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CLONING AND EXPRESSION OF DEHYDROQUINATE DEHYDRATASE, SHIKIMATE DEHYDROGENASE AND GLUCOSE DEHYDROGENASE GENES FROM *Gluconobacter oxydans* 621H

Miss Chayatip Insomphun

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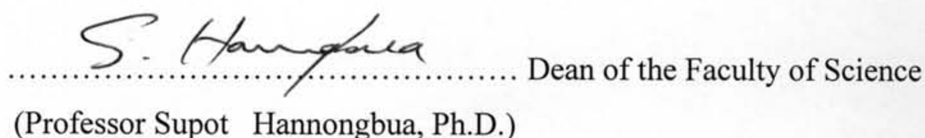
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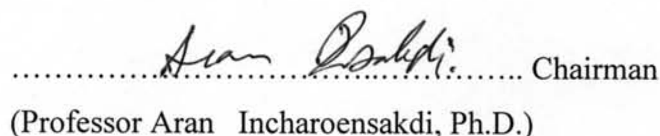
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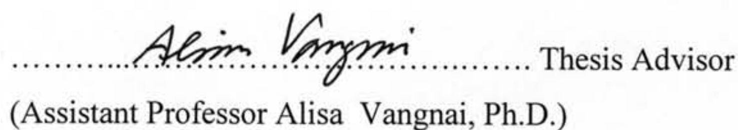
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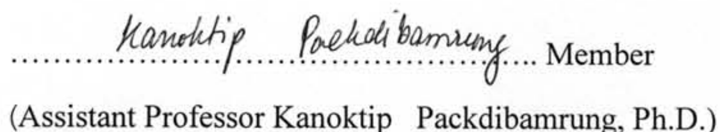
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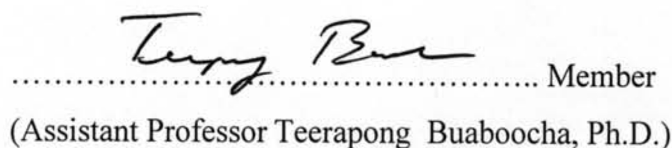
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ชญานิ์ทิพ อินสมพันธ์: การโคลนและแสดงออกของยีนดีไฮโดรควิเนตดีไฮดราเตส ยีนชิคิเมตดีไฮโดรจีเนส และยีนกลูโคสดีไฮโดรจีเนสจาก *Gluconobacter oxydans* 621H (CLONING AND EXPRESSION OF DEHYDROQUINATE DEHYDRATASE, SHIKIMATE DEHYDROGENASE AND GLUCOSE DEHYDROGENASE GENES FROM *Gluconobacter oxydans* 621H) อ. ที่ปรึกษา : ผศ.ดร. อลิสา วังใน, 182 หน้า .

ชิคิเมตและดีไฮโดรชิคิเมต ถูกนำมาใช้เป็นวัตถุดิบตั้งต้นเพื่อผลิตสารในอุตสาหกรรมหลายประเภทอย่างกว้างขวาง ยังผลให้เกิดการศึกษาและการพัฒนากระบวนการผลิตของสารดังกล่าว ในการศึกษานี้ได้ทำการโคลนยีนดีไฮโดรควิเนตดีไฮดราเตส (*dqd*, GOX0437) และยีนชิคิเมตดีไฮโดรจีเนส (*skdh*, GOX0859 และ GOX1959) จากเชื้อกลูโคโนแบคเตอร์สายพันธุ์ 621H และทำการแสดงออกที่มากเกินปกติในเชื้ออีโคไลสายพันธุ์ BL21(DE3) โดยใช้ pET-21a เป็นเวคเตอร์แสดงออก จากผลการแสดงออกของ *E. coli* BL21 (DE3)/pET-*dqd* พบว่า เมื่อทำการแสดงออกที่ 30 องศาเซลเซียส เอนไซม์มีแอคติวิตี (10.80 หน่วยต่อมิลลิกรัม) สูงกว่าเมื่อทำการแสดงออกที่ 37 องศาเซลเซียส 4 เท่า สำหรับการแสดงออกของ *skdh* (GOX0859) ค่าแอคติวิตีของ *E. coli* BL21 (DE3)/pET-GOX0859 มีค่าต่ำ (0.048 หน่วยต่อมิลลิกรัม) และไม่แตกต่างจากเชื้ออีโคไลสายพันธุ์ BL21(DE3) ดังนั้นยีน *skdh* (GOX0859) จึงถูกโคลนเข้าเวคเตอร์แสดงออก pCold I และแสดงออกร่วมกับ chaperone vector (pG-KJE8) เพื่อปรับปรุงแอคติวิตีของเอนไซม์ SKDH ซึ่งจากผลการแสดงออกร่วมกันพบว่ายีน *skdh* (GOX0859) ไม่มีแอคติวิตีของเอนไซม์ SKDH ในทางกลับกันการแสดงออกของ *E. coli* BL21 (DE3)/pET-GOX1959 เอนไซม์ SKDH มีแอคติวิตีสูงขึ้นอย่างเห็นได้ชัด (92.49 หน่วยต่อมิลลิกรัม) เมื่อใช้ชิคิเมตและ $NADP^+$เป็นสับสเตรทและโคแฟคเตอร์ตามลำดับ การแสดงออกนี้ทำให้เอนไซม์ SKDH มีแอคติวิตีสูงขึ้นจากเดิม 15 เท่า ดังนั้นจึงได้ทำเอนไซม์ SKDH (GOX1959) ให้บริสุทธิ์ด้วย Ni-NTA agarose คอลัมน์เพื่อหาค่าจลนพลศาสตร์ โดยค่า K_m และค่า V_{max} สำหรับชิคิเมตและ $NADP^+$ มีค่าเท่ากับ 250 ไมโครโมลาร์, 168.4 ไมโครโมลต่อนาทีมิลลิกรัมโปรตีน และ 51.7 ไมโครโมลาร์, 384.6 ไมโครโมลต่อนาทีมิลลิกรัมโปรตีน ตามลำดับ ยีน *skdh* (GOX1959) ได้ถูกแสดงออกในเชื้อกลูโคโนแบคเตอร์สายพันธุ์ IFO3244 โดยใช้ pSG8 เป็นเวคเตอร์แสดงออกส่งผลให้แอคติวิตีของเอนไซม์ SKDH มีค่าสูงขึ้น 10 เท่า นอกจากนี้มีการนำ $NADP^+$ กลับมาใช้อีกครั้ง โดยทำการแสดงออกร่วมกันของ ยีน *gdh* (GOX2015) ซึ่งถอดรหัสให้เอนไซม์กลูโคสดีไฮโดรจีเนสและยีน skdh (GOX1959) ในเวคเตอร์ pET-21a ภาวะที่เหมาะสมในการแสดงออกยีนทั้งสองนี้ร่วมกัน คือ เลี้ยงในอาหาร LB ปริมาตร 100 มิลลิลิตร ที่อุณหภูมิ 37 องศาเซลเซียส และเหนี่ยวนำให้เกิดการแสดงออกด้วย 0.2 มิลลิโมลาร์ IPTG เพื่อที่จะปรับปรุงแอคติวิตีของเอนไซม์ GDH ให้สูงขึ้น ซึ่งให้แอคติวิตีของ SKDH (GOX0859) และ GDH สูงถึง 279.24 หน่วยต่อมิลลิกรัม และ 1.03 หน่วยต่อมิลลิกรัม อย่างไรก็ตามเมื่อนำ pET-GOX1959 มาแสดงออกร่วมกับเวคเตอร์ pACGD ซึ่งมียีน *gdh* จากเชื้อบาซิลัส สายพันธุ์ *megaterium* ในเชื้ออีโคไลสายพันธุ์ BL21(DE3) ไม่ได้ช่วยเพิ่มแอคติวิตีของ GDH

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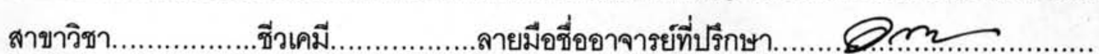

สาขาวิชา................ชีวเคมี................ลายมือชื่ออาจารย์ที่ปรึกษา...............................

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CHAYATIP INSOMPHUN: CLONING AND EXPRESSION OF DEHYDROQUINATE DEHYDRATASE, SHIKIMATE DEHYDROGENASE AND GLUCOSE DEHYDROGENASE GENES FROM *Gluconobacter oxydans* 621H. THESIS ADVISOR: ASST. PROF. ALISA VANGNAI, Ph.D., 183 pp.

Shikimate and dehydroshikimate have been widely used as starting material for several industries. Consequently, shikimate and dehydroshikimate production have been studied and developed. In this research, *dehydroquinate dehydratase* (*dqd*, GOX0437) and two *shikimate dehydrogenase* (*skdh*, GOX0859 and GOX1959) genes from *Gluconobacter oxydans* 621H were cloned and overexpressed in *Escherichia coli* BL21 (DE3) by using pET-21a vector. The pET-*dqd* expression result showed that the DQD activity when cultured at 30°C was 4-fold (10.80 U/mg) higher than that when cultured at 37°C. Gene expression of *E. coli* BL21 (DE3)/pET-GOX0859 showed very low SKDH activity (0.047 U/mg) which was fairly similar to that of *E. coli* wild type strain. Therefore, *skdh* (GOX0859) was subcloned into pCold I vector and co-expressed with pG-KJE8 chaperone vector to improve SKDH activity. From co-expression result, *skdh* (GOX0859) did not show SKDH activity. On the other hand, the expression of *E. coli* BL21 (DE3)/pET-GOX1959 exhibited significant SKDH activity (92.49 U/mg) with shikimate and $NADP^+$ as a substrate and a cofactor, respectively. This expression enhanced SKDH activity by 15 fold. Then, the overexpressed SKDH (GOX1959) was purified using Ni-NTA agarose column and determined for its kinetic parameters. K_m and V_{max} for shikimate and $NADP^+$ were 250 μM, 168.4 μmole/min.mg protein and 51.7 μM, 384.6 μmole/min.mg protein, respectively. Furthermore, homologus expression of *skdh* (GOX1959) was carried out using *G. oxydans* IFO3244 and pSG8 vector resulting in 10 times increasing SKDH activity. $NADP^+$ regeneration was performed by co-expression of *gdh* (GOX2015) encoding glucose dehydrogenase with *skdh* (GOX1959) in pET-21a. The conditions for *gdh* and GOX1959 co-expression were optimized. The SKDH and GDH activity was 2.3 fold increased (98.97 up to 227.90 U/mg) and 5.3 fold increased (0.15 up to 0.79 U/mg), respectively, when grown in 100-ml LB at 37°C, and induced with 0.2 mM IPTG at OD_{600} 0.54. To improve GDH activity, pET-GOX1959 was co-expressed with pACGD vector harboring *gdh* gene from *Bacillus megaterium* in *E. coli* BL21 (DE3). However, the GDH activity was not observed.

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Field of study.......Biochemistry........Advisor's signature....Alisa Vangnai....

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ABBREVIATIONS

A	Absorbance
BSA	bovine serum albumin
cm	centimeter
^{o}C	Degree Celsius
Da	Dalton
DNA	deoxyribonucleic acid
DQD	Dehydroquinate dehydratase
et al.	Et. Alii (latin), and others
GDH	Glucose dehydrogenase
IPTG	Isopropylthiogalactoside
Kb	kilobase
k_{cat}	catalytic constant
K_m	Michaelis constant
M	Molar
mA	miliampare
mg	milligram
min	minute
ml	milliliter
mM	milimolar
MW	molecular weight
ng	nanogram
nm	nanometer
OD	Optical density

PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
rpm	revolution per minute
SDS	sodium dodecyl sulphate
TEMED	N,N,N',N'-Tetramethylene ethylene diamine
V	volt
v/v	volume by volume
V_{max}	maximal velocity
w/v	weight by volume
µg	Microgram
µl	Microlitre