การโคลนยีนและลักษณะสมบัติของแอล-ไลซีน 6-ดีไฮโดรจิเนสจาก *Achromobacter denitrificans* สำหรับการผลิตกรดแอล-พิพีคอลิก

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GENE CLONING AND CHARACTERIZATION OF L-LYSINE 6-DEHYDROGENASE FROM *Achromobacter denitrificans* FOR L-PIPECOLIC ACID PRODUCTION

Mr. Prakarn Ruldeekulthamrong

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

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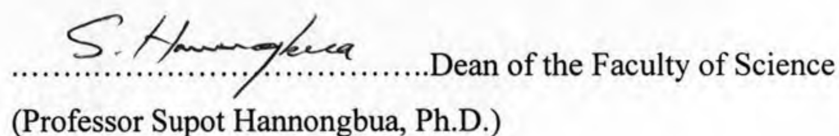
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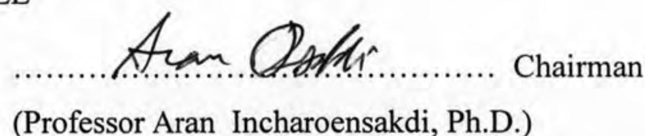
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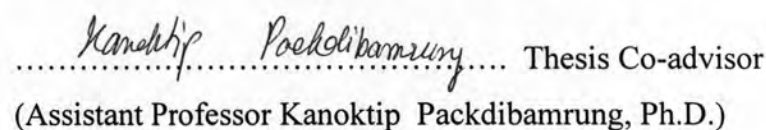
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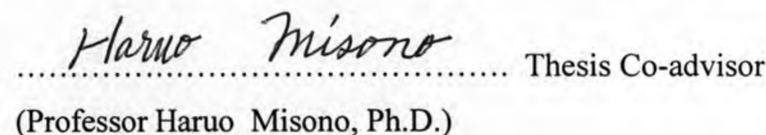
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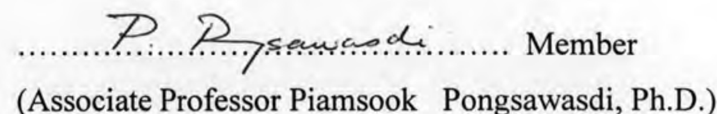
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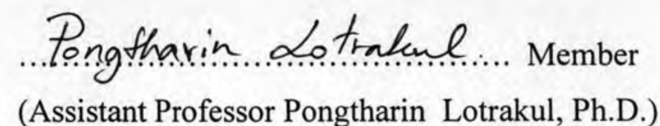
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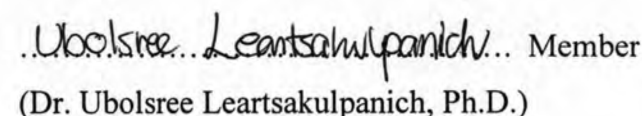
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ประกานต์ ฤดีกุลธำรง: การโคลนยีน และ ลักษณะสมบัติของแอล-ไลซีน 6-ดีไฮโดรจิเนสจาก *Achromobacter denitrificans* สำหรับการผลิตกรดแอล-พิพีคอลิก (GENE CLONING AND CHARACTERIZATION OF L-LYSINE 6-DEHYDROGENASE FROM *Achromobacter denitrificans* FOR L-PIPECOLIC ACID PRODUCTION) อ. ที่ปรึกษา: รศ. ดร. ศิริพร สิทธิประณีต, 247 หน้า.

ไลซีน 6-ดีไฮโดรจิเนส (EC 1.4.1.18) เร่งปฏิกิริยาการดึงหมู่อะมิโนจากแอล-ไลซีนให้ได้ผลิตภัณฑ์ คือ ไพเพอริดีน-6-คาร์บอกซีเลต และแอมโมเนีย ซึ่งเป็นปฏิกิริยาที่ไม่ย้อนกลับ และต้องการไพริดีนนิวคลีโอไทด์เป็นโคเอนไซม์ งานวิจัยนี้ได้ทำการคัดกรองแบคทีเรียจากดินที่ผลิตไลซีน 6-ดีไฮโดรจิเนส จากการระบุสายพันธุ์ พบว่าเป็น *Achromobacter denitrificans* เมื่อทำเอนไซม์ให้บริสุทธิ์พบว่าเอ็นไซม์มีความบริสุทธิ์ขึ้น 175 เท่า และมีแอคติวิตีคงเหลือ 34 เปอร์เซ็นต์ จากนั้นทำการหาลำดับกรดอะมิโนบางส่วนภายในสายของเอนไซม์ เพื่อนำมาใช้ในออกแบบไพรเมอร์สำหรับการเพิ่มปริมาณบางส่วนของยีนด้วยเทคนิคพีซีอาร์และใช้เทคนิค inverse PCR เพื่อให้ได้ลำดับของยีนไลซีน 6-ดีไฮโดรจิเนสที่สมบูรณ์ แล้วทำการโคลนยีนเข้าสู่เซลล์เจ้าเรือน *E. coli* BL21(DE3) โดยใช้พลาสมิด pET-17b เป็นพาหะ ภาวะที่เหมาะสมในการแสดงออกของยีนไลซีน 6-ดีไฮโดรจิเนส คือ การเหนี่ยวนำด้วย IPTG ความเข้มข้น 0.2 มิลลิโมลาร์เป็นเวลา 8 ชั่วโมง โดยสารละลายเอนไซม์หยาบจากรีคอมบิแนนท์โคลนมีแอคติวิตีจำเพาะสูงกว่าเอนไซม์จาก *A. denitrificans* 6 3 เท่า การทดสอบความเสถียรของการแสดงออกของยีนไลซีน 6-ดีไฮโดรจิเนส พบว่าการเพาะเชื้อต่อช่วงรีคอมบิแนนท์โคลน 80 ครั้ง ยังคงมีแอคติวิตีของเอนไซม์ 100 เปอร์เซ็นต์ เมื่อทำรีคอมบิแนนท์เอนไซม์ให้บริสุทธิ์ พบว่าเอนไซม์มีแอคติวิตีคงเหลือ 47 เปอร์เซ็นต์และมีความบริสุทธิ์เพิ่มขึ้น 2.8 เท่า เอนไซม์มีน้ำหนักโมเลกุลประมาณ 240,000 ดาลตันประกอบด้วย 6 หน่วยย่อยที่มีน้ำหนักโมเลกุลเท่ากัน เอนไซม์มีความจำเพาะต่อ แอล-ไลซีน และ NAD^+ สูงมาก pH และอุณหภูมิที่เหมาะสมในการเร่งปฏิกิริยาคือ 9.3 และ 50 องศาเซลเซียส ตามลำดับ เอนไซม์มีความเสถียรช่วง pH 7.5 ถึง 8.0 ค่า K_m ต่อแอล-ไลซีน และ NAD^+ ของเอนไซม์ในรูปไดเมอร์และเฮกซะเมอร์เท่ากับ 11.11, 0.138, 8.62 และ 0.092 มิลลิโมลาร์ ตามลำดับ เพื่อการผลิตกรดแอล-พิพีคอลิก ยีนของไพโรลีน-5-คาร์บอกซิเลตรีดักเทส ซึ่งเร่งปฏิกิริยาการเปลี่ยนไพเพอริดีน-6-คาร์บอกซีเลตเป็นกรดแอล-พิพีคอลิกจาก *Bacillus cereus* ATCC 11778 ได้ถูกทรานสฟอร์มร่วมกับยีนไลซีน 6-ดีไฮโดรจิเนสโดยใช้พลาสมิดพาหะ pET-17b ภาวะที่เหมาะสมในการแสดงออกของยีนทั้งสองคือ การเหนี่ยวนำด้วย IPTG ความเข้มข้น 0.1 มิลลิโมลาร์เป็นเวลา 3 ชั่วโมง สารละลายเอนไซม์หยาบจากรีคอมบิแนนท์โคลนมีแอคติวิตีจำเพาะของ ไลซีน 6-ดีไฮโดรจิเนส และไพโรลีน-5-คาร์บอกซิเลตรีดักเทส สูงกว่าเอนไซม์หยาบจาก *A. denitrificans* และ*B. cereus* 25 และ 11 เท่าตามลำดับ ในการผลิตกรดแอล-พิพีคอลิกพบว่า การผลิตเกิดขึ้นสูงสุดเมื่อทำให้ผนังเซลล์มีความสามารถในการซึมผ่านมากขึ้นโดยบ่มรีคอมบิแนนท์เซลล์กับ xylene เป็นเวลา 5 นาที จากนั้นจึงนำเซลล์ที่ได้มาบ่มกับแอล-ไลซีน ความเข้มข้น 200 มิลลิโมล่าร์ในสารละลายปฏิกิริยาที่ประกอบด้วยบัฟเฟอร์ Tris-HCl pH 9.0 ความเข้มข้น 200 มิลลิโมล่าร์เป็นเวลา 24 ชั่วโมง โดยกรดแอล-พิพีคอลิกที่ได้มีความเข้มข้นสูงสุด 77 มิลลิโมลาร์หรือ 9.9 กรัมต่อลิตร

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PRAKARN RULDEEKULTHAMRONG: GENE CLONING AND CHARACTERIZATION OF L-LYSINE 6-DEHYDROGENASE FROM *Achromobacter denitrificans* FOR L-PIPECOLIC ACID PRODUCTION. THESIS ADVISOR: ASSOC. PROF. SIRIPORN SITTIPRANEED, Ph.D., 247 pp.

NAD^+-dependent lysine 6-dehydrogenase (Lys 6-DH, EC 1.4.1.18) catalyzes the irreversible oxidative deamination of L-lysine to form ammonia and piperidiene-6-carboxylate. A Lys 6-DH producing bacterium strain K-1, screened from soil sample, was identified as *Achromobacter denitrificans*. Lys 6-DH from *A. denitrificans* K-1 was purified 175 fold with a 34% yield. The amino acid sequences of internal peptide fragments of Lys 6-DH were determined and used for degenerated primer design. The partial nucleotide sequence of the lysine 6-dehydrogenase gene (*lys 6-dh*) was investigated by PCR technique. Using inverse PCR, the whole *lys 6-dh* gene was obtained. The gene was cloned and expressed in *E. coli* BL 21(DE3) using expression vector, pET-17b. The optimum condition for *lys 6-dh* gene expression was induction with 0.2 mM IPTG for 4 hours. The specific activity of crude recombinant enzyme was 63 fold higher than that of the enzyme from *A. denitrificans*. After daily subculture for 80 days, the *lys 6-dh* gene expression in *E. coli* BL 21(DE3) remained 100% of that of the parent. Recombinant enzyme was purified 2.8 fold with a 47% yield. The enzyme had a molecular mass about 240,000 Da with 6 identical subunits. The enzyme had high substrate specificity on L-lysine and NAD^+. The optimum pH and temperature were 9.3 and 50°C, respectively. The enzyme was stable over a pH range from 7.5 to 8.0. The apparent K_m value for L-lysine and NAD^+ of dimeric and hexameric form of this enzyme were 11.11, 0.138, 8.62 and 0.092 mM, respectively. For L-pipecolic acid production, the pyrroline-5-carboxylate reductase gene (*p5cr*) from *Bacillus cereus* ATCC 11778, encoding pyrroline-5-carboxylate reductase catalyses piperidiene-6-carboxylate to L-pipecolic acid, was co-existed with the *lys 6-dh* gene and transformed into *E. coli* BL21(DE3) using pET-17b. The optimum condition for both genes expression was induction with 0.1 mM IPTG for 3 hours. The specific activity of lysine 6-dehydrogenase and pyrroline-5-carboxylate reductase form crude enzyme were 25 and 11 folds higher than those of the enzyme from *A. denitrificans* and *B. cereus*, respectively. The highest production of L-pipecolic acid was obtained when *E. coli* cells was treated by xylene for 5 min. to increase cell permeability and then incubated in the reaction mixture consisited of 200 mM L-ysine in 200 mM Tris-HCl buffer, pH 9.0 for 24 hours. The amount of L-pipecolic acid production was 77 mM (9.9 g/l).

Field of study.....Biological Science....

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LIST OF ABBREVIATIONS

A	absorbance, 2′-deoxyadenosine (in a DNA sequence)
ADP	adenine dinucleotide phosphate
AlaDH	alanine dehydrogenase
AspDH	aspartic dehydrogenase
bp	base pairs
BLAST	Basic Local Alignment Search Tool
BSA	bovine serum albumin
C	2′-deoxycytidine (in a DNA sequence)
^{o}C	degree Celsius
Da	Dalton
DEAE	diethylaminoethyl
DNA	deoxyribonucleic acid
dNTP	2′-deoxynucleoside 5′-triphosphate
DTT	dithiothreitol
EC	Enzyme Commission
EDTA	ethylene diamine tetraacetic acid
ELSD	evaporative light scattering detector
G	2′-deoxyguanosine (in a DNA sequence)
GluDH	glutamate dehydrogenase
GlyDH	glycine dehydrogenase
HPLC	high-performance liquid chromatography
HCl	hydrochloric acid
IPTG	isopropyl-thiogalactoside
kb	kilobase pairs in duplex nucleic acid, kilobases in single-standed nucleic acid
KCl	potassium chloride
kDa	kiloDalton
K_m	Michaelis constant
KOH	potassium hydroxide

l	liter
LAT	Lysine aminotransferase
lat	Lysine aminotransferase gene
LB	Luria-Bertani
LeuDH	leucine dehydrogenase
LysDH	lysine dehydrogenase
Lys 2-DH	lysine 2-dehydrogenase
Lys 6-DH	lysine 6-dehydrogenase
lys 6-dh	lysine 6-dehydrogenase gene
μg	microgram
μl	microliter
μM	micromolar
M	mole per liter (molar)
mA	milliampere
mg	milligram
min	minute
ml	milliliter
mM	millimolar
M_r	relative molecular mass
MW	molecular weight
N	normal
NAD^+	nicotinamide adenine dinucleotide (oxidized)
NADH	nicotinamide adenine dinucleotide (reduced)
ng	nanogram
nm	nanometer
$(NH_4)_2SO_4$	ammonium sulfate
OD	optical density
P-5-C	pyrroline-5-carboxylate
P5CR	pyrroline-5-carboxylate reductase
p5cr	pyrroline-5-carboxylate reductase gene
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction

PheDH	phenylalanine dehydrogenase
pmol	picomole
PMSF	phenyl methyl sulfonyl fluoride
RNase	ribonuclease
SDS	sodium dodecyl sulfate
SerDH	serine dehydrogenase
T	2′-deoxythymidine (in a DNA sequence)
TB	Tris-borate buffer
TE	Tris-EDTA buffer
TEMED	*N, N, N′, N′*-tetramethyl ethylene diamine
TLC	thin-layer liquid chromatography
T_m	melting temperature, melting point
TrpDH	tryptophan dehydrogenase
UV	ultraviolet
V	voltage
ValDH	valine dehydrogenase
v/v	volume by volume
w/w	weight by weight