

การปรับปรุงการผลิตแอล-ฟีนิลอะลานีนใน *Escherichia coli*
โดยกระบวนการวิศวกรรมเมแทบอลิซึม



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IMPROVEMENT OF L-PHENYLALANINE PRODUCTION IN *Escherichia coli*
BY METABOLIC ENGINEERING PROCESS

Miss Mayura Thongchuang

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Biotechnology

Faculty of Science

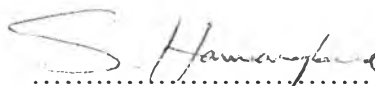
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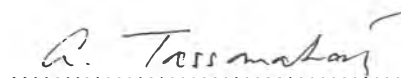
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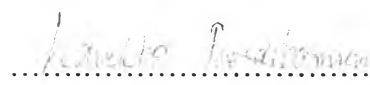
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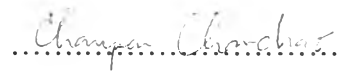

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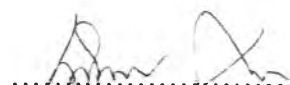

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แอล-ฟีนิลอะลานีน (L-Phe) เป็นกรดอะมิโนจำเป็นชนิดหนึ่ง ใช้เป็นสารตั้งต้นในการผลิตสารสำคัญในทางการแพทย์ และอุตสาหกรรมอาหาร เช่น แอสปาร์แตม ความต้องการใช้แอสปาร์แตมที่เพิ่มมากขึ้นนำไปสู่การเพิ่มการผลิต L-Phe ในจุลชีพโดยเฉพาะอย่างยิ่งใน *E. coli* งานวิจัยนี้มีเป้าหมายในการใช้กระบวนการวิศวกรรมเมแทบอลิซึมในการปรับปรุงการผลิต L-Phe จากกลีเซอรอล ได้ทำการโคลนยีนฟีนิลอะลานีนดีไฮโดรจีเนส (*phedh*) จาก *Bacillus lentus* เข้าสู่ *E. coli* BL21(DE3) โดยใช้ expression vector pRSFDuet-1 (*pPheDH*) เพื่อเพิ่มการสร้าง L-Phe จากฟีนิลไพรูเวทในขั้นตอนสุดท้ายของการสังเคราะห์ผลิตภัณฑ์ พบว่าการผลิต L-Phe โดยโคลน *pPheDH* ในอาหารที่มีแหล่งคาร์บอนเป็นกลีเซอรอลซึ่งเป็นผลพลอยได้จากการผลิตไบโอดีเซลมีข้อจำกัด ซึ่งอาจเกิดในขั้นตอนการขนส่งกลีเซอรอลเข้าสู่เซลล์ การส่ง L-Phe ออกนอกเซลล์ หรือการควบคุมในขั้นตอนต่างๆ ของการสังเคราะห์ L-Phe ดังนั้น จึงทำการโคลนยีนต่างๆ ที่เข้ารหัสให้โปรตีนที่สำคัญในวิถีการสังเคราะห์ L-Phe การนำกลีเซอรอลเข้าเซลล์และการนำ L-Phe ออกจากเซลล์ร่วมกับยีน *phedh* ได้แก่ *aroB*, *aroL*, *glpF*, *glpK*, *pheA*, *tktA* และ *yddG* ซึ่งเข้ารหัสให้ 3-dehydroquinate synthase, shikimate kinase II, glycerol facilitator, glycerol kinase, chorismate mutase/prephenate dehydratase, transketolase และ aromatic amino acid exporter ตามลำดับ โดยโคลน *pPTFBLY* ซึ่งประกอบด้วยยีน *phedh*, *tktA*, *glpF*, *aroB*, *aroL* และ *yddG* มีอัตราการผลิต L-Phe สูงที่สุดคือ 3.36 มิลลิกรัมต่อลิตรต่อชั่วโมงซึ่งสูงกว่าที่ได้จากโคลน *pPheDH* ถึง 2.1 เท่าโดยความเข้มข้นของ L-Phe ที่ 240 ชั่วโมงหลังจากวัดค่าการดูดกลืนแสงที่ 600 นาโนเมตรได้ 0.6 เท่ากับ 429 มิลลิกรัมต่อลิตร เมื่อทำการเลี้ยงโคลน *pPYF* ในถังหมักขนาด 5 ลิตร (อุณหภูมิ 37 °C อัตราการให้อากาศ 1 ปริมาตรอากาศต่อปริมาตรอาหารต่อนาที ความเร็วในการกวน 400 รอบ ต่อนาที) พบว่าในการหมักแบบกะผลิต L-Phe สูงสุดที่ 56 ชั่วโมงเท่ากับ 366 มิลลิกรัมต่อลิตร ส่วนในการหมักแบบกึ่งกะที่มีการเติมกลีเซอรอลสองครั้ง ที่ 40 และ 60 ชั่วโมง ตามลำดับ พบว่าปริมาณ L-Phe สูงสุดเท่ากับ 445 มิลลิกรัมต่อลิตร การหมักแบบกึ่งกะโดยเติมกลีเซอรอลครั้งเดียวที่ 40 ชั่วโมงได้ปริมาณ L-Phe สูงสุดเท่ากับ 545 มิลลิกรัมต่อลิตร ดังนั้นโคลน *pPYF* ให้ปริมาณ L-Phe เมื่อทำการผลิตในระดับถังหมักสูงกว่าการผลิตในระดับขวดเย้าประมาณ 2.0 เท่า

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MAYURA THONGCHUANG : IMPROVEMENT OF L-PHENYLALANINE PRODUCTION IN *Escherichia coli* BY METABOLIC ENGINEERING PROCESS.
 ADVISOR : ASST. PROF. KANOKTIP PACKDIBAMRUNG, Ph.D.,
 CO-ADVISOR: PROF. PIAMSOOK PONGSAWADSI, Ph.D., PROF. YUSUF CHISTI, Ph.D., 220 pp.

L-Phenylalanine (L-Phe), an essential amino acid, is mainly used as a reactant for production of important substances in medicine and food industry such as aspartame. An increase in demand for aspartame leads to the improvement of L-Phe production in well-known microorganisms, especially *Escherichia coli*. This work aims to improve L-Phe production from glycerol by metabolic engineering process. Phenylalanine dehydrogenase gene (*phedh*) from *Bacillus lentus* was cloned into pRSFDuet-1 (pPheDH) and expressed in *E. coli* BL21(DE3) to increase the conversion of phenylpyruvate to L-Phe in the last step of L-Phe biosynthesis pathway. The recombinant pPheDH clone had limited ability to produce L-Phe when the cells were grown in the minimal medium containing glycerol, a by-product from biodiesel production, as carbon source, possibly due to poor glycerol uptake, low ability to excrete L-Phe, or tightly regulated steps in L-Phe biosynthesis. Accordingly, various genes encoding pivotal proteins involving in L-Phe biosynthesis pathway, glycerol uptake and L-Phe excretion were co-expressed with *phedh* gene. These genes were *aroB*, *aroL*, *glpF*, *glpK*, *pheA*, *tktA* and *yddG* which encode 3-dehydroquinate synthase, shikimate kinase II, glycerol facilitator, glycerol kinase, chorismate mutase/prephenate dehydratase, transketolase and aromatic amino acid exporter, respectively. The recombinant pPTFBLY clone containing *phedh*, *tktA*, *glpF*, *aroB*, *aroL* and *yddG* genes had the highest L-Phe production rate of 3.36 mg/L/h which was 2.1-fold higher than that of pPheDH clone and the L-Phe concentration of 429 mg/L was attained at 240 h after the optical density at 600 nm reached 0.6. In a 5 L stirred-tank fermenter (37 °C, an aeration rate of 1 vvm, an agitation speed of 400 rpm), the L-Phe production was carried out using pPYF clone. For batch fermentation, the maximum concentration of L-Phe (366 mg/L) was achieved at 56 h. In fed-batch fermentation with addition of new glycerol medium at 40 h and 60 h, the highest yield of L-Phe production of 445 mg/L was obtained. Moreover, one time feeding of glycerol at 40 h resulted in the maximum L-Phe titer of 545 mg/L. The maximum L-Phe production of pPYF clone in the fermenter was nearly 2.0-fold higher than that in shake flask.

Field of Study: ...Biotechnology.... Student's Signature.....*Mayura Thongchuang*.....
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A	absorbance, 2'-deoxyadenosine (in a DNA sequence)
AroB	3-dehydroquinate synthase
<i>aroB</i>	3-dehydroquinate synthase gene
AroL	shikimate kinase II
<i>aroL</i>	shikimate kinase II gene
AroF	L-tyrosine-feedback inhibited DAHP synthase
<i>aroF</i>	L-tyrosine-feedback inhibited DAHP synthase gene
<i>aroF^{fbr}</i>	L-tryptophan-feedback resistant DAHP synthase gene
AroG	L-phenylalanine-feedback inhibited DAHP synthase
<i>aroG</i>	L-phenylalanine-feedback inhibited DAHP synthase gene
<i>aroG^{fbr}</i>	L-phenylalanine-feedback resistant DAHP synthase gene
AroH	L-tryptophan-feedback inhibited DAHP synthase
<i>aroH</i>	L-tryptophan-feedback inhibited DAHP synthase gene
bp	base pairs
BSA	bovine serum albumin
C	2'-deoxycytidine (in a DNA sequence)
° C	degree Celsius
Da	Dalton
DNA	deoxyribonucleic acid
dNTP	2'-deoxynucleoside 5'-triphosphate
EC	Enzyme Commission
EDTA	ethylene diamine tetraacetic acid
G	2'-deoxyguanosine (in a DNA sequence)
GlpF	glycerol facilitator
<i>glpF</i>	glycerol facilitator gene
GlpFK	glycerol facilitator and glycerol kinase
<i>glpFK</i>	glycerol facilitator and glycerol kinase genes
GlpK	glycerol kinase
<i>glpK</i>	glycerol kinase gene

HPLC	high-performance liquid chromatography
IPTG	isopropyl- β -D-thiogalactoside
kb	kilobase pairs in duplex nucleic acid, kilobases in single-stranded nucleic acid
kDa	kiloDalton
K_m	Michaelis constant
KOH	potassium hydroxide
L	liter
L-phenylalanine	L-Phe
L-tryptophan	L-Trp
L-tyrosine	L-Tyr
LB	Luria-Bertani
μ g	microgram
μ L	microliter
μ M	micromolar
M	mole per liter (molar)
mg	milligram
min	minute
mL	milliliter
mM	millimolar
MW	molecular weight
N	normal
NAD ⁺	nicotinamide adenine dinucleotide (oxidized)
NADH	nicotinamide adenine dinucleotide (reduced)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
ng	nanogram
nm	nanometer
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PheA	chorismate mutase/ prephenate dehydratase
<i>pheA</i>	chorismate mutase/ prephenate dehydratase gene

<i>pheA</i> ^{fb}	feedback resistant chorismate mutase/ prephenate dehydratase
PheDH	phenylalanine dehydrogenase
<i>phedh</i>	phenylalanine dehydrogenase gene
PMSF	phenyl methyl sulfonyl fluoride
RNase	ribonuclease
SDS	sodium dodecyl sulfate
T	2'-deoxythymidine (in a DNA sequence)
TE	Tris-EDTA buffer
TEMED	<i>N, N, N', N'</i> -tetramethyl ethylene diamine
TktA	transketolase
<i>tktA</i>	transketolase gene
T_m	melting temperature, melting point
UV	ultraviolet
v/v	volume by volume
vvm	volumes of air per volume of liquid per minute
w/w	weight by weight
YddG	aromatic amino acid exporter
<i>yddG</i>	aromatic amino acid exporter gene