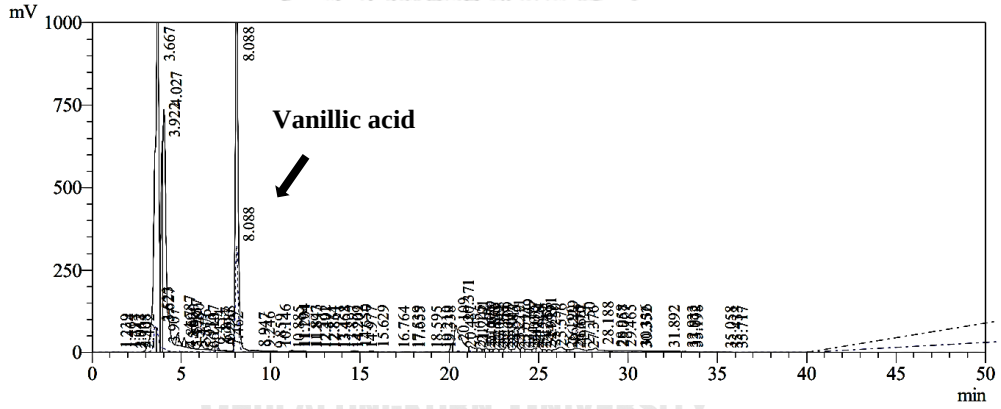


Figure A48 HPLC chromatogram obtained from batch vanillic acid production in shake flask scale using the resting cell of recombinant *E. coli* BL21(DE3)/pETDuet-*padC-vdh*/pCDFDuet-*ado* (OD_{600} = 40), conducted by glycine-NaOH buffer (pH 10), 20 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 48 h.

HPLC chromatogram of the effect of different pH on fed-batch vanillic acid production

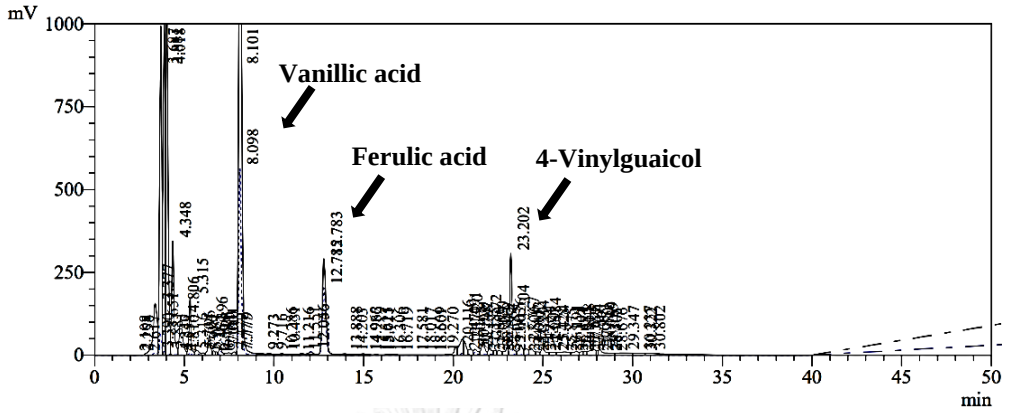


Figure A49 HPLC chromatogram obtained from fed-batch vanillic acid production in shake flask scale using the resting cell of recombinant *E. coli* BL21(DE3)/pETDuet-*padC-vdh*/pCDFDuet-*ado* (OD_{600} = 40), conducted by Tris-HCl buffer (pH 8), 4 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 48 h.

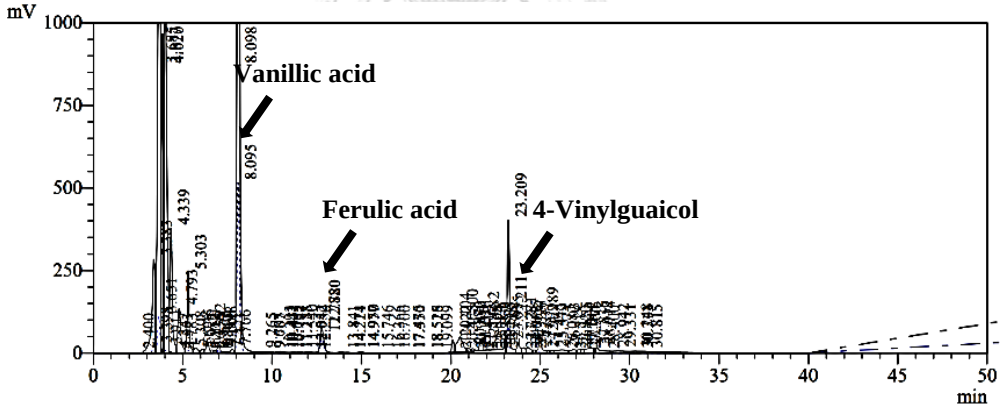


Figure A50 HPLC chromatogram obtained from fed-batch vanillic acid production in shake flask scale using the resting cell of recombinant *E. coli* BL21(DE3)/pETDuet-*padC-vdh*/pCDFDuet-*ado* (OD_{600} = 40), conducted by glycine-NaOH buffer (pH 9), 4 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 48 h.

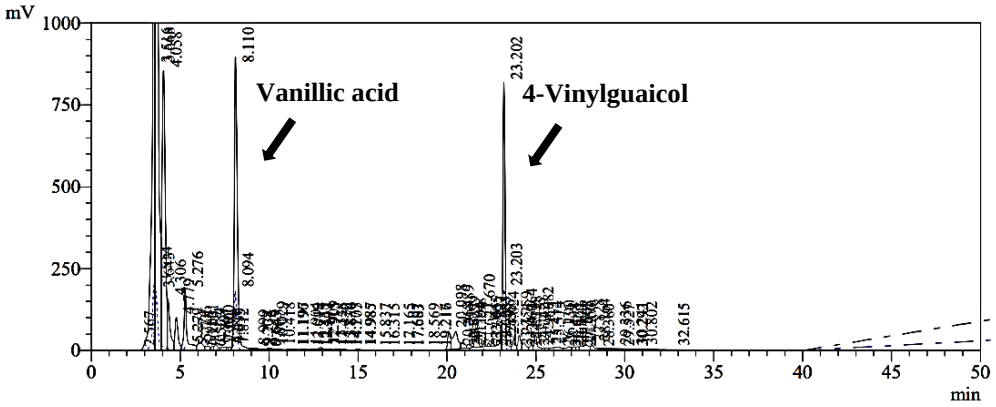


Figure A51 HPLC chromatogram obtained from fed-batch vanillic acid production in shake flask scale using the resting cell of recombinant *E. coli* BL21(DE3)/pETDuet-*padC-vdh*/pCDFDuet-*ado* (OD_{600} = 40), conducted by glycine-NaOH buffer (pH 10), 4 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 48 h.

HPLC chromatogram of vanillic acid production by a two-step bioprocess using the recombinant E. coli BL21(DE3) resting cell in shake flask scale

First-step production: bioconversion of caffeic acid to ferulic acid

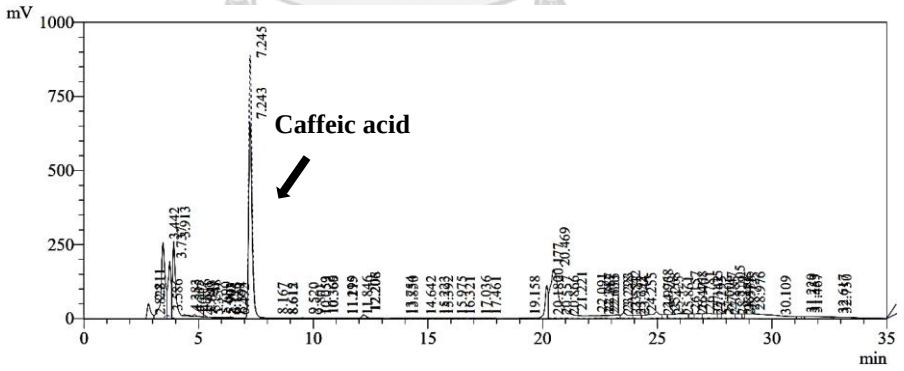


Figure A52 HPLC chromatogram obtained from the batch shake flask production of ferulic acid in two-step bioprocess for vanillic acid production using the resting cell of recombinant *E. coli* BL21(DE3)/pRSFDuet-*comt* (OD_{600} = 40), conducted by potassium phosphate buffer (pH 7.5), 2 mM of caffeic acid as substrate, 2 mM of DL-methionine as methyl donor, 200 rpm, and 26℃ at 0 h.

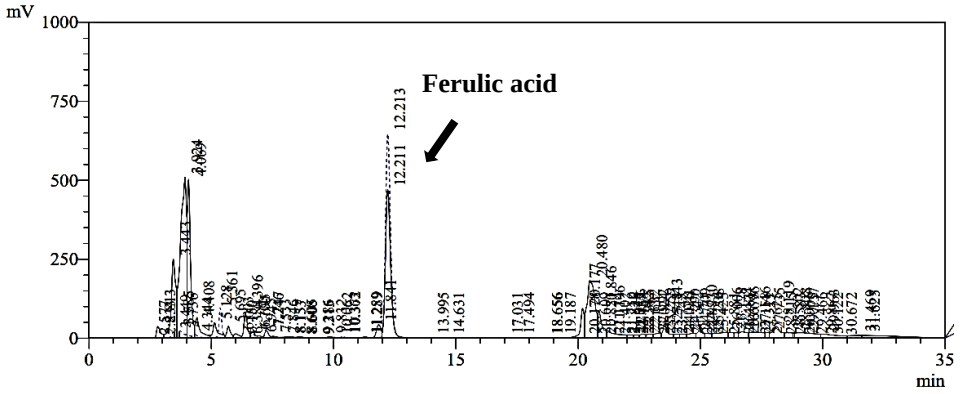


Figure A53 HPLC chromatogram obtained from the batch shake flask production of ferulic acid in two-step bioprocess for vanillic acid production using the resting cell of recombinant *E. coli* BL21(DE3)/pRSFDuet-*comt* (OD_{600} = 40), conducted by potassium phosphate buffer (pH 7.5), 2 mM of caffeic acid as substrate, 2 mM of DL-methionine as methyl donor, 200 rpm, and 26℃ at 36 h.

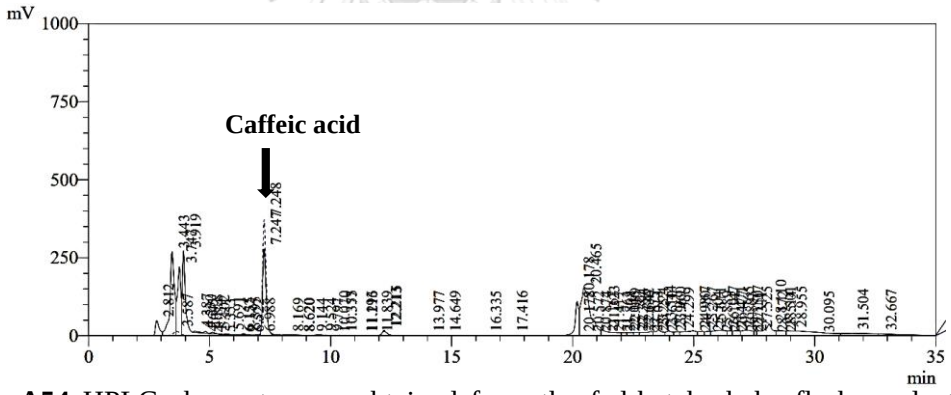


Figure A54 HPLC chromatogram obtained from the fed-batch shake flask production of ferulic acid in two-step bioprocess for vanillic acid production using the resting cell of recombinant *E. coli* BL21(DE3)/pRSFDuet-*comt* (OD_{600} = 40), conducted by potassium phosphate buffer (pH 7.5), 2 mM of caffeic acid as substrate, 2 mM of DL-methionine as methyl donor, 200 rpm, and 26℃ at 0 h.

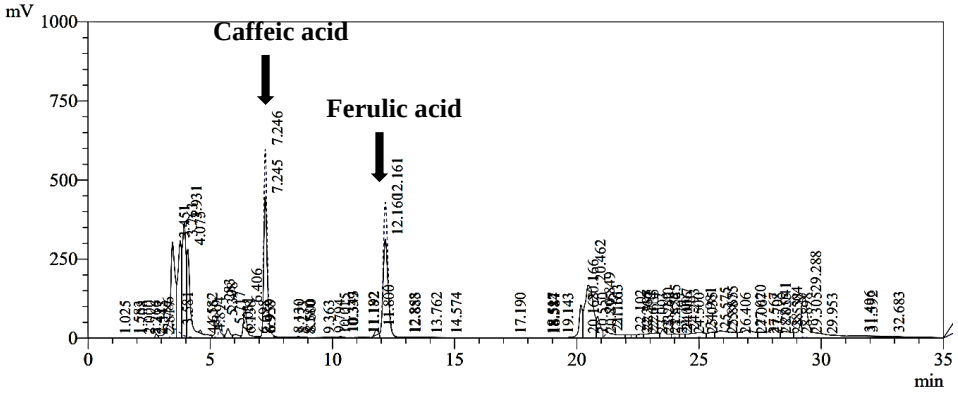


Figure A55 HPLC chromatogram obtained from the fed-batch shake flask production of ferulic acid in two-step bioprocess for vanillic acid production using the resting cell of recombinant *E. coli* BL21(DE3)/pRSFDuet-*comt* (OD_{600} = 40), conducted by potassium phosphate buffer (pH 7.5), 2 mM of caffeic acid as substrate, 2 mM of DL-methionine as methyl donor, 200 rpm, and 26℃ at 12 h.

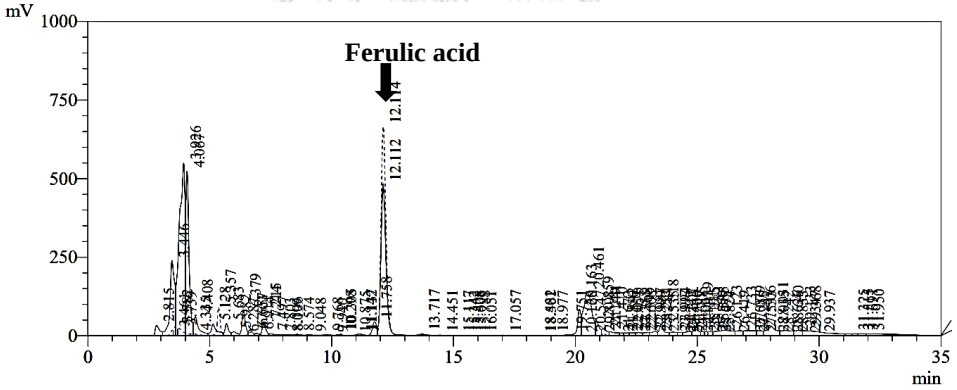


Figure A56 HPLC chromatogram obtained from the fed-batch shake flask production of ferulic acid in two-step bioprocess for vanillic acid production using the resting cell of recombinant *E. coli* BL21(DE3)/pRSFDuet-*comt* (OD_{600} = 40), conducted by potassium phosphate buffer (pH 7.5), 2 mM of caffeic acid as substrate, and 2 mM of DL-methionine as methyl donor, 200 rpm, and 26℃ at 36 h.

Second-step production: bioconversion of ferulic acid to vanillic acid

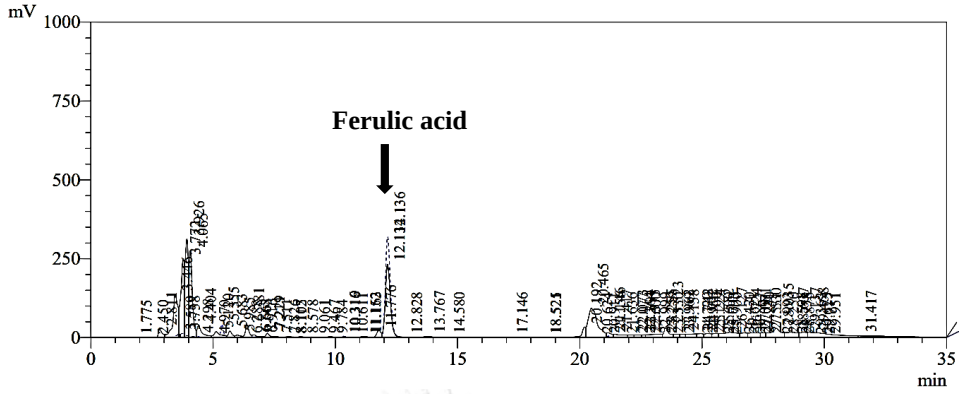


Figure A57 HPLC chromatogram obtained from the shake flask production of vanillic acid in two-step bioprocess using the resting cell of recombinant *E. coli* BL21(DE3) empty vector (OD_{600} = 40), conducted by Tris-HCl buffer (pH 8) and 1 mM of ferulic acid from the first-step bioprocess as substrate, 200 rpm, and 37℃ at 0 h.

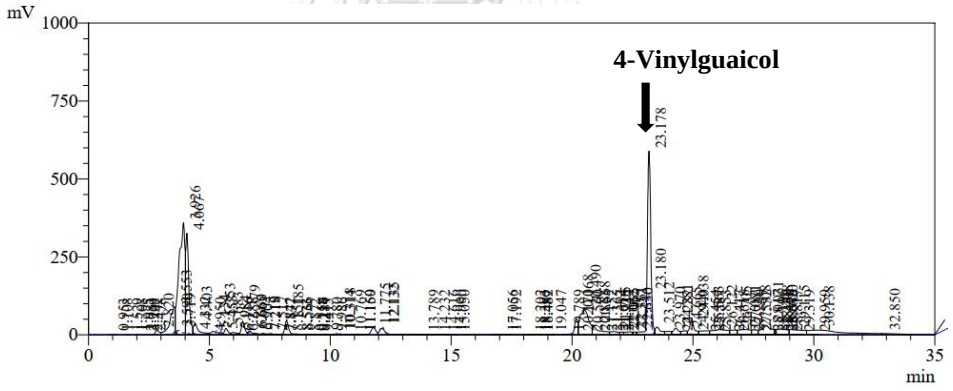


Figure A58 HPLC chromatogram obtained from the shake flask production of vanillic acid in two-step bioprocess using the resting cell of recombinant *E. coli* BL21(DE3)/pETDuet-*padC-vdh*/pCDFduet-*ado* (OD_{600} = 40), conducted by Tris-HCl buffer (pH 8) and 1 mM of ferulic acid from the first-step bioprocess as substrate, 200 rpm, and 37℃ at 0 h.

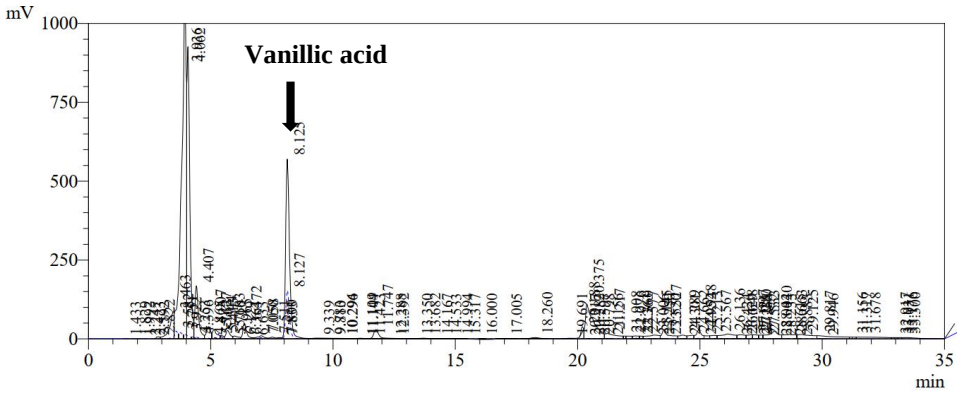


Figure A59 HPLC chromatogram obtained from the shake flask production of vanillic acid in two-step bioprocess using the resting cell of recombinant *E. coli* BL21(DE3)/pETDuet-*padC-vdh*/pCDFduet-*ado* (OD600 = 40), conducted by Tris-HCl buffer (pH 8) and 1 mM of ferulic acid from the first-step bioprocess as substrate, 200 rpm, and 37℃ at 48 h.

HPLC chromatogram of the effect of IPTG concentration on vanillic acid production in shake flask scale using growing cell of recombinant *E. coli* BL21(DE3) carrying pETDuet-*padC-vdh* and pCDFDuet-*ado*

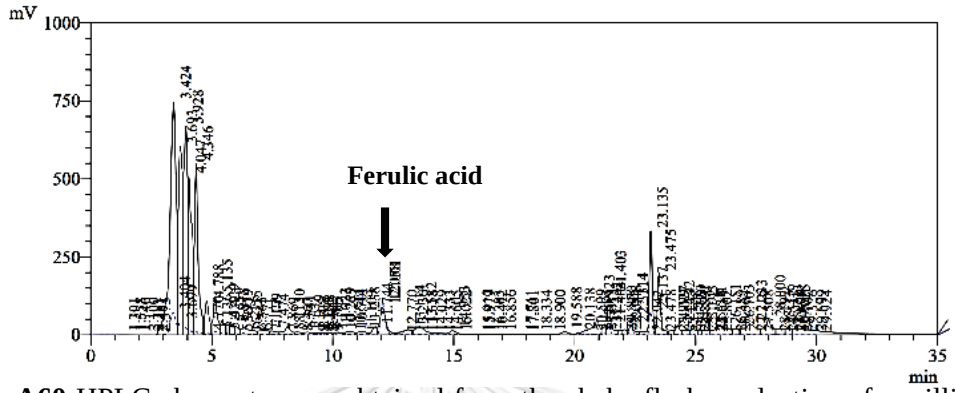


Figure A60 HPLC chromatogram obtained from the shake flask production of vanillic acid using the growing cell of recombinant *E. coli* BL21(DE3)/pETDuet-*padC-vdh*/pCDFDuet-*ado* in 50 mL of LB medium without supplementation of inducer for protein expression, conducted by 1% inoculum, 1 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 67 h.

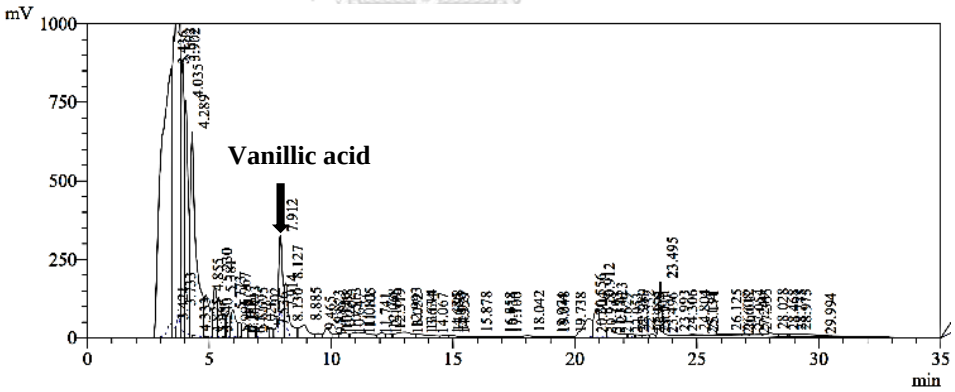


Figure A61 HPLC chromatogram obtained from the shake flask production of vanillic acid using the growing cell of recombinant *E. coli* BL21(DE3)/pETDuet-*padC-vdh*/pCDFduet-*ado* in 50 mL of LB medium, induced protein expression with 0.2 mM of IPTG, conducted by 1% inoculum, 1 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 67 h.

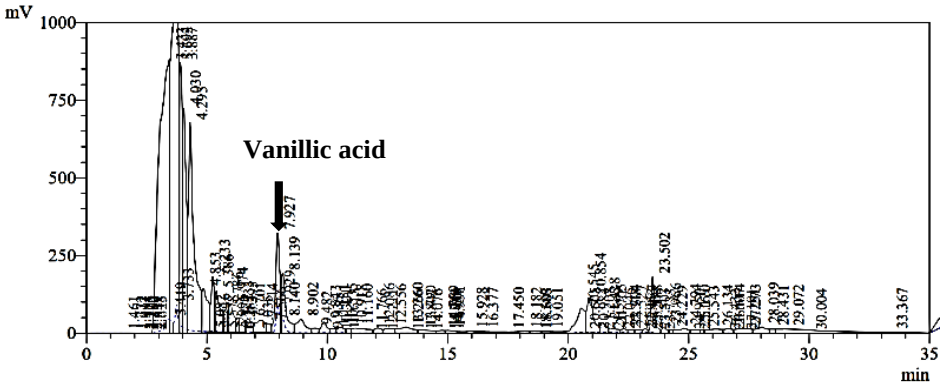


Figure A62 HPLC chromatogram obtained from the shake flask production of vanillic acid using the growing cell of recombinant *E. coli* BL21(DE3)/pETDuet-*padC*-*vdh*/pCDFDuet-*ado* in 50 mL of LB medium, induced protein expression with 0.4 mM of IPTG, conducted by 1% inoculum, 1 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 67 h.

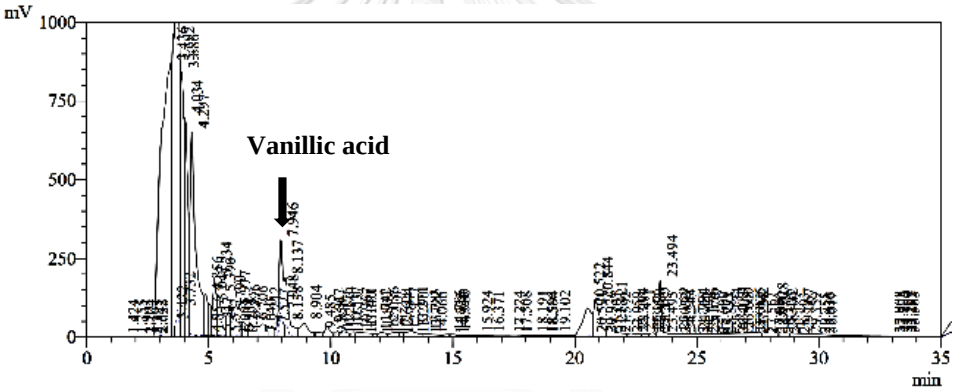


Figure A63 HPLC chromatogram obtained from the shake flask production of vanillic acid using the growing cell of recombinant *E. coli* BL21(DE3)/pETDuet-*padC*-*vdh*/pCDFDuet-*ado* in 50 mL of LB medium, induced protein expression with 1.2 mM of IPTG, conducted by 1% inoculum, 1 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 67 h.

HPLC chromatogram of the effect of carbon source on vanillic acid production in shake flask scale using growing cell of recombinant *E. coli* BL21(DE3) carrying pETDuet-*padC-vdh* and pCDFDuet-*ado*

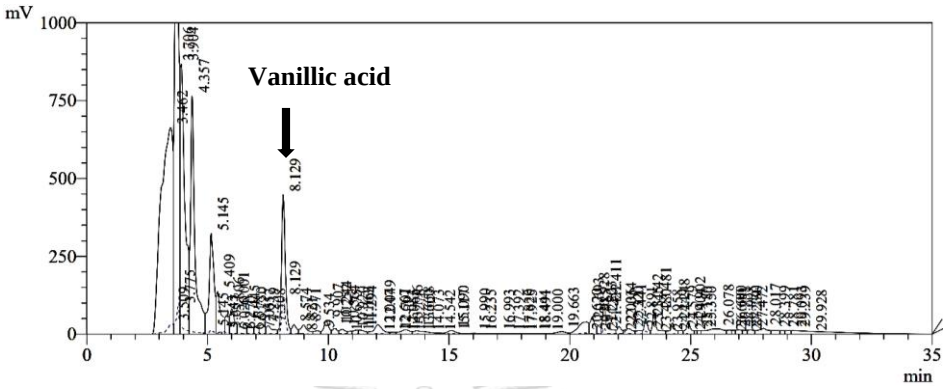


Figure A64 HPLC chromatogram obtained from the shake flask production of vanillic acid using the growing cell of recombinant *E. coli* BL21(DE3)/pETDuet-*padC-vdh*/pCDFDuet-*ado* in 50 mL of LB medium without supplementation of carbon source, conducted by 1% inoculum, 0.2 mM of IPTG as inducer, 1 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 67 h.

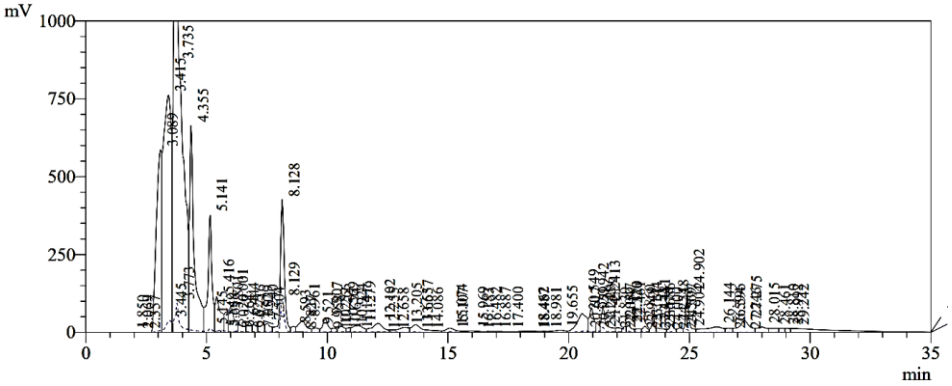


Figure A65 HPLC chromatogram obtained from the shake flask production of vanillic acid using the growing cell of recombinant *E. coli* BL21(DE3)/pETDuet-*padC-vdh*/pCDFDuet-*ado* in 50 mL of LB medium supplemented with 1 g/L of glucose as carbon source, conducted by 1% inoculum, 0.2 mM of IPTG as inducer, 1 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 67 h.

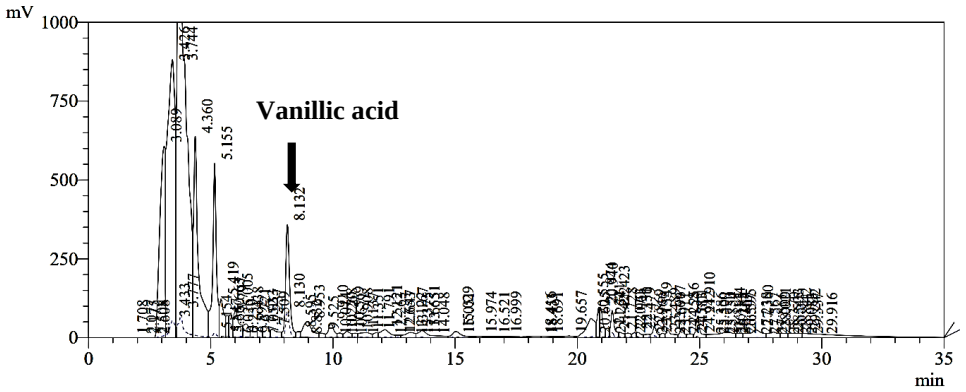


Figure A66 HPLC chromatogram obtained from the shake flask production of vanillic acid using the growing cell of recombinant *E. coli* BL21(DE3)/pETDuet-*padC*-*vdh*/pCDFDuet-*ado* in 50 mL of LB medium supplemented with 5 g/L of glucose as carbon source, conducted by 1% inoculum, 0.2 mM of IPTG as inducer, 1 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 67 h.

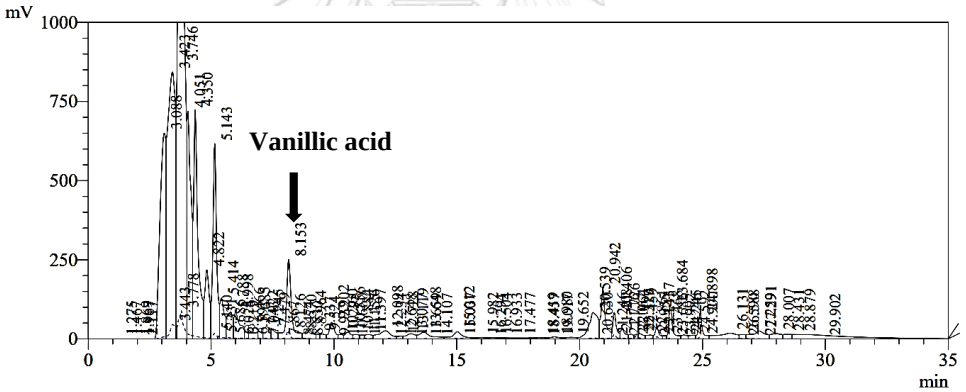


Figure A67 HPLC chromatogram obtained from the shake flask production of vanillic acid using the growing cell of recombinant *E. coli* BL21(DE3)/pETDuet-*padC*-*vdh*/pCDFDuet-*ado* in 50 mL of LB medium supplemented with 10 g/L of glucose as carbon source, conducted by 1% inoculum, 0.2 mM of IPTG as inducer, 1 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 67 h.

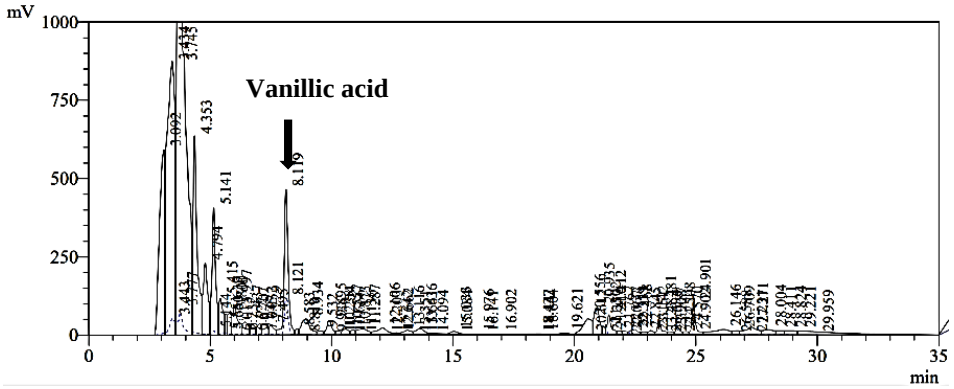


Figure A68 HPLC chromatogram obtained from the shake flask production of vanillic acid using the growing cell of recombinant *E. coli* BL21(DE3)/pETDuet-*padC-vdh*/pCDFduet-*ado* in 50 mL of LB medium supplemented with 1 g/L of glycerol as carbon source, conducted by 1% inoculum, 0.2 mM of IPTG as inducer, 1 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 67 h.

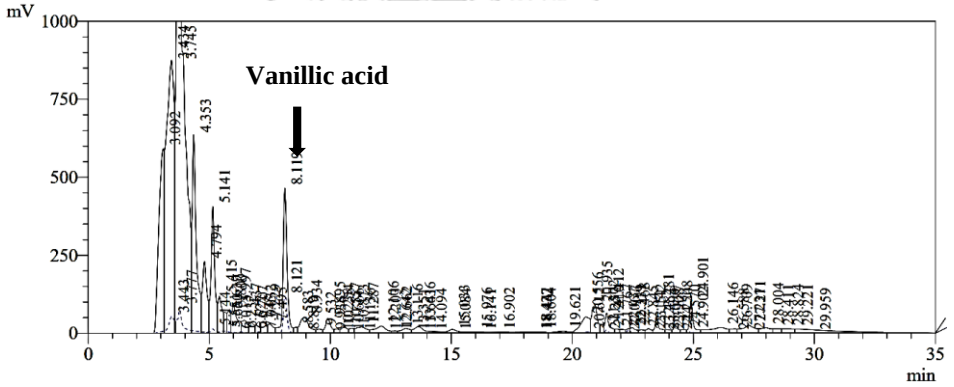


Figure A69 HPLC chromatogram obtained from the shake flask production of vanillic acid using the growing cell of recombinant *E. coli* BL21(DE3)/pETDuet-*padC-vdh*/pCDFDuet-*ado* in 50 mL of LB medium supplemented with 5 g/L of glycerol as carbon source, conducted by 1% inoculum, 0.2 mM of IPTG as inducer, 1 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 67 h.

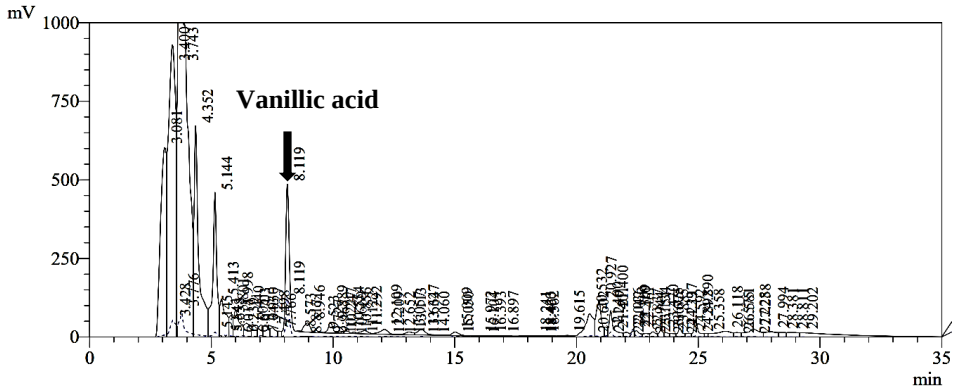


Figure A70 HPLC chromatogram obtained from the shake flask production of vanillic acid using the growing cell of recombinant *E. coli* BL21(DE3)/pETDuet-*padC*-*vdh*/pCDFDuet-*ado* in 50 mL of LB medium supplemented with 10 g/L of glycerol as carbon source, conducted by 1% inoculum, 0.2 mM of IPTG as inducer, 1 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 67 h.

HPLC chromatogram of the effect of medium on vanillic acid production in shake flask scale using growing cell of recombinant *E. coli* BL21(DE3) carrying pETDuet-*padC-vdh* and pCDFDuet-*ado*

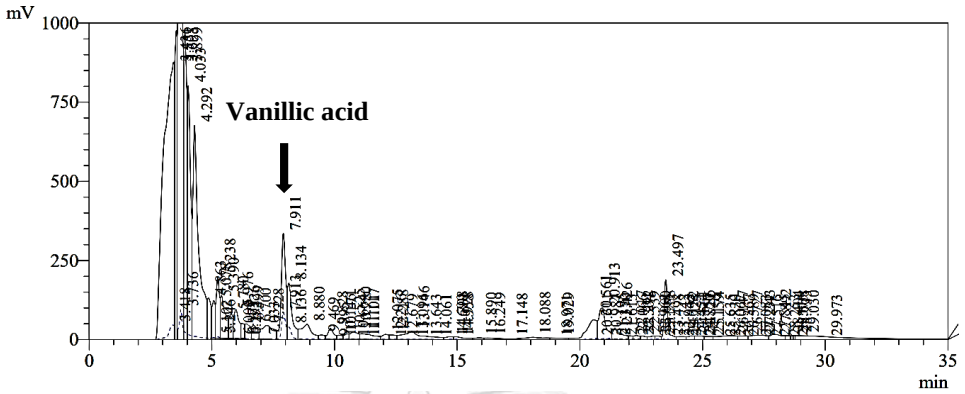


Figure A71 HPLC chromatogram obtained from the shake flask production of vanillic acid using the growing cell of recombinant *E. coli* BL21(DE3)/pETDuet-*padC-vdh*/pCDFDuet-*ado* in 50 mL of LB medium supplemented with 1 g/L of glycerol as carbon source, conducted by 1% inoculum, 0.2 mM of IPTG as inducer, 1 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 67 h.

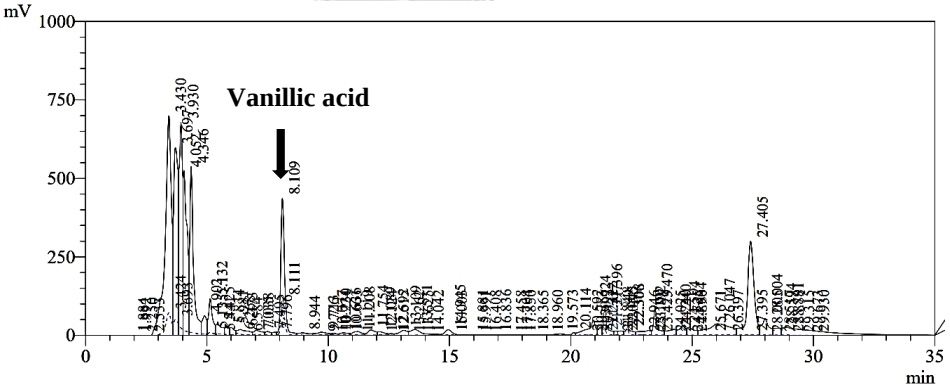


Figure A72 HPLC chromatogram obtained from the shake flask production of vanillic acid using the growing cell of recombinant *E. coli* BL21(DE3)/pETDuet-*padC-vdh*/pCDFDuet-*ado* in 50 mL of 2XLB medium supplemented with 1 g/L of glycerol as carbon source, conducted by 1% inoculum, 0.2 mM of IPTG as inducer, 1 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 67 h.

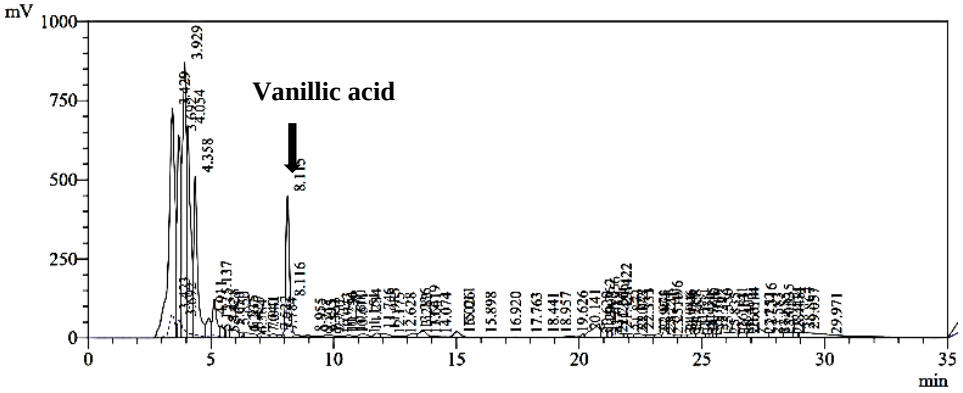


Figure A73 HPLC chromatogram obtained from the shake flask production of vanillic acid using the growing cell of recombinant *E. coli* BL21(DE3)/pETDuet-*padC*-*vdh*/pCDFDuet-*ado* in 50 mL of fermentation medium supplemented with 1 g/L of glycerol as carbon source, conducted by 1% inoculum, 0.2 mM of IPTG as inducer, 1 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 67 h.

HPLC chromatogram of the effect of different inducers on vanillic acid production in shake flask scale using growing cell of recombinant E. coli BL21(DE3) carrying pETDuet-padC-vdh and pCDFDuet-ado

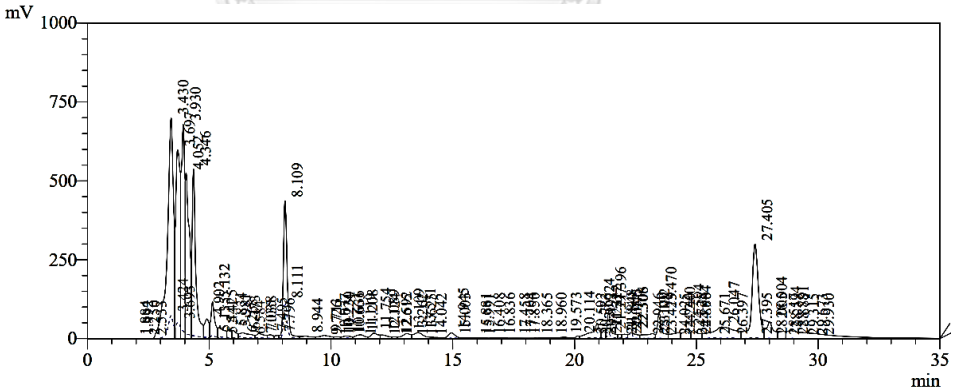


Figure A73 HPLC chromatogram obtained from the shake flask production of vanillic acid using the growing cell of recombinant *E. coli* BL21(DE3)/pETDuet-*padC*-*vdh*/pCDFDuet-*ado* in 50 mL of fermentation medium supplemented with 1 g/L of glycerol as carbon source, conducted by 1% inoculum, 0.2 mM of IPTG as inducer, 1 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 67 h.

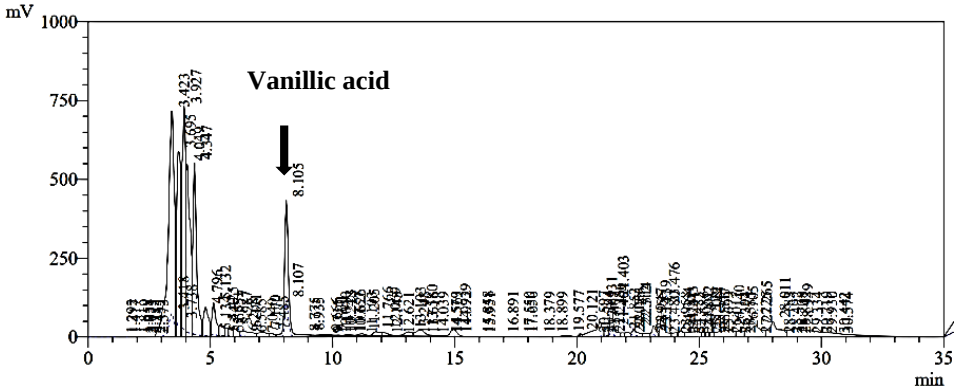


Figure A74 HPLC chromatogram obtained from the shake flask production of vanillic acid using the growing cell of recombinant *E. coli* BL21(DE3)/pETDuet-*padC*-*vdh*/pCDFDuet-*ado* in 50 mL of fermentation medium supplemented with 1 g/L of glycerol as carbon source, conducted by 5 g/L of lactose as inducer, 1 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 67 h.

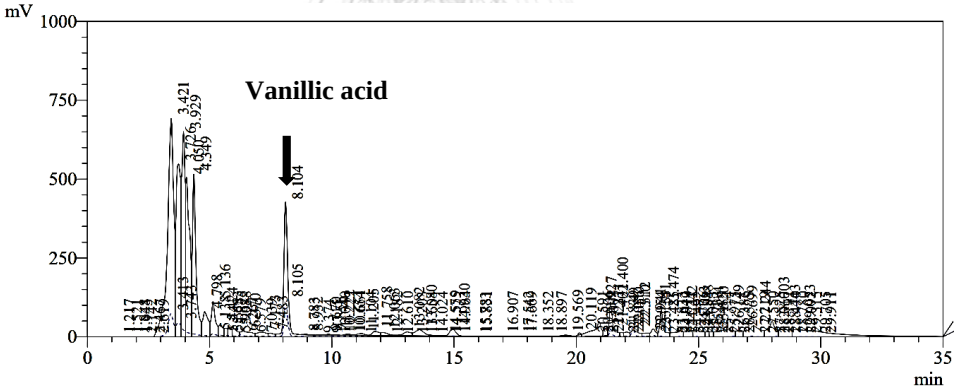


Figure A75 HPLC chromatogram obtained from the shake flask production of vanillic acid using the growing cell of recombinant *E. coli* BL21(DE3)/pETDuet-*padC*-*vdh*/pCDFDuet-*ado* in 50 mL of fermentation medium supplemented with 1 g/L of glycerol as carbon source, conducted by 1% inoculum, 10 g/L of lactose as inducer, 1 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 67 h.

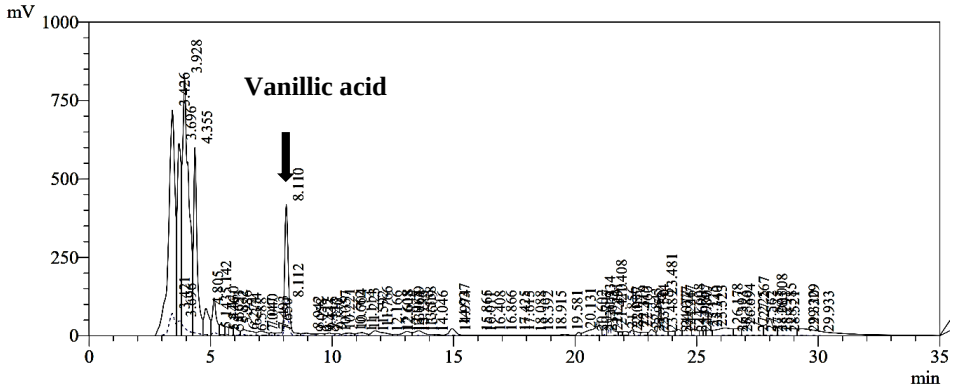


Figure A76 HPLC chromatogram obtained from the shake flask production of vanillic acid using the growing cell of recombinant *E. coli* BL21(DE3)/pETDuet-*padC*-*vdh*/pCDFDuet-*ado* in 50 mL of fermentation medium supplemented with 1 g/L of glycerol as carbon source, conducted by 1% inoculum, 5 g/L of galactose as inducer, 1 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 67 h.

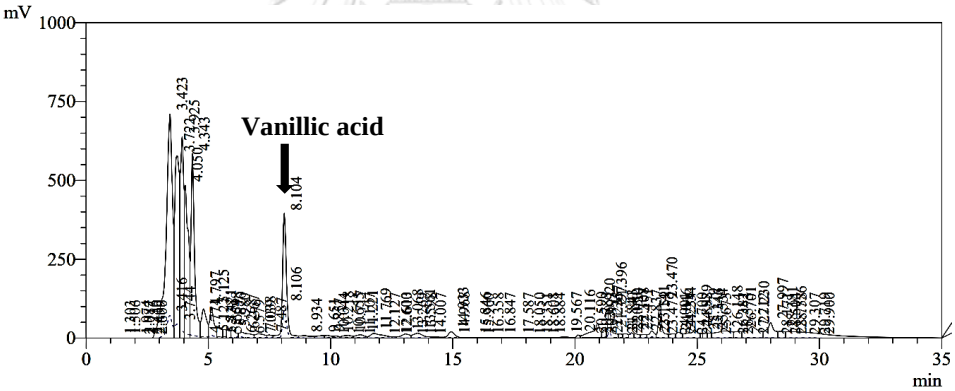


Figure A77 HPLC chromatogram obtained from the shake flask production of vanillic acid using the growing cell of recombinant *E. coli* BL21(DE3)/pETDuet-*padC*-*vdh*/pCDFDuet-*ado* in 50 mL of fermentation medium supplemented with 1 g/L of glycerol as carbon source, conducted by 1% inoculum, 10 g/L of galactose as inducer, 1 mM of ferulic acid as substrate, 200 rpm, and 37℃ at 67 h.

HPLC chromatogram of effect of inducer type on vanillic acid production in 5-L fermenter using *E. coli* BL21(DE3)/pETDuet-*padc*-*vdh*/pCDFDuet-*ado*

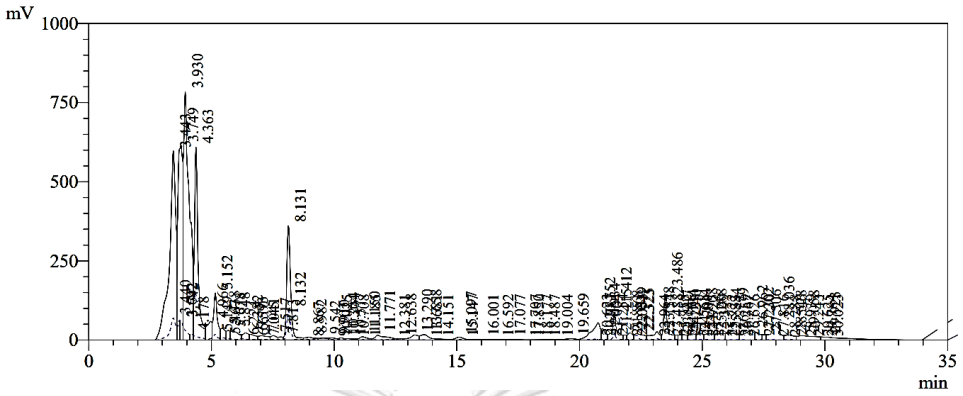


Figure A78 HPLC chromatogram obtained from the 5-L fermenter production of vanillic acid using the growing cell of recombinant *E. coli* BL21(DE3)/pETDuet-*padC*-*vdh*/pCDFDuet-*ado* in 3 L of fermentation medium supplemented with 1 g/L of glycerol as carbon source, conducted by 1% inoculum, 0.2 mM of IPTG as inducer, 1 mM of ferulic acid as substrate, 1 vvm of aeration rate, pH 8, 200 rpm, and 37℃ at 67 h.

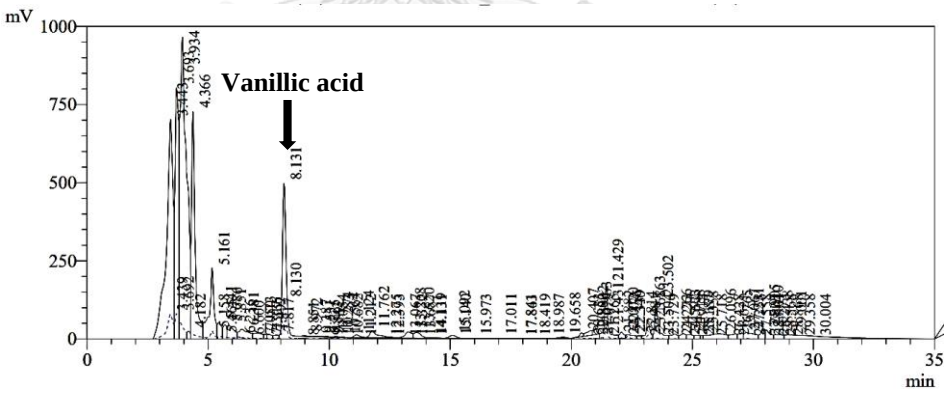


Figure A79 HPLC chromatogram obtained from the 5-L fermenter production of vanillic acid using the growing cell of recombinant *E. coli* BL21(DE3)/pETDuet-*padC*-*vdh*/pCDFDuet-*ado* in 3 L of fermentation medium supplemented with 1 g/L of glycerol as carbon source, conducted by1% inoculum, 5 g/L of lactose as inducer, 1 mM of ferulic acid as substrate, 1 vvm of aeration rate, pH 8, 200 rpm, and 37℃ at 67 h.

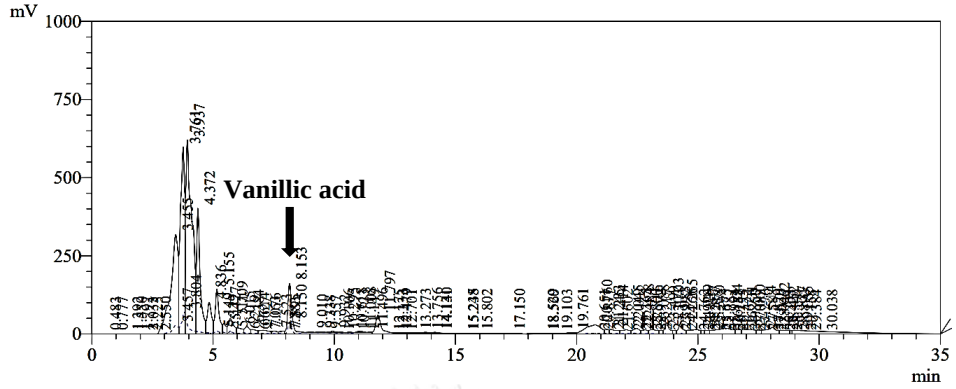


Figure A80 HPLC chromatogram obtained from the 5-L fermenter production of vanillic acid using the growing cell of recombinant *E. coli* BL21(DE3)/pETDuet-*padC*-*vdh*/pCDFDuet-*ado* in 3 L of fermentation medium supplemented with 1 g/L of glycerol as carbon source, conducted by 1% inoculum, 10 g/L of lactose as inducer, 1 mM of ferulic acid as substrate, 1 vvm of aeration rate, pH 8, 200 rpm, and 37℃ at 67 h.

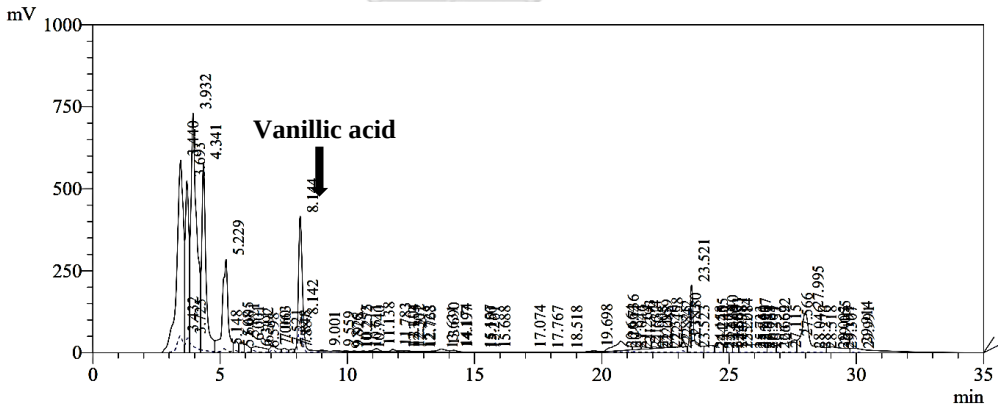


Figure A81 HPLC chromatogram obtained from the 5-L fermenter production of vanillic acid using the growing cell of recombinant *E. coli* BL21(DE3)/pETDuet-*padC*-*vdh*/pCDFDuet-*ado* in 3 L of fermentation medium supplemented with 1 g/L of glycerol as carbon source, conducted by 1% inoculum, 5 g/L of galactose as inducer, 1 mM of ferulic acid as substrate, 1 vvm of aeration rate, pH 8, 200 rpm, and 37℃ at 67 h.

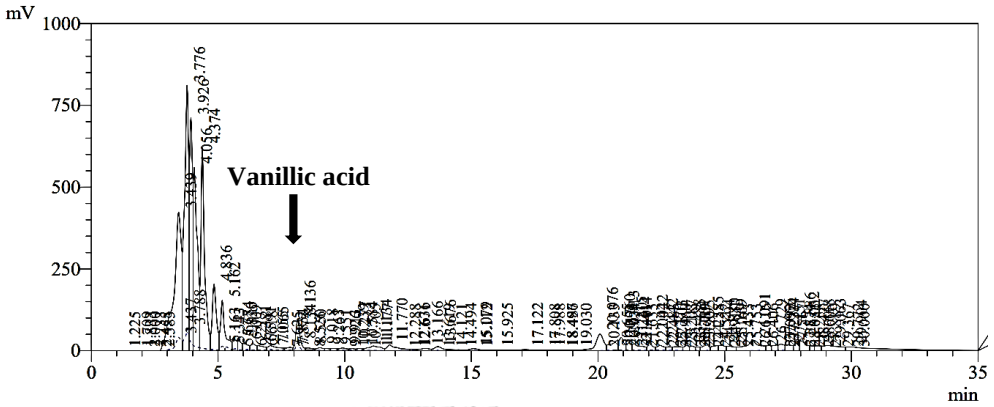


Figure A82 HPLC chromatogram obtained from the 5-L fermenter production of vanillic acid using the growing cell of recombinant *E. coli* BL21(DE3)/pETDuet-*padC*-*vdh*/pCDFDuet-*ado* in 3 L of fermentation medium supplemented with 1 g/L of glycerol as carbon source, conducted by 1% inoculum, 10 g/L of galactose as inducer, 1 mM of ferulic acid as substrate, 1 vvm of aeration rate, pH 8, 200 rpm, and 37℃ at 67 h.

HPLC chromatogram of effect of inoculum percentage on vanillic acid production in 5-L fermenter using *E. coli* BL21(DE3)/pETDuet-*padc*-*vdh*/pCDFD-*ado*

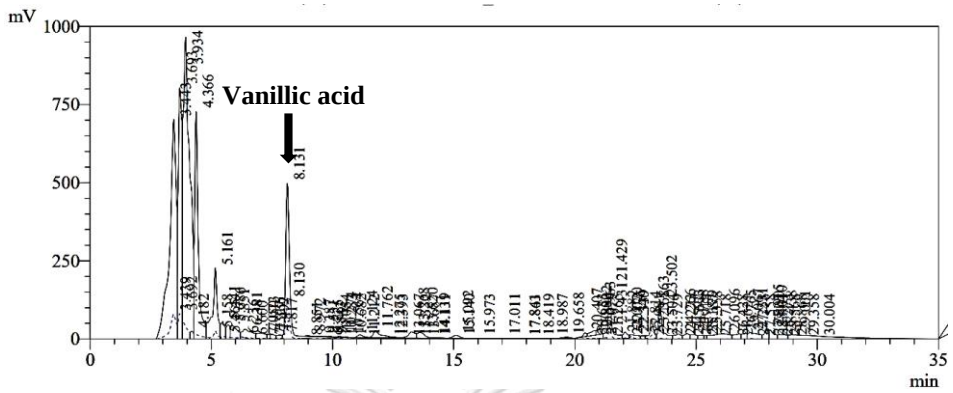


Figure A83 HPLC chromatogram obtained from the 5-L fermenter production of vanillic acid using the growing cell of recombinant *E. coli* BL21(DE3)/pETDuet-*padC*-*vdh*/pCDFDuet-*ado* in 3 L of fermentation medium supplemented with 1 g/L of glycerol as carbon source, conducted by 1% inoculum, 5 g/L of lactose as inducer, 1 mM of ferulic acid as substrate, 1 vvm of aeration rate, pH 8, 200 rpm, and 37℃ at 67 h.

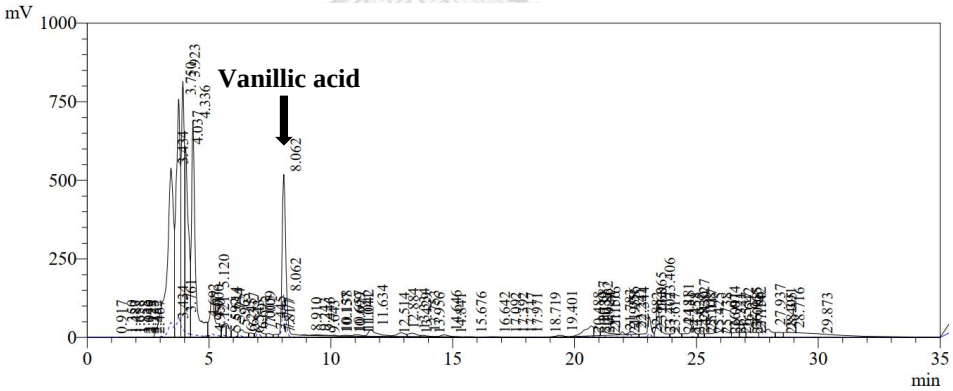


Figure A84 HPLC chromatogram obtained from the 5-L fermenter production of vanillic acid using the growing cell of recombinant *E. coli* BL21(DE3)/pETDuet-*padC*-*vdh*/pCDFDuet-*ado* in 3 L of fermentation medium supplemented with 1 g/L of glycerol as carbon source, conducted by 5% inoculum, 5 g/L of lactose as inducer, 1 mM of ferulic acid as substrate, 1 vvm of aeration rate, pH 8, 200 rpm, and 37℃ at 67 h.

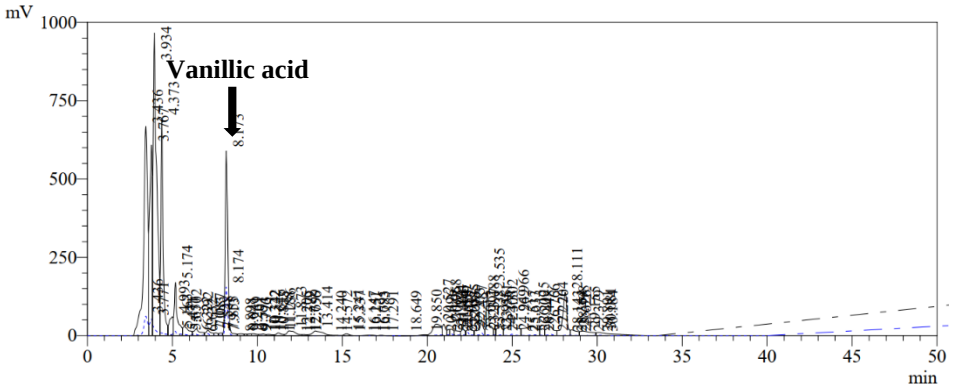


Figure A85 HPLC chromatogram obtained from the 5-L fermenter production of vanillic acid using the growing cell of recombinant *E. coli* BL21(DE3)/pETDuet-*padC*-*vdh*/pCDFDuet-*ado* in 3 L of fermentation medium supplemented with 1 g/L of glycerol as carbon source, conducted by 10% inoculum, 5 g/L of lactose as inducer, 1 mM of ferulic acid as substrate, 1 vvm of aeration rate, pH 8, 200 rpm, and 37℃ at 67 h.

APPENDIX B

STANDARD GRAPH OF *E. coli* CELL DRY WEIGHT

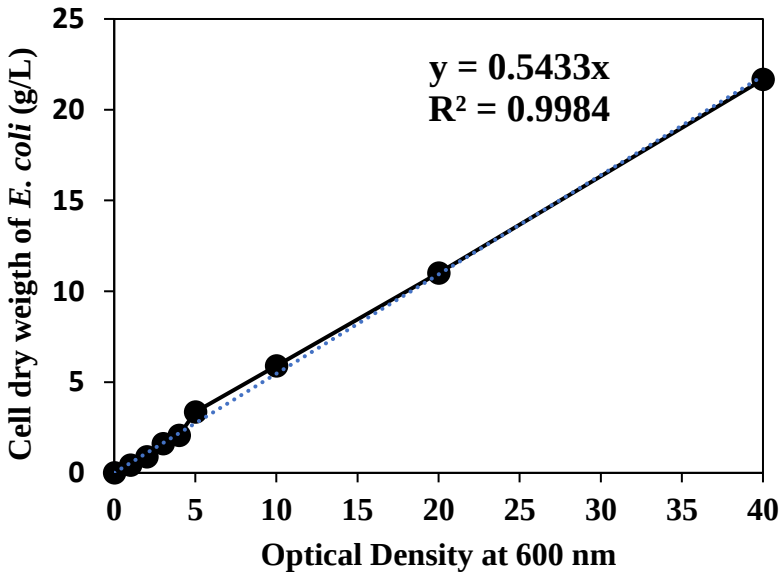


Figure B. Standard graph of *E. coli* cell dry weight against OD 600 nm

APPENDIX C

CALCULATION OF KINETIC VALUES

C1 Yield (% molar)

$$\textbf{Yield (\% molar)} = \frac{\text{Final vanillic acid titer (mM)}}{\text{Initial substrate} - \text{ Final substrate (mM)}} \times 100$$

C2 Productivity (mg/L/h)

$$\textbf{Productivity (mg/L/h)} = \frac{\text{Final vanillic acid titer (mg/L)}}{\text{Fermentation time (h)}}$$

VITA

NAME	Mr. Jedsadakorn Ninrat
DATE OF BIRTH	14 March 1996
PLACE OF BIRTH	Bangkok, Thailand
INSTITUTIONS ATTENDED	Bachelor's Degree in Biochemistry, Faculty of science, Chulalongkorn University in 2017.
HOME ADDRESS	1279/152, Moo 6, Thai Ban Mai, Mueang Samut Prakan, Samutprakan, Thailand, 10270.
PUBLICATION	Kotchaplai, P., Ninrat, J., Mahipant, G., & Vangnai, A. S. (2022). Involvement of Cytochrome P450 in Organic-Solvent Tolerant Bacillus subtilis GRSW1-B1 in Vanillin Production via Ferulic Acid Metabolism. Fermentation, 8(10), 508. Ninrat, J., Kotchaplai, P, & Vangnai, A. S. Production of vanillic acid from ferulic acid using genetically modified Escherichia coli. The 33rd Annual Meeting of Thai Society for Biotechnology and International Conference (TSB2021), 25 November 2021 , Hybrid Conference, Bangkok, Thailand