

การแสดงออกของไลเพสจาก *CANDIDA RUGOSA* เพื่อการใช้ประโยชน์ในอุตสาหกรรม



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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต

สาขาวิชาเทคโนโลยีชีวภาพ

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ปีการศึกษา 2552

ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย



4 8 7 3 8 4 4 2 2 3

**OVEREXPRESSION OF LIPASES FROM *CANDIDA RUGOSA*
FOR INDUSTRIAL APPLICATIONS**

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

Faculty of Science

Chulalongkorn University

Academic year 2009

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522344

รุ่งทิwa เปี่ยมทองคำ : การแสดงออกของไลเปสจาก *CANDIDA RUGOSA* เพื่อการใช้ประโยชน์ในอุตสาหกรรม (OVEREXPRESSION OF LIPASES FROM *CANDIDA RUGOSA* FOR INDUSTRIAL APPLICATIONS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รองศาสตราจารย์ ดร. วรวุฒิ จุฬาลักษณ์านุกูล, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: Professor Alain Marty, 155 หน้า.

ไลเปสมีความสามารถที่หลากหลายในการเป็นตัวเร่งปฏิกิริยาโดยเฉพาะไลเปสจาก *Candida rugosa* ในงานวิจัยนี้ได้ทำการศึกษาไลเปสจาก *Candida rugosa* สายพันธุ์ดั้งเดิม และ *C. rugosa* สายพันธุ์ที่แสดงออกบนยีนส์ Lip1, Lip3 และ Lip4 ไลเปสทั้ง 4 ชนิดมีความจำเพาะในการเร่งปฏิกิริยาต่อสารตั้งต้นที่ต่างกัน การศึกษาขั้นแรกเป็นการศึกษาการแสดงออกของไลเปสยีนส์โดยใช้ยีสต์ *Yarrowia lipolytica* สายพันธุ์ JMY1212 เป็นเจ้าบ้านในการแสดงออกของไลเปสจาก *C. rugosa* ทั้งสามยีนส์ (Lip1, Lip3 และ Lip4) จากการศึกษาคุณสมบัติเฉพาะพบว่า Lip1 และ Lip3 มีความจำเพาะต่อสารตั้งต้นกรดไขมันที่มีความยาวอะตอมคาร์บอนขนาดกลาง (C8-C12) ในขณะที่ Lip4 มีความจำเพาะต่อกรดไขมันที่มีความยาวอะตอมคาร์บอน C18:1 และเป็นครั้งแรกที่มีการนำไลเปสทั้งสามชนิดมาทำปฏิกิริยาในการสังเคราะห์กรดไขมันสายยาวไม่อิ่มตัวชนิดโอเมก้า-3 (*cis*-5, 8, 11, 14, 17-eicosapentaenoic acid (EPA) และ *cis*-4, 7, 10, 13, 16, 19-docosahexaenoic acid (DHA)) ซึ่งมีคุณค่าทางโภชนาการ ผลการศึกษาพบว่าไลเปสทั้งสามชนิดสามารถแยก DHA ได้ความบริสุทธิ์มากกว่า 90 เปอร์เซ็นต์ จากปฏิกิริยาไฮโดรลิซิส (97, 100 และ 93 % สำหรับ Lip1, Lip3 และ Lip4 ตามลำดับ) และเพิ่มความเข้มข้นของ DHA ให้มากกว่า 60 เปอร์เซ็นต์ จากความเข้มข้นเริ่มต้นที่ 25 เปอร์เซ็นต์ นอกจากนี้ Lip4 ยังสามารถแยก EPA จากเอสเทอร์ของ EPA ได้ 60 เปอร์เซ็นต์ (13 และ 16 เปอร์เซ็นต์สำหรับ Lip1 และ Lip3) ส่วนที่สองเป็นการพัฒนาความสามารถในการไฮโดรลิซิสสารผสมของเอสเทอร์ชนิด (*R, S*)-2-bromo aryl acetic acid โดยใช้ไลเปสจาก *C. rugosa* พบว่า Lip1 และ Lip3 มีความจำเพาะในการทำปฏิกิริยากับสารตั้งต้น เอสเทอร์ชนิด *S* ($E > 300$) ในขณะที่ Lip4 มีความจำเพาะในการทำปฏิกิริยากับสารตั้งต้นเอสเทอร์ชนิด *R* ($E=15$) ซึ่งจากการศึกษาในเชิงลึกและโมเดลสามมิติพบว่าการเปลี่ยนแปลงกรดอะมิโนที่ตำแหน่ง 296 บนยีนส์ Lip1 และ Lip4 เป็นตำแหน่งที่สำคัญต่อความจำเพาะของการเร่งปฏิกิริยา และการศึกษาการผลิตไบโอดีเซลจากไลเปสตรังรูป พบว่า การใช้วัสดุค้ำจุณ NKA-9 ในการผลิตไลเปสตรังรูปจาก *C. rugosa* ทำปฏิกิริยาทรานส์เอสเทอร์ฟิเคชันโดยใช้อัตราส่วนน้ำมันต่อเมทานอล 1:4 และทำปฏิกิริยาที่อุณหภูมิ 30 องศาเซลเซียส สามารถสังเคราะห์เมทิลเอสเทอร์ได้ 75 เปอร์เซ็นต์

สาขาวิชา เทคโนโลยีชีวภาพ

ปีการศึกษา 2552

ลายมือชื่อนิสิต..... *รุ่งทิwa เปี่ยมทองคำ*

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4873844223 : MAJOR BIOTECHNOLOGY

KEYWORDS : Lipase, *Candida rugosa*, expression system, mutagenesis, molecular modelisation, resolution of racemic mixture, purification of DHA, biodiesel production

RUNGTIWA PIAMTONGKAM : OVEREXPRESSION OF LIPASES FROM *CANDIDA RUGOSA* FOR INDUSTRIAL APPLICATIONS. THESIS
ADVISOR: ASSOC. PROF. WARAWUT CHULALAKSANANUKUL, Ph.D.
THESIS CO-AVISOR: PROF. ALAIN MARTY, 155 pp.

Lipases are the most studied enzymes and the most used in industry. In this work, we were interested in four lipases of industrial interest. There are belonging to the lipase family of *Candida rugosa* (wild type, Lip1, Lip3 and Lip4). We first tested a new expression system, a specific strain of *Yarrowia lipolytica*, for expression of *C. rugosa*. This strain JMY1212 enables integration to be targeted to a special locus of the *Y. lipolytica* genome. We demonstrated that it is the first expression system in which it is possible to compare statistically variant activities directly from the supernatant of the culture. Three lipases of *C. rugosa* were cloned successfully in this strain and their activities and specificities with respect to fatty acid chain lengths were studied. Lip1 and Lip3 have specificity for the fatty-acids of medium chain (C8-C12) whereas Lip4 prefers C18: 1. Moreover, for the first time, purification, from a mixture of ethyl esters issued from fish oil, polyunsaturated fatty acids (PUFAs); *cis*-5, 8, 11, 14, 17-eicosapentaenoic acid (EPA) and *cis*-4, 7, 10, 13, 16, 19-docosahexaenoic acid (DHA), molecules with health benefits, was realised with the three *C. rugosa* lipases, separately. Whatever the enzyme the recovery of DHA is superior to 90 % (97, 100 and 93 % for Lip1, Lip3 and Lip4 respectively). The maximal DHA purity ~60 % was obtained with Lip3 and Lip4, with an initial ethyl ester mixture containing 25% DHA. A remarkable difference between these enzymes lies in the fact that Lip4 is able to better hydrolyse the EPA esters (60% against 13% and 16% respectively for Lip1 and Lip3). Lip4 is also able to hydrolyse DHA (7% against 3 and 0 % for Lip1 and Lip3 respectively). The second part of this work was devoted to the improvement of the enantioselectivity of the two enzymes studied with respect to the resolution of a racemic mixture of pharmaceutical industry, the *R, S* esters of 2-bromo aryl acetic acid. The 3 lipases of *C. rugosa* proved to be remarkable from the point of view of enantioselectivity. In spite of their high homology, their specificity is different. Lip1 and Lip3 are completely specific for the *S* enantiomer ($E > 300$), whereas Lip4 is *R* specific ($E = 15$). The molecular docking of the *S* and *R* enantiomers in the active site of Lip1 and Lip4 lipases enables the observed differences in specificity to be better understood and targets for site-directed mutagenesis to be proposed. We demonstrated that the nature of the amino acid present in position 296 is crucial for the discrimination of these enzymes. The third part of reaction was biodiesel production from immobilized *C. rugosa* lipase. Optimum conditions were as follow; substrate molar ratio was Palm oil to methanol; 1:4, Lipase was immobilized on macroporous resin namely, NKA-9. Optimum temperature was 30 °C for transesterification. The result obtained 75 % of methyl ester production.

Field of Study : Biotechnology

Academic Year : 2009

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ACKNOWLEDGEMENTS

I would like to express my deepest gratitude and appreciation to my adviser Associate Professor Dr. Warawut Chulalaksananukul for his great advice, encouragement and his kind support, suggestion and help throughout my study.

I would like to express my deepest appreciation and gratitude to my co-adviser Professor Alain Marty for his invaluable guidance advice, suggestion, correction, comment, encouragement, morale support and mercy throughout the process of this research.

I also would like to express gratitude extended to Associate Professor Dr. Preeda Boon-long, Associate Professor Dr. Sirirat Rengpipat, Associate Professor Dr. Orathai Chavalparit and Assistant Professor Dr. Suphang Chulalaksananukul, as a chairman and members of thesis committee, respectively. All of whom have made valuable comments and suggestion and also dedicating valuable time for thesis examination.

Special thanks are also extending to Dr. Sophie Duquesne, Dr. Florence Bordes, Dr. Emmanuelle Cambon, Dr. Isabelle André, Dr. Sandrine Laguerre and Dr. Sophie Barbe for their help, kind assistance, suggestion and friendship throughout my research time at INSA-Toulouse. And my express thanks to Etienne Sevarac, Dr. Olivier Galy, Leticia Casas, Ivanna Rivera and all members of EAD1 for their kindness help, guidance and friendship during my stay in France.

I am also thankful to all members of Biofuel by Biocatalyst Research Unit, Chulalongkorn University and friends for any help during my thesis.

I would like to acknowledge The Royal Golden Jubilee Ph.D. Program (RGJ), Thailand Research Fund and France Embassy for their financial support during my thesis and extend my thankful to Laboratoire d'Ingénierie des Systèmes Biologiques et des Procédés (LISBP) de l'INSA de Toulouse, France for facilitate experiments in laboratory.

Finally, the greatest gratitude and indebtedness is expressed to my lovely family, my parents, my aunt, my brother and my loved ones, for their unlimited love, care, encouragement, understanding, morale support, financial support and never leave me alone.

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LIST OF ABBREVIATIONS

2M2B :	2-methyl-2-butanol
BSA :	Bovine Serum Albumine
CRL :	<i>Candida rugosa</i> lipase
CV :	Coefficient of variant
DHA :	Docosahexaenoic acid
DNA :	Deoxyribonucleic acid
DNS :	3,5-dinitrosalicylic acid
dNTP :	Deoxyribonucleotide
E :	Enantioselectivity
ee _s :	enantiomeric excess of substrate
ee _p :	enantiomeric excess of product
EDTA :	Ethylene diamine tetraacetic acid
EPA:	Eicosapentaenoic acid
G :	Gibbs' Free Energy
H :	Enthalpy
LB :	Luria-Bertani culture media
LiAc :	Lithium acetate
OD :	Optical Density
PCR :	Polymerase Chain Reaction
PDB :	<i>Para</i> -nitrophenol
<i>p</i> NPB :	<i>Para</i> -nitrophenyl butyrate
SDS :	Sodium dodecyl sulfate
T :	Temperature
Tris :	Trishydroxymethylaminomethane
Vi :	initiale velocity