รายงานวิจัยฉบับสมบูรณ์

ชื่อภาษาไทย: การผลิต conjugated linoleic acids จากน้ำมันเมล็ดทานตะวันโดยใช้ไลเปสร่วมกับแบคทีเรีย *Lactobacillus*

ชื่อภาษาอังกฤษ: Production of conjugated linoleic acids from sunflower oil by lipase couple with *Lactobacillus*

โดย

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กุมภาพันธ์ พ.ศ. 2558

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การผลิต conjugated linoleic acids จากน้ำมันเมล็ดทานตะวันโดยใช้ไลเปสร่วมกับแบคทีเรีย *Lactobacillus* ในขั้นต้นได้ทำการคัดเลือกเอนไซม์ที่เหมาะสมต่อการย่อยสลายของน้ำมัน พบว่าการใช้ไลเปสที่ผลิตจากยีสต์สามารถย่อยสลายน้ำมันได้มากกว่าการใช้ไลเปสจากแหล่งอื่น โดยเฉพาะการใช้ *Candida rugosa* lipase ในการย่อยสลายน้ำมันให้ระดับการย่อยสูงสุด คือร้อยละ 67.12 หลังจากนั้นจึงศึกษาสภาวะที่เหมาะสมต่อกระบวนการดังกล่าว ปัจจัยต่าง ๆ ที่เกี่ยวข้องกับกระบวนการนี้จะถูกคัดเลือกโดยใช้แผนการทดลองแบบแฟคทอเรียลบางส่วน (Fractional factorial design) ซึ่งตัวแปรที่เกี่ยวข้องได้แก่ อัตราส่วนปริมาณของเอนไซม์ต่อน้ำมัน อัตราส่วนปริมาณของบัฟเฟอร์ต่อน้ำมัน ค่าพีเอชเริ่มต้น อุณหภูมิ และเวลาในการทำปฏิกิริยา เมื่อวิเคราะห์ผลด้วยวิธีการทางสถิติ พบว่าทุกตัวแปรมีผลต่อระดับการย่อยสลายของน้ำมันอย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) ยกเว้นอุณหภูมิในการทำปฏิกิริยา จากนั้นจึงประเมินสภาวะที่เหมาะสมของกระบวนการดังกล่าวโดยวิธีพื้นผิวตอบสนอง (Response surface methodology) ด้วยแผนการทดลอง central composite design พบว่าตัวแปรที่มีผลอย่างมีนัยสำคัญต่ออัตราการย่อยสลายของน้ำมัน คือ อัตราส่วนปริมาณของเอนไซม์ต่อน้ำมัน อัตราส่วนปริมาณของบัฟเฟอร์ต่อน้ำมัน และค่าพีเอชเริ่มต้น ($p < 0.05$) เมื่อวิเคราะห์สมการถดถอยพบว่าสมการที่ใช้ในการพยากรณ์มีความแม่นยำ ($p < 0.05$) และมีความเหมาะสม ($R^2 = 0.89$) นอกจากนี้เมื่อทำการทวนสอบสมการดังกล่าว พบว่าความคลาดเคลื่อนระหว่างระดับการย่อยสลายที่พยากรณ์ และระดับการย่อยสลายที่ได้จากการทดลอง มีค่าน้อยกว่าร้อยละ 5 สภาวะที่เหมาะสมต่อระดับการย่อยสลายร้อยละ 76.06 ได้แก่ อัตราส่วนปริมาณของเอนไซม์ต่อน้ำมัน คือ 750.28 ยูนิตต่อกรัม อัตราส่วนปริมาณของบัฟเฟอร์ต่อน้ำมัน คือ 4.0 กรัมต่อกรัม พีเอชเริ่มต้นของปฏิกิริยา คือ 6.70 อุณหภูมิของปฏิกิริยา คือ 40 องศาเซลเซียส และเวลาในการทำปฏิกิริยา คือ 19 ชั่วโมง เพื่อปรับปรุงความบริสุทธิ์ของกรดลิโนเลอิกที่ได้จึงนำผลิตภัณฑ์ที่ได้จากการย่อยสลายน้ำมันมาเพิ่มความบริสุทธิ์ด้วย

วิธีการ urea complex fractionation ทำให้ความบริสุทธิ์ของกรดลิโนเลอิกเพิ่มขึ้นถึงร้อยละ 70.07 การศึกษาลำดับต่อมานำแบคทีเรียกรดแลคติกจำนวน 12 สายพันธุ์มาคัดเลือกสายพันธุ์ที่มีความสามารถในการผลิตกรดคอนจูเกตลิโนเลอิกในอาหารเหลวชนิด De Man-Rogosa-Sharpe (MRS) ที่มีองค์ประกอบของกรดลิโนเลอิก พบว่า *Lactobacillus plantarum* TISTR 543 สามารถผลิตกรดคอนจูเกตลิโนเลอิก เท่ากับ 0.04 มิลลิกรัมต่อมิลลิลิตร หลังจากนั้นจึงศึกษาผลของความเข้มข้นกรดลิโนเลอิกต่อการเจริญของแบคทีเรียชนิดนี้ พบว่าการเพิ่มความเข้มข้นของกรดลิโนเลอิกทำให้แบคทีเรียลดการเจริญ โดยที่แบคทีเรียชนิดนี้จะสามารถเจริญได้ในอาหารเหลวชนิด MRS ที่มีความเข้มข้นของกรดลิโนเลอิกได้ถึงระดับ 2.0 มิลลิกรัมต่อมิลลิลิตร การศึกษาขั้นสุดท้ายคือการผลิตกรดคอนจูเกตลิโนเลอิกจากน้ำมันทานตะวันโดยการใช้ไลเปสร่วมกับแบคทีเรียกรดแลคติก พบว่าสามารถผลิตกรดคอนจูเกตลิโนเลอิกได้ 85.0 มิลลิกรัมต่อกรัมน้ำมัน ดังนั้นกระบวนการเตรียมกรดคอนจูเกตลิโนเลอิกโดยกระบวนการทางชีวภาพจึงมีความเป็นไปได้ โดยข้อเสนอด้านการวิจัยในอนาคตคือ ควรปรับปรุงวิธีการเตรียมกรดลิโนเลอิกจากน้ำมันเมล็ดทานตะวันให้มีประสิทธิภาพมากขึ้นโดยใช้ตัวเร่งปฏิกิริยาที่มีความเหมาะสม ควรศึกษาสภาวะที่เหมาะสมต่อการผลิต และวิธีการที่เหมาะสมในการเก็บเกี่ยวกรดคอนจูเกตลิโนเลอิกเพื่อเพิ่มผลผลิตของกระบวนการผลิตต่อไป

Keywords: Conjugated linoleic acids, ไลเปส, *Lactobacillus*, น้ำมันเมล็ดทานตะวัน, แบคทีเรียกรด แลคติก

Abstract

Project Code : MRG5380116

Project Title : Production of conjugated linoleic acids from sunflower oil by lipase couple with *Lactobacillus*

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In present study, bioprocess for the preparation of conjugated linoleic acids (CLAs) was demonstrated. Initially, enzymatic preparation and enrichment of linoleic acid (LA) were examined. Enzyme screening for oil hydrolysis was investigated. The results indicated that using lipase from yeast as a biocatalyst exhibited the higher rate of oil hydrolysis than using lipases from other sources. Particularly, utilizing *Candida rugasa* lipase gave the highest hydrolysis rate of 67.12%. Then, optimization of the enzymatic process was performed. Several variables involving in the enzymatic process were screened to identify significant variables through fractional factorial design. The investigated variables were enzyme to oil ratio, buffer to oil ratio, initial pH, reaction temperature and reaction time. Statistical analysis indicated that most of the variables were statistically significant ($p < 0.05$) except for reaction temperature. Then, the optimal conditions for oil hydrolysis were examined. Response surface methodology, based on central composite design, was adopted. According to statistical analysis, the variables significantly affecting the degree of oil hydrolysis were buffer to oil ratio, enzyme to oil ratio and initial pH ($p < 0.05$). Regression analysis suggested that the predictive model was adequate precision ($p < 0.05$) and satisfactory level of coefficient of determination ($R^2 = 0.89$). In addition, verification of the statistical model suggested that difference between the predicted and actual values of the hydrolysis degree was less than 5%. The optimal conditions to obtain oil hydrolysis degree of 76.06% were achieved as the following conditions, enzyme to oil ratio of 750.28 U/g, buffer to oil ratio of 4.0 (w/w), initial pH of 6.70, reaction time of 19 h and reaction temperature of 40 $^{\circ}$C. In order to improve the purity of LA, the hydrolysate was enriched using urea complex fractionation. The purity of LA could be enriched to 70.07%. Consequently, the potential lactic acid bacteria (LAB) were selected to examine capability of CLA

production. 12 strains of the selected LAB were cultured in De Man-Rogosa-Sharpe (MRS) broth supplemented with LA. *Lactobacillus plantarum* TISTR 543 was found to be an appropriate CLA producer. Content of CLA production from the LAB was 0.04 mg/mL. Then, the effect of LA concentration on growth of the LAB was evaluated. The results suggested that increase in concentration of LA deteriorated the growth and this bacterium was able to grow in MRS supplemented with LA concentration up to 2.0 mg/mL. Finally, production of CLAs from sunflower oil using lipase couple with the appropriate LAB was evaluated. Capability of CLA production from sunflower oil using this bioprocess was 85.0 mg/g. Therefore, bio-based preparation of CLAs from oil could be established. Forthcoming studies are required in order to enhance the capability of CLA production. For example, improvement of LA preparation from sunflower oil could be achieved using more suitable catalysts. Optimization of the reaction conditions for CLA production and the purification approach of the resulting CLAs could enhance productivity of the overall process.

Keywords: Conjugated linoleic acids, lipase, *Lactobacillus*, sunflower oil, lactic acid bacteria

บทสรุปย่อผู้บริหาร (Executive Summary)

“การผลิต conjugated linoleic acid จากน้ำมันเมล็ดทานตะวันโดยใช้ไลเปสร่วมกับแบคทีเรีย *Lactobacillus*”

Conjugated linoleic acids (CLAs) เป็นกลุ่มไอโซเมอร์ของ linoleic acid ซึ่งประกอบด้วยคาร์บอนจำนวน 18 หน่วย ตำแหน่งที่เกิดพันธะคู่จะเชื่อมต่อด้วยพันธะเดียว (–C=C-C=C–) ในรูปแบบที่เป็น cis- หรือ trans- ซึ่งไอโซเมอร์ที่พบมากที่สุดคือ cis-9, trans-11 และ trans-10, cis-12 โดย CLAs มีส่วนช่วยเสริมสุขภาพ และช่วยป้องกันโรคต่าง ๆ ได้ เช่น ความดันโลหิตสูง เบาหวาน และโรคมะเร็งบางชนิด เป็นต้น โดยเฉพาะ trans-10, cis-12-CLA มีส่วนช่วยลดไขมันในร่างกาย ในขณะที่ cis-9, trans-11-CLA มีคุณสมบัติช่วยป้องกันมะเร็งบางชนิด (Bhattacharya et al., 2006) อาหารที่มี linoleic acid เป็นองค์ประกอบจะถูกเปลี่ยนไปเป็น CLAs ได้จากการทำงานของจุลินทรีย์ที่พบภายในร่างกาย และยังพบ CLAs ในเนื้อ และนมของสัตว์เคี้ยวเอื้องได้เช่นกัน (Ogawa et al., 2005) อย่างไรก็ตาม ปริมาณของ CLAs ที่ได้รับจากอาหารนั้น มักจะไม่มากพอที่จะช่วยป้องกันโรคได้ จึงจำเป็นต้องได้รับ CLAs เพิ่มเติมจากแหล่งอื่น ๆ ในบรรดาจุลินทรีย์ที่มีศักยภาพในการผลิต CLAs *Lactobacillus* นับเป็น lactic acid bacteria กลุ่มหนึ่งที่มีศักยภาพสูงในการผลิต CLAs ซึ่งสามารถพัฒนาไปสู่การผลิตในเชิงพาณิชย์ได้ (Andrade et al., 2012) โดยพบว่า *Lactobacillus* ที่สามารถผลิต CLAs ได้จากกรดไขมันที่เกี่ยวข้อง ได้แก่ linoleic acid และ ricinoleic acid แต่จุลินทรีย์กลุ่มนี้ไม่สามารถใช้น้ำมันในการผลิต CLAs ได้โดยตรง (Ogawa et al., 2005)

อย่างไรก็ตาม การผลิต CLAs จากกรดไขมันดังกล่าวอาจไม่สามารถพัฒนาไปสู่การผลิตในเชิงพาณิชย์ได้ เนื่องจากข้อจำกัดด้านปริมาณ และราคาของวัตถุดิบ ดังนั้นการผลิต CLAs จากน้ำมัน (triglyceride) ที่มีกรดไขมันดังกล่าวเป็นองค์ประกอบจึงน่าจะเป็นทางเลือกหนึ่ง น้ำมันจากเมล็ดทานตะวันนับเป็นวัตถุดิบชนิดหนึ่งที่เหมาะสม เนื่องจากน้ำมันชนิดนี้มี linoleic acid เป็นกรดไขมันหลัก จึงทำให้การเตรียม linoleic acid เข้มข้นเพื่อนำไปใช้ในการผลิต CLAs ดำเนินการได้ง่ายขึ้น ซึ่งการทำให้กรดไขมันเข้มข้น (fatty acid enrichment) หรือทำให้บริสุทธิ์เพิ่มขึ้นนิยมใช้ไลเปสในการเร่งปฏิกิริยาเนื่องจากเป็นเอนไซม์กลุ่มหนึ่งที่มีความจำเพาะในหลายระดับได้แก่ ความเพาะต่อตำแหน่งหรือชนิดของกรดไขมัน (Hayes, 2004) การคัดเลือกไลเปสที่มีความเหมาะสมต่อการผลิต linoleic acid จึงมีบทบาทสำคัญในกระบวนการนี้ ดังนั้นผลการศึกษานี้จึงน่าจะเป็นการเพิ่มทางเลือกในใช้วัตถุดิบสำหรับผลิต CLAs โดยจุลินทรีย์ และเป็นการสร้างองค์ความรู้พื้นฐานในการพัฒนาการผลิต CLAs โดยกระบวนการทางชีวภาพต่อไป

วัตถุประสงค์ของการวิจัย งานวิจัยครั้งนี้จึงเป็นการศึกษาชนิดของไลเปส และวิธีการที่เหมาะสมต่อการเตรียม linoleic acid เข้มข้นจากน้ำมันเมล็ดทานตะวัน และศึกษาความสามารถของจุลินทรีย์กลุ่ม *Lactobacillus* ในการผลิต CLAs จาก linoleic acid เข้มข้น รวมทั้งความเป็นไปได้ในการผลิต CLAs จากน้ำมันเมล็ดทานตะวันโดยใช้ไลเปสร่วมกับจุลินทรีย์กลุ่ม *Lactobacillus*

ผลการดำเนินงานวิจัย พบว่าการใช้ไลเปสที่ผลิตจากยีสต์สามารถย่อยสลายน้ำมันได้มากกว่าการใช้ไลเปสจากแหล่งอื่น โดยเฉพาะการใช้ *Candida rugosa* lipase ในการย่อยสลายน้ำมันให้ระดับการย่อยสูงสุด คือ ร้อยละ 67.12 หลังจากนั้นจึงศึกษาสภาวะที่เหมาะสมต่อกระบวนการดังกล่าว ปัจจัย

ต่างๆ ที่เกี่ยวข้องกับกระบวนการนี้จะถูกคัดเลือกโดยใช้แผนการทดลองแบบแฟคทอเรียลบางส่วน (Fractional factorial design) ซึ่งตัวแปรที่เกี่ยวข้องได้แก่ อัตราส่วนปริมาณของเอนไซม์ต่อน้ำมัน อัตราส่วนปริมาณของบัฟเฟอร์ต่อน้ำมัน ค่าพีเอชเริ่มต้น อุณหภูมิและเวลาในการทำปฏิกิริยา เมื่อวิเคราะห์ผลด้วยวิธีการทางสถิติพบว่า ทุกตัวแปรมีผลต่อระดับการย่อยสลายของน้ำมันอย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) ยกเว้นอุณหภูมิในการทำปฏิกิริยา จากนั้นจึงประเมินสภาวะที่เหมาะสมของกระบวนการดังกล่าวโดยวิธีพื้นผิวตอบสนอง (Response surface methodology) ด้วยแผนการทดลอง central composite design พบว่าตัวแปรที่มีผลอย่างมีนัยสำคัญต่ออัตราการย่อยสลายของน้ำมันคือ อัตราส่วนปริมาณของเอนไซม์ต่อน้ำมัน อัตราส่วนปริมาณของบัฟเฟอร์ต่อน้ำมัน และค่าพีเอชเริ่มต้น ($p < 0.05$) เมื่อวิเคราะห์สมการถดถอย พบว่าสมการที่ใช้ในการพยากรณ์มีความแม่นยำ ($p < 0.05$) และมีความเหมาะสม ($R^2 = 0.89$) นอกจากนี้เมื่อทำการทวนสอบสมการดังกล่าว พบว่าความคลาดเคลื่อนระหว่างระดับการย่อยสลายที่พยากรณ์และระดับการย่อยสลายที่ได้จากการทดลองน้อยกว่าร้อยละ 5 สภาวะที่เหมาะสมต่อระดับการย่อยสลายร้อยละ 76.06 ได้แก่ อัตราส่วนปริมาณของเอนไซม์ต่อน้ำมัน คือ 750.28 ยูนิตต่อกรัม, อัตราส่วนปริมาณของบัฟเฟอร์ต่อน้ำมันคือ 4.0 กรัมต่อกรัม พีเอชเริ่มต้นของปฏิกิริยาคือ 6.70 อุณหภูมิของปฏิกิริยาคือ 40 องศาเซลเซียส และเวลาในการทำปฏิกิริยาคือ 19 ชั่วโมง เพื่อปรับปรุงความบริสุทธิ์ของกรดลิโนเลอิกที่ได้จึงนำผลิตภัณฑ์ที่ได้จากการย่อยสลายน้ำมัน มาเพิ่มความบริสุทธิ์ด้วยวิธีการ urea complex fractionation ทำให้ความบริสุทธิ์ของกรดลิโนเลอิกเพิ่มขึ้นถึงร้อยละ 70.07

การศึกษาลำดับต่อมาจึงนำแบคทีเรียกรดแลคติกจำนวน 12 สายพันธุ์มาคัดเลือกสายพันธุ์ที่มีความสามารถในการผลิตกรดคอนจูเกตลิโนเลอิกในอาหารเหลวชนิด De Man-Rogosa-Sharpe (MRS) ที่มีองค์ประกอบของกรดลิโนเลอิก พบว่า *Lactobacillus plantarum* TISTR 543 สามารถผลิตกรดคอนจูเกตลิโนเลอิกเท่ากับ 0.04 มิลลิกรัมต่อมิลลิลิตร หลังจากนั้นจึงศึกษาผลของความเข้มข้นกรดลิโนเลอิกต่อการเจริญของแบคทีเรียชนิดนี้ พบว่าการเพิ่มความเข้มข้นของกรดลิโนเลอิกทำให้แบคทีเรียลดการเจริญ โดยที่แบคทีเรียชนิดนี้จะสามารถเจริญได้ในอาหารเหลวชนิด MRS ที่มีความเข้มข้นของกรดลิโนเลอิกได้ถึงระดับ 2.0 มิลลิกรัมต่อมิลลิลิตร การศึกษาขั้นสุดท้ายคือ การผลิตกรดไขมันคอนจูเกตลิโนเลอิกจากน้ำมันทานตะวันโดยการใช้ไลเปสร่วมกับแบคทีเรียกรดแลคติก พบว่าสามารถผลิตกรดคอนจูเกตลิโนเลอิกได้ 85.0 มิลลิกรัมต่อกรัมน้ำมัน ดังนั้นกระบวนการเตรียมกรดคอนจูเกตลิโนเลอิกโดยกระบวนการทางชีวภาพจึงมีความเป็นไปได้

ข้อเสนอด้านการวิจัย

1) ควรปรับปรุงวิธีการเตรียม linoleic acid จากน้ำมันเมล็ดทานตะวันให้มีประสิทธิภาพมากขึ้นโดยใช้ตัวเร่งปฏิกิริยาที่เหมาะสม

2) ควรศึกษาสภาวะที่เหมาะสมต่อการผลิต CLAs จากน้ำมันเมล็ดทานตะวันโดยใช้ไลเปสร่วมกับจุลินทรีย์กลุ่ม *Lactobacillus*

3) ควรศึกษาวิธีการที่เหมาะสมในการเก็บเกี่ยว CLAs ที่ได้จากกระบวนการทางชีวภาพดังกล่าว

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CHAPTER I
Introduction

1.1. Rationale and background

Conjugated linoleic acids (CLAs) refer a mixture of positional and geometrical isomers of linoleic acid (LA) with conjugated double bonds and have attracted much attention because of their health benefits such as anti-carcinogenesis, anti-atherogenesis, growth promotion and reduction of body fat (Bhattacharya et al., 2006; Koba and Yanagita, 2014). Generally, natural CLAs are the intermediates of LA biohydrogenation to stearic acid (SA) by ruminal bacteria. The amount of natural CLAs is very low and insufficient effect on health benefit. To overcome this problem, beneficial CLAs can be produced by alkaline isomerization of oil with high content of LA such as safflower, sunflower and soybean oil. Since unfavorable CLAs obtaining from chemical process exhibit adversely to physiological effect, CLAs producing from bioprocess could be used for medical and nutraceutical products (Ogawa et al., 2005).

In addition, CLAs were found to be produced from LA and oil with high content of LA by several microorganisms (Andrade et al., 2012; Ogawa et al., 2005; Van Nieuwenhove et al., 2012). Among of them, Lactic acid bacteria (LAB) are the alternative CLAs producer because they could produce linoleic acid isomerase (LAI). Isomerase activity was observed in several groups of LABs. The well-known groups of the potential LAB are *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Pediococcus* and *Streptococcus*. Ability of the potential LAB to produce CLAs depends on the nature of each strain (Andrade et al., 2012). CLAs were found to be obtained from *Enterococcus* sp., *Lactobacillus* sp., *Lactococcus* sp., *Pediococcus* sp. and *Streptococcus* sp. in the range of 0.05-3.45 mg/mL (Ogawa et al., 2005). Using plant seed oil instead of LA for CLA production could reduce the cost of production because plant seed oil was found to have high content of LA at 48.30-74.0% (Firestone, 2006), In this case, oil has to hydrolyze to generate LA. Then, it is converted to CLAs by the potential LAB. Many reports revealed that several oil could be used as a substrate to produce CLAs (Abd El-Salam et al., 2010; Puniya et al., 2008; Wang et al., 2007; Xu et al., 2004). For example, sesame oil was hydrolyzed by lipase. Then, LA was converted to CLAs by *Pediococcus* sp. GS4 (Dubey et al., 2012). CLA production from sunflower oil was achieved by

enzymatic hydrolysis and conversion of LA was performed by *Lb. brevis* and *Propionibacterium freudenreichii* (Puniya et al., 2008; Wang et al., 2007).

Normally, production of fatty acids is conducted by chemical and enzymatic hydrolysis of the oil. Conventional hydrolysis of triacylglycerides must be operated under the high temperature (250 °C) and pressure (50 bars) or using alkaline as a catalyst. This method leads to a formation of undesirable products and high energy consumption (Hayes, 2004). Then, the desired fatty acid is separated and purified (Yuji, 2005). On the other hand, enzymatic approach is an attractive method because it can be performed under mild condition as well as a high purity of product is usually obtained (Hayes, 2004; Piazza and Foglia, 2007; Yuji, 2005). Naturally, oil is usually hydrolyzed into free fatty acids and glycerol by lipase. In addition, these enzymes are also able to catalyze various reactions such as esterification and transesterification. Catalytic ability of lipase is specific to acyl group, position and stereoisomer of the substrate (Hayes, 2004). According to their unique characteristics, preparation of free fatty acids usually uses lipase as a biocatalyst.

In order to enhance productivity, optimization of the production is required. Traditionally, optimization of bioprocess is carried out by one factor at a time method. This method requires large number of experimental trials and it could not describe interaction of different variables as well as it is not assured to reach the optimal condition. In order to overcome these limitations, response surface methodology (RSM) is widely applied. It is a collection of statistical and mathematical techniques useful for development, improvement, and optimization of the process (Montgomery, 2001). RSM consists of various designs, central composite design (CCD) is widely used because it reduces the number of experiment treatments, covers wide range and describes the interaction of the variables (Bezerra et al., 2008). In this study, a bioprocess for CLA production from sunflower oil was established. In order to achieve this goal, optimal preparation of LA concentrates needs to be developed. In this case, suitable biocatalyst and optimal conditions for oil hydrolysis must be examined. Then, the potential LAB strain was used for CLA production.

1.2 Objectives of the research project

1.2.1 To examine the suitable type of lipase for oil hydrolysis

1.2.2 To optimize the enzymatic hydrolysis of sunflower oil in order to prepare LA from sunflower oil

1.2.3 To identify potential LAB for CLA production

1.2.4 To establish bioprocess for CLA production from sunflower oil using lipase couple with potential LAB

1.3. Scope and limitation of the research project

This study was divided in to 4 parts according to the objectives. Initially, the effect of lipase species on degree of hydrolysis was examined for selection of the suitable biocatalyst. Then, enzymatic hydrolysis was optimized for preparation of LA concentrates. Consequently, screening the potential LAB was performed to identify the candidate strain. Finally, bioprocess for the production of CLA from sunflower oil was demonstrated.

1.4. Anticipated outcomes

1.4.1. Suitable biocatalyst for oil hydrolysis was identified.

1.4.2. Enzymatic preparation of LA concentrates from sunflower oil was achieved.

1.4.3. Appropriate LAB strain for CLA preparation was obtained.

1.4.4. Bioprocess for CLA production from sunflower oil was established.

1.4.5. The results of this study could be used to present and publish in international conferences and scientific journal.

CHAPTER II

Literature Review

2.1. Fatty acids

2.1.1. Nomenclature of fatty acids

Traditionally, the nomenclature of the fatty acids was based on a trivial name because of the convenience and simplicity. However, this name does not express the chemical structure. Recently, the nomenclature of the fatty acids is classified according to their chemical characteristics.

2.1.1.1. Systematic nomenclature

The systematic name of the fatty acids is derived from IUPAC nomenclature of the organic chemistry. Fatty acids are named systematically as carboxylic acids and the number of carbon atoms. The ending name of the fatty acids is changed to indicate the number of double bounds in their chemical structure. In case of unsaturated fatty acids, the ending name is -anoic acid, but it is changed to -enoic, -adienoic, -atrienoic, -atetraenoic, -apentaenoic and –ahexaenoic acid in order to indicate the presence of one to six double bonds (Scrimgeour and Harwood, 2007). The double bonds configuration is revealed by *Z-/E-* or *cis-*/*trans-* notation. Therefore, the systematic name of fatty acids is an excellent description of the fatty acids, but it is inconvenient for general use. In order to avoid this limitation, the shorthand name is accepted. The two numbers separated by a colon for the chain length and a number of the double bonds, respectively (Ruiz-Rodriguez et al., 2010; Scrimgeour and Harwood, 2007)

2.1.1.2. The Delta-*x* nomenclature

The delta-x nomenclature is based on the position and configuration of the double bond related to the carboxyl end. Position of double bond in structures of the fatty acids is indicated by Δ*x*, where *x* is the double bond nearest the carboxyl end. Each double bond is preceded by a *cis-* or *trans-* prefix, which presents the conformation of the molecule around the bond (Ruiz-Rodriguez et al., 2010; Scrimgeour and Harwood, 2007).

2.1.1.3. The ω-*x* or n-*x* nomenclature

This name is counted double bond from the methyl end and written in n-*x* or ω-*x*, where *x* is the double bond nearest the methyl end. Generally, this nomenclature is

recommended and widely used in the biomedical and nutritional terms (Ruiz-Rodriguez et al., 2010; Scrimgeour and Harwood, 2007).

2.1.2. The structures of fatty acids

Typically, fatty acids, a monocarboxylic acid with a long aliphatic chain, contain an even chain length between C_4 to C_{22}. The double bonds of fatty acids are occurred by desaturizing at specific positions related to the carboxyl group. Generally, fatty acids are known as the building block of lipid and mainly originated from triacylglycerols or phospholipids (Scrimgeour and Harwood, 2007). Numerous fatty acids are usually classified by chain lengths, positions, configurations of carbon atoms, types of unsaturation and a range of additional substituents along with the aliphatic chain (Scrimgeour and Harwood, 2007). The fatty acids commonly differ in chains lengths. The short- and medium-chain fatty acids are fatty acids containing aliphatic chain length of 4 to 14 carbons (C_4-C_{14}), respectively (Beermann et al., 2003). The long-chain fatty acids refers the fatty acids containing aliphatic chain more than 16 to 21 carbons (C_{16}-C_{21}) (Scrimgeous, 2005). Moreover, the fatty acids containing aliphatic chain longer than 22 carbons are very long chain fatty acids (Frank, 2004; Ruiz-Rodriguez et al., 2010). In addition, fatty acids can be categorized as saturated, monounsaturated or polyunsaturated fatty acids based on the number of double bonds.

2.1.2.1. Saturated fatty acids

Saturated fatty acids are defined as a homologous series of monocarboxylic acids with the straight chains of the carbon atoms and they have no double bonds in the carbon chains. Natural saturated fatty acids are found in the esterified forms. The general formula is $CH_3(CH_2)_nCOOH$ (Christie, 2003). Normally, the saturated fatty acids consist of the odd- and even- numbered homologues ranging from 2 to 36 carbon atoms (Table 2.1).

2.1.2.2. Monoenoic acids

Monoenoic acids are straight-chain carbon containing one double bond an even number of the carbon atoms and recognized as monounsaturated fatty acids. The general structure of monoenoic acids is $CH_3(CH_2)_xCH=CH(CH_2)_yCOOH$ (Christie, 2003). The monounsaturated fatty acids exist in *cis*-(*Z*) and *trans*-(*E*) configuration. The *cis* configuration is mostly found in natural oil and fat such as oleic acid (OA) but the *trans* configuration is not commonly found. The monoenoic fatty acids from natural source

are found in esterified form and they contain 16 and 18 carbon atoms (Table 2.2) (Christie, 2003; Scrimgeour and Harwood, 2007).

Table 2.1 Nomenclature and chemical structure of the major saturated fatty acids

Systematic Names	Trivial Names	Shorthand Names	Structures
Dodecanoic acid	Lauric acid	12:0	$CH_3(CH_2)_{l0}COOH$
Tetradecanoic acid	Myristic acid	14:0	$CH_3(CH_2)_{12}COOH$
Hexadecanoic acid	Palmitic acid	16:0	$CH_3(CH_2)_{14}COOH$
Octadecanoic acid	Stearic acid	18:0	$CH_3(CH_2)_{16}COOH$
Eicosanoic acid	Arachidic acid	20:0	$CH_3(CH_2)_{18}COOH$

(Christie, 2003; Scrimgeour and Harwood, 2007)

Table 2.2 Nomenclature and chemical structure of the major monoenoic fatty acids

Systematic Names	Trivial Names	Shorthand Names	Omega-Names	Structures
Z-9-hexadecenoic acid	Palmitoleic Acid	16:1, *9c*	7	$CH_3(CH_2)_5CH=CH(CH_2)_7COOH$
Z-9-octadecenoic acid	Oleic acid	18:1, *9c*	9	$CH_3(CH_2)_7CH=CH(CH_2)_7COOH$
E-11-octadecenoic acid	Vaccenic acid	18:1, *11t*	7	$CH_3(CH_2)_5CH=CH(CH_2)_9COOH$
Z-13-docosenoic acid	Erucic acid	22:1, *13c*	9	$CH_3(CH_2)_7CH=CH(CH_2)_{11}COOH$

(Scrimgeour and Harwood, 2007)

2.1.2.3. Methylene-interrupted polyunsaturated fatty acids

Polyunsaturated fatty acids (PUFAs) refer a group of fatty acids containing two or more double bonds and existing *cis(Z)*-configuration. The double bond in PUFAs is methylene-interrupted pattern (Frank, 2004; Scrimgeour and Harwood, 2007). The general formula is $CH_3(CH_2)_m(CH=CHCH_2)_x(CH_2)_nCOOH$ (Christie, 2003). The principal PUFAs are divided into omega-3 and omega-6 family depending on the position of double bond related to the methyl end (Table 2.3). Generally, the properties of PUFAs are the low melting point and susceptible to oxidative deterioration or auto-oxidation (Christie, 2003; Scrimgeour and Harwood, 2007).

Table 2.3 Nomenclature and chemical structures of the important PUFAs

Systematic Names	Trivial Names	Shorthand Names	Omega-Names	Structures
Z, Z-9, 12-octadecadienoic acid	Linoleic Acid	18:2, *9c,12c*	6	$CH3(CH_2)_3(CH_2CH=CH)_2(CH_2)_7COOH$
Z, Z, Z-6, 9, 12-octadecatrienoic acid	ɣ-linoleic acid	18:3, *6c, 9c,12c*	6	$CH_3(CH_2)_3(CH_2CH=CH)_3(CH_2)_4COOH$
Z, Z, Z, Z-5, 8, 11, 14-eicosatetraenoic acid	Arachidonic Acid	20:4, *5c, 8c,11c,14c*	6	$CH_3(CH_2)_3(CH_2CH=CH)_4(CH_2)_3COOH$
Z, Z, Z-9, 12, 15-octadecatrienoic acid	α-linolenic acid	18:3, *9c,12c, 15c*	3	$CH_3(CH_2CH=CH)_3(CH_2)_7COOH$
Z, Z, Z, Z, Z-5, 8, 11, 14, 17-eicosapentaenoic acid	EPA	20:5, *5c, 8c, 11c,14c, 17c*	3	$CH_3(CH_2CH=CH)_5(CH_2)_3COOH$
Z, Z, Z, Z, Z, Z-4, 7, 10, 13, 16, 19-docosahexaenoic acid	DHA	22:6, *4c, 7c,10c,13c, 16c,19c*	3	$CH_3(CH_2CH=CH)_6CH_2)_2COOH$

(Scrimgeour and Harwood, 2007)

2.1.2.4. Conjugated acids

Conjugated fatty acids are mainly PUFAs with two or more conjugated doubles. The double bonds of PUFAs are transformed by a shift of isolated double bonds towards a structure in which unsaturated centers are immediately adjacent to each other (Scrimgeour and Harwood, 2007). Generally, they are also found as an intermediate or by-product of the reaction in some plants and animals (Table 2.4).

Table 2.4 Nomenclature and the sources of some conjugated fatty acids

Shorthand Names	Trivial Names	Sources
18:2 8*t*, 10*t*		
18:2 9*t*, 11*t*		
18:2 9*c*, 11*c*	Conjugated linoleic acid	Ruminant fat
18:2 9*c*, 11*t*		*Chilopsis linearis*
18:2 10*t*, 12*t*		
18:2 10*t*, 12*c*		
18:2 10*c*, 12*c*		
18:3 8*t*, 10*t*, 12*t*	ß-calendic acid	*Calendula officinalis*
18:3 8*t*, 10*t*, 12*c*	Calendic acid	*Calendula officinalis*
18:3 8*c*, 10*t*, 12*c*	Jacaric acid	*Jacaranda mimosifolia*
18:3 9*t*, 11*t*, 13*t*	ß-eleostearic acid	*Aleurites fordii*
18:3 9*c*, 11*t*, 13*t*	α-eleostearic acid	*Aleurites fordii* *Parinarium* spp., *Momordica* sp.
18:3 9*t*, 11*t*, 13*c*	Catalpic acid	*Catalpa* spp.
18:3 9*c*, 11*t*, 13*c*	Punicic acid	*Punica granatum*, *Momordica balsamina*

(Scrimgeour and Harwood, 2007)

2.1.3. Production of the fatty acids

Production of the fatty is related to the reactions of the carboxyl and ester groups in oil. These reactions include hydrolysis, esterification, alcoholysis, acidolysis and interesterification. Starting material of reaction may be acids, ester, or triacylglycerols (Frank, 2004; Scrimgeous, 2005). The production of the fatty acids is made by two methods, chemical and biological methods, depending on the type of the catalysts. The fatty acids and their derivatives are widely used as a feedstock in food, cosmetic, pharmaceutical and oleochemical manufactures.

2.1.3.1. Chemical production of the fatty acids

This process utilizes a reaction of the carboxyl and ester groups catalyzed by acid, base, or physical condition. The products of the reaction depend on the type of the primary feedstock and reaction such as glycerol, fatty acids or methyl esters. Several reports revealed that the valuable products are produced by this process. For example, Olutoye, Wong, Chin and Hameed (2014) demonstrated the production of methyl esters

from low cost palm fatty acid distillate by esterification with methanol. They found that, ZrFeTiO was an efficient catalyst. The conditions of reaction were performed by catalyst loading of 3% (w/w), methanol to the oil ratio of 3:1 (w/w) under the reaction temperature of 170 °C for 5 h. Under these conditions, the conversion rate of 96.50% was obtained. Klinkesorn et al., (2004) studied the enrichment of EPA and DHA from tuna oil using sodium methoxide as a catalyst by tranesterification. They demonstrated that the reaction conditions were successfully to produce tuna oil enriched with EPA and DHA when conditions were carried out by reactant of 1.50 % (w/w), molar ratio of oil and omega 3 PUFA of 1/4 (w/w) under the reaction temperature of 80 °C for 5 h. In case of the fatty acid production by hydrolysis, triacylglycerols were used as starting materials and were catalyzed by chemical compounds, high pressure and temperature. These processes are known as an alkaline hydrolysis and high-pressure splitting.

- Alkaline hydrolysis

In this process, triacylglycerides are hydrolyzed by high concentration of sodium hydroxide solution at 70-100 °C. The resulting products are glycerol and alkaline salt of fatty acids. Then, the acidification of the alkaline salt is performed by adding the hydrochloric acid. The main disadvantages of this process are the impurity of the final products such as odor and coloration, neutralization of excess acids as well as disposal of large amount of salts (Frank, 2004; Scrimgeous, 2005).

- High pressure splitting

High pressure splitting or Colgate-Emery process is an efficient process for a large-scale production of free fatty acids from fat and oil. This process is a continuous counter current process using water as a reactant. The high temperature and pressure of this process is around 225-230 °C and 30-70 bar, respectively (Attarakih et al., 2012). The reaction time of the process is around 2-3 h with the high yield of 98-99% (Sonntag, 1979). The main disadvantages of this process are occurrence of the undesirable reaction, high energy consumption and unexpected products (Frank, 2004; Scrimgeous, 2005).

2.1.3.2. Biological production of the fatty acids

The production of free fatty acids produced by the chemical process has several disadvantages such as high energy consumption, undesirable products, and polluting environment. To overcome these problems, the use of biocatalysts such as enzyme has received great attention because high purity of products, mild conditions, and energy saving are usually obtained. Bioprocess for fatty acid production is based on the use of

the specific enzymes as biocatalyst. Typically, lipase is able to hydrolyze oil to fatty acids and it is also able to catalyze several reactions such as esterification and transesterification. Many reports proposed the application of lipase for production of valuable fatty acids. For example, Sabeder et al. (2006) studied the enzymatic production of the fatty acid sugar esters from fructose with the fatty acids in the organic medium. The highest yield of the fructose palmitate in 2-methyl 2-butanol was 78% when conditions were performed using the substrates of 10 % (w/w), the molecular sieves of 12.10 % (w/w) under the reaction temperature of 60°C and the stirring rate of 600 rpm for 72 h. Wang et al. (2012) developed the production of the glycerides with the highly concentrated omega-3 PUFAs (DHA and EPA) from the high-acid crude fish oil. The designed process consisted of the enzymatic deacidification, PUFAs-enriched fish oil by alkaline catalysis, concentration of the resulting products by molecular distillation and enzymatic catalysis. The immobilized *Candida antarctica* lipase was used as the biocatalyst. The final products contained 74.6 % of DHA and 5.5 % of EPA. Zhao et al. (2014) reported the preparation of biodiesel from soybean oil using lipase-catalyzed transesterification with blended alcohols as acyl acceptors. They concluded that *C. antarctica* lipase was found to be an efficient biocatalyst for this report. The highest yield of biodiesel was 95% (w/w) when the conditions were carried out using an enzyme loading of 5–10 % (w/w), the reaction temperature of 30 °C and the methanol of 60% (mol).

2.1.4. Downstream process for the production of fatty acids

2.1.4.1. Crystallization

Crystallization is a classical process for fractionation of fatty acids. Separation of the fatty acids or triacylglycerols is based on their melting point. The dry fractionation is also known as the low-temperature crystallization. The aim of this method is enrichment of the fatty acids or triacylglycerols by removing the high melting point components at the low temperature about -70 °C by using dry ice as refrigerant. Alternatively, the solvent fractionation (crystallization) involves the use of organic solvents such as acetone or hexane in order to improve the yield of each fraction. This method is carried out at the low temperature (Frank, 2004; Wanasundara et al., 2005).

2.1.4.2. Distillation under the reduced pressure

Distillation is a process of separating the component substances from a liquid mixture. The separation of these component substances depends on the boiling points, vaporization, and condensation of the individual component. This procedure requires

high temperature and pressure. Under these conditions, the oxidation, polymerization and isomerization of double bonds are occurred. To overcome of the limitation, the fractional distillation under the reduced pressure was performed. The reduced pressure can be used to isolate the highly unsaturated acids such as monomeric, dimeric, polymeric materials and separation of monoacylglycerols. However, undesired products may be occurred (Frank, 2004; Wanasundara et al., 2005).

2.1.4.3. Urea complex fractionation

Urea fractionation is a potential technique for isolating fatty acids and fatty acid methyl esters. Typically, the urea crystallizes in a tightly packed tetragonal structure with diameter channels of 5.67 °A and it precipitates in a hexagonal structure with channels of 8-12 °A in the presence of the long straight-chain molecules (Wanasundara et al., 2005). These channels attach with aliphatic chain length more than 6 carbon atoms (Gu et al., 2009) and they are trapped with urea using Vander Waals force, London dispersion force and induced electrostatic attraction (Swern, 1964). In addition, aliphatic chain length with double bonds in molecule increases bulk of the molecule and reduces likelihood of its inclusion with urea (Schlenk, 1953). Therefore, the fatty acids and their methyl esters are separated based on the presence of the multiple double bonds in the configuration of the fatty acid molecule. Saturated fatty acids are more complex with the urea as compared with monoenoic acids and PUFAs. Since, the saturated acids prefer inclusion with the urea, the saturated fatty acids are mostly found in the urea complex fractionation (UCF), and monounsaturated fatty acids and PUFAs mainly remain in the non-urea complex fractionation (NUCF) (Frank, 2004; Wanasundara et al., 2005). Several reports revealed the application of the urea complex fractionation to enrich and purify the valuable fatty acids.

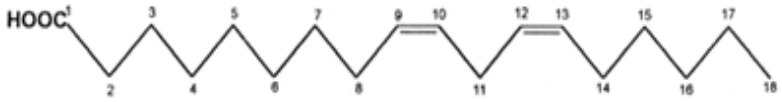
The following examples were summarized. Liu et al. (2006) studied that the concentration of DHA and EPA from the tuna oil by the urea complex fractionation techniques. The total content of DHA and EPA were enriched to 85.02% and 25.10% when the conditions were a urea to fatty acid ratio of 15 (mol/mol), a crystallization temperature of −5 °C and a crystallization time of 20 h. Wu et al. (2008) revealed that LA from the sunflower oil was separated and purified by the urea complex fractionation. The purity and the recovery of LA were reasonably obtained when the purity of LA was 87.80%, and the recovery was 83.40% at the urea to fatty acids ratio of 0.94 (w/w), the ethanol to urea ratio of 5 (v/w), the crystallization temperature of 18 °C and the crystallization time of 5 h. Gu et al. (2009) developed the concentration of

LA of the perilla oil by the gradient cooling urea inclusion. They found that the maximum content of LA was 81.90% (w/w) at the urea to fatty acid ratio of 3 (w/v), the solvent to fatty acids ratio of 7 (v/w), the reaction temperature of 74.85 °C, the crystallization temperature and the time of -20°C and 12 h, respectively. Ahmed et al. (2009) proposed the urea crystallization process to enrich GLA in the lipid extracted from *Mucor zychae*. They concluded that two-stage urea crystallization processes gave the GLA purity and the recovery of 92.70% and 69.0%, respectively. The two stage urea crystallization conditions to enrich GLA were the fatty acid ethyl ester to urea ratio of 1:3 (w/w) and the crystallization temperature of 0 °C.

2.2. Linoleic acid

Linoleic acid (LA), one of PUFAs, consists of 18 carbon atoms with two double bonds at 9 and 12 positions. It is recognized as a member of the essential fatty acids because the human bodies cannot synthesis this acid. Therefore, LA is classified as an omega-6 family according to the methyl end of carbon chain. The characteristics of this acid are described in Table 2.5.

Table 2.5 The characteristics of linoleic acid

General information	Description
Chemical structure	
Systematic name	*Z*,*Z*-9,12-Octadecadienoic acid
Color	Colorless to yellowish oil
Density	0.90 g/cm^3
Molecular weight	280.40 g/mol
Boiling point	230 °C
Melting point	-5 °C

(Scrimgeour and Harwood, 2007)

2.2.1. Benefits of LA

LA is used as a precursor for biosynthesis of valuable fatty acids such as ɣ-linolenic acid (GLA), Arachidinic acid (AA) and some prostaglandins. Deficiency of

LA causes numerous abnormalities on human health like diabetic neuropathy, rheumatoid arthritis, cardiovascular and autoimmune disorders (Das, 2006; Simopoulos, 1979). Currently, LA has gain considerable attention in cosmetic industries because of its beneficial properties on the skin such as anti-inflammatory, acne reduction and moisture retention (Letawe et al., 1998; Zhao et al., 2005). In addition, it can inhibit melanogenesis and be used to treat melasma patients (Lee et al., 2002). Moreover, LA is used as a precursor for the production of the conjugated linoleic acids (CLAs) through the biological process by the microbial isomerization (Andrade et al., 2012; Ogawa et al., 2005).

2.2.2. Pathway of LA synthesis

Generally, LA is synthesized in animal and plant tissue. The double bond is inserted by Δ12-desaturase at the Δ12 position in OA (Scrimgeour and Harwood, 2007). The main substrate for Δ12-desaturase is located in the endoplasmic reticulum of the cells of animal and plant tissue. Then, LA is converted to GLA by Δ6-desaturase. Consequently, GLA becomes elongation and desaturation to AA by elongase and Δ5-desaturase. The sequential action of LA is shown in Figure 2.1.

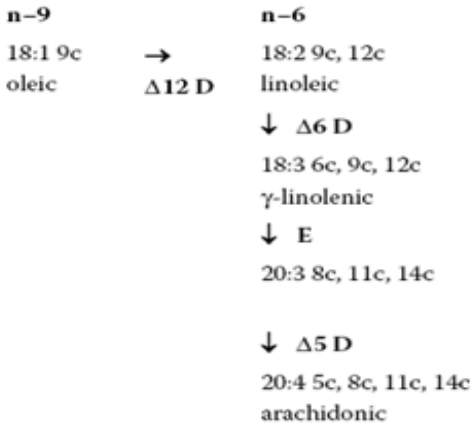


Figure 2.1 Biosynthesis of an omega-6 family in animal and plant tissue (D = desaturase and E = elongase) (Scrimgeour and Harwood, 2007)

2.2.3. Sources of LA

Generally, LA is found in the liquid of the cell membranes and it is plentiful in nature. It is mostly found in triacylglycerides of plant seed oil such as corn, soybean,

sunflower and safflower oil (Scrimgeous, 2005). Table 2.6 shows composition of the fatty acids in the various plant seed oil.

Table 2.6 The fatty acids composition of some plant seed oil

Plant seed oil	Content of fatty acids % (w/w)			
	C 16:0	C18:1	C18:2	C18:3
Corn oil	13	31	52	1
Soybean oil	11	22	53	8
Sunflower oil	6	18	69	-
Safflower oil	6	11	74	-

(Firestone, 2006)

2.2.4. Production of LA from plant oil

Production of LA by lipase-catalyzed hydrolysis is not widely used in the commercial industry. However, the conventional process leads to high energy consumption and impurity of the products. A few publications have described in this topic and reviewed. Sehanputri and Hill (1999) demonstrated the hydrolysis of corn oil in the hollow fiber reactor. The immobilized lipase from *Pseudomonas* sp. was found to be efficient biocatalyst. They showed that the high content of LA was obtained at 30 °C and buffer pH values of 7.0. Brijwani and Vadlani (2010) revealed the hydrolysis of corn distillers dried grain oil using *C. rugosa* lipase. They concluded that the hydrolysis degree was 96% and the high content of LA was more than 60% (w/w) when using enzyme concentration of 60 KU/L, substrate concentration of 0.09 M and the stirring rate of 750 rpm at 40 °C for 6 h.

2.3. Conjugated linoleic acids

Conjugated linoleic acids (CLAs) are a group of the positional and geometric conjugated dienoic isomers of LA. The position of the conjugated double bonds may diverge from 7 to 14 carbon atoms. This includes all possible geometric configurations of *cis–trans*, *trans–cis*, *cis–cis* and *trans–trans* isomers (Bessa et al., 2000). Generally, they are able to absorb light at an ultraviolet wavelength of 233-234 nm because CLAs contain the conjugated dienes in their conformation (Riel, 1963). Theoretically, 54 isomers of CLAs are possible, but only about 28 isomers of CLAs are able to be produced by the natural and chemical processes. However, the bioactive isomers of

CLAs are *cis*-9, *trans*-11-CLA, and *tran*-10, *cis*-12-CLA (Bhattacharya et al., 2006). The chemical structure of CLAs are given in Figure 2.2

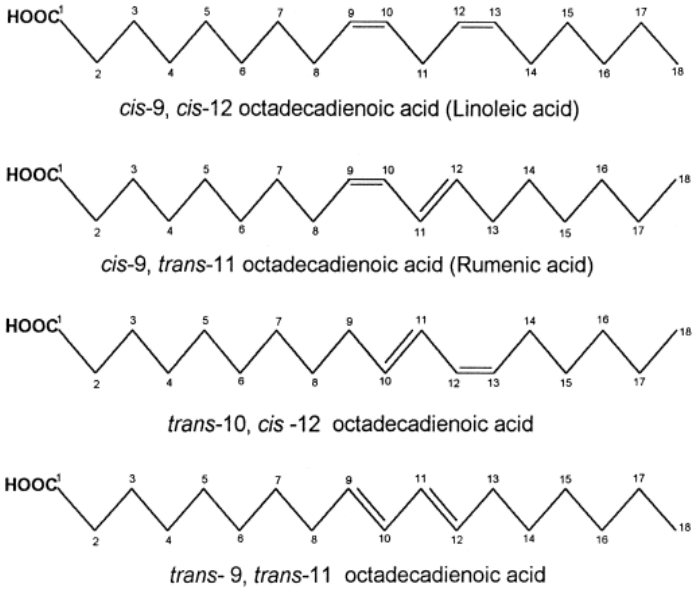


Figure 2.2 The chemical structure of LA and bioactive CLAs (Bessa et al., 2000)

2.3.1. Health benefits of CLAs

CLAs were firstly discovered by the Dr. Pariza's group at the University of Wisconsin-Madison. They found that the extract of grilled-ground beef extract showed the anti-mutagenic activity (Ha et al., 1987). Consequently, CLAs have gain considerable attention due to their biological properties on health such as anti-carcinogenesis and anti-obesity (Bhattacharya et al., 2006; Koba and Yanagita, in press). In addition, some isomers inhibit platelet aggregation and have an anti-proliferative effect (Al-Madaney et al., 2003; Koba and Yanagita, in press).

2.3.1.1. Anti-carcinogenesis

One possible mechanism of the anti-carcinogenesis activity, CLAs can activate peroxisome proliferator-activated receptors (PPARs). The activated PPARɤ induces apoptosis and inhibits proliferation of prostate, breast colon, and gastric cancer cells while the activated PPARα and PPARɤ can also inhibit nuclear factors of (NF)-κB and activate the protein activation of (AP1) (Koba and Yanagita, in press).

2.3.1.2. Anti-obesity

It has been reported that CLAs have the anti-obesity and hypolipidemic effects in animals such as mice, rats and pigs. The anti-obesity effect was initially reported by Park et al. (1995). The intake of 0.5 % CLAs in ICR-male and -female mice (50% of *cis*-9, *trans*-11-CLA and 50% of *trans*-10, *cis*-12-CLA) results in decreasing the body fat mass and increasing the lean body mass. The multiple mechanisms of the anti-obesity are the increment of the energy expenditure, the reduction of the lipid accumulating in the adipose tissues and/or adipocytes, the enhancement of the adipocyte apoptosis, the modulation of the adipokines and cytokines such as leptin, TNF- a adiponectin, or interleukins, the improvement of the fatty acid β-oxidation in skeletal muscles, brown adipose and oxygen consumption (Park and Pariza, 2007). In addition, CLAs may also be an activation of PPARs and induce PPARs-regulated lipolytic genes, including carnitine-palmitoyl transferase, acyl CoA oxidase and uncoupling protein in liver, and muscle and brown adipose tissue (Koba and Yanagita, in press).

2.3.1.3. Anti-diabetic effects

CLAs may normalize glucose metabolism and act as an agonist of PPARɤ. The activation of PPARɤ could increase plasma adiponectin concentration and ameliorate hyperinsulinemia. Moreover, *trans*-10, *cis*-12-CLA was found to be the specific action of the anti-diabetic effects (Koba and Yanagita, in press; Park, 2009).

2.3.1.4. Anti-hypertension

CLAs can also exhibit the anti-hypertensive effect because they may stimulate the production of the physiologically active adipocytokines such as adiponectin, leptin, and angiotensinogen. The *trans*-10, *cis*-12-CLA was the main isomer affecting to the anti-hypertension rather than *cis*-9, *trans*-11-CLA (Koba and Yanagita, in press; Park, 2009).

2.3.1.5. Anti-atherosclerosis

CLAs are effectively anti-atherogenic dietary fatty acids in animal models of atherosclerosis by the activation of PPARs. The multiple mechanisms of the anti-atherosclerosis are decreasing in levels of the atherogenic lipoprotein plasma, increasing in high density lipoprotein cholesterol (HDL) by increasing the apo A-I and apo A-II synthesis. In addition, the repression of the nuclear NF_kB and apo A-I transcription activity could be decreased by the vascular inflammation (Koba and Yanagita, in press; Park, 2009).

2.3.1.6. Immune and inflammatory responses

Koba and Yanagita (impress) revealed that CLAs inhibit pro-inflammatory cytokines and improve immunization related to the response modulating the key inflammatory mediators such as TNF-α, anti-inflammatory cytokines, prostaglandins, and nitric oxide. In the case of human studies, CLAs may give positive influence the immunization and inflammatory functions by modulating antibody production, decreasing TNF-ɤ and IFN-ɤ and reducing mitogen induced by the T-cell activation.

2.3.2. Source of CLAs

CLAs are mainly found in ruminant and dairy products such as meat, milk, dairy food, and some plant seed oil. The unique characteristic of natural CLAs consists of mainly bioactive CLAs. In the case of the ruminant products, *cis*-9, *trans*-11-CLA presents around 80-90% of the total CLAs, and *trans*-10, *cis*-12-CLA contains only 3-5% of the total CLAs. However, the natural CLAs are very low concentration in the range of 2-5 mg/g of the total fat (Koba and Yanagita, in press). For large-scale production, CLAs are produced by alkaline isomerization from oil with high content of LA used as a starting material (Scrimgeour and Harwood, 2007). This process produces the bioactive isomers in amount of 40-45% of the total CLAs and unexpected isomers (Bhattacharya et al., 2006).

2.3.3. Production and biosynthesis of CLAs

2.3.3.1. Chemical production

- Alkaline isomerization

CLAs can be obtained from alkaline isomerization of LA or plant seed oil with high content of LA such as soybean, sunflower, and safflower oil. LA or plant seed oil is heated in the solution of the sodium hydroxide with the ethylene glycol at 180 °C for 2 h under an inert atmosphere (O'Quinn et al., 2007). This method was described by a prototrophic shift mechanism (Moore, 1939). However, the production of CLAs by this process requires the isolation and enrichment step (O'Quinn et al., 2007).

- Homo and Heterogeneous catalysts

The homo- and heterogeneous catalysts are used to accelerate the process of the CLA production. The homogeneous metal catalyst is broadly used to produce CLAs such as the Wilkinson's catalyst ($RhCL(PPh_3)_3$). However, limitation of this method is the difficulty to remove and reuse the catalysts from the products (Behr et al., 2013). Hydrogenation of triacylglycerols with high content of unsaturated fatty acids, isomerization, and double migration can occur by using heterogeneous catalysts such as

nickel, palladium, platinum, and rhodium. The possible mechanism is known as the metal hydride addition, elimination and the α-allyl complex mechanism. However, the main disadvantages of these catalysts are that the cost of the catalysts is expensive and the catalysts are very toxic (Kitayama et al., 1996).

2.3.3.2. Biological synthesis

In natural, CLAs are mainly found in ruminant and dairy product due to biohydrogenation of PUFAs. Most PUFAs are derived from animal forages and concentrated feeds with high content of LA and GLA. Generally, these PUFAs are found in form of triacylglycerols. This form is hydrolyzed by lipase from a rumen microorganism. Therefore, LA and GLA are gained and used as starting materials for biohydrogenation by rumen microorganism (Andrade et al., 2012; Van Nieuwenhove et al., 2012). CLAs are produced as the first intermediate of biohydrogenation of LA into the stearic acid (SA). The first step of biohydrogenation is the transferring double bond at the carbon-12 position to the carbon-11 position done by linoleate isomerase (LAI; EC 5.3.1.5). The second step is the rapid conversion to *trans*-11 C18:1 (*trans*-vaccenic acid; TVA) by the reduction mechanism and the TVA is further hydrogenated to SA. Among the rumen microorganisms, *Butyrinvibrio fibrisolvents* is the main responsible for this process. Additionally, another CLA synthesis pathway, the TVA in the mammary gland was desaturased by Δ9-desaturase (Andrade et al., 2012; Van Nieuwenhove et al., 2012). Biosynthesis pathways of CLAs in the rumen and the mammary gland tissues of ruminant are given in Figure 2.3.

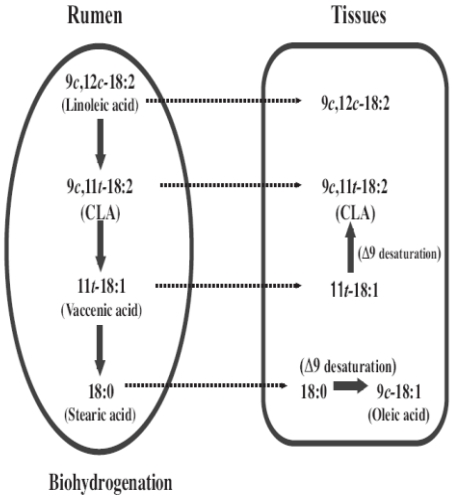


Figure 2.3 Biosynthesis pathways of CLAs in the rumen and the mammary gland tissues of the ruminant (Koba and Yanagita, 2014)

2.3.4. CLA production by LAI from LAB

Butyrivibrio fibrisolvens was initially reported as the CLA-producing microorganism. Moreover, microorganism from the dairy products and human and animal intestine was indicated as the efficient CLA-producing bacteria such as *Bifidobacteria* sp., *Propionibacterium* sp. and LAB such as the groups of *Enterococcus, Lactobacillus, Lactococcus, Pediococcus* and *Streptococcus* are able to produce CLAs (Ogawa et al., 2005). Among these microorganisms, CLAs are occurred due to biohydrogenation using LAI (Andrade et al., 2012; Van Nieuwenhove et al., 2012). A list of CLA-producing microorganisms is shown in Table 2.7. However, the limitations of this method are low concentration of LA due to low solubility and the high toxic of the fatty acid, the low concentration and productivity of CLAs as well as the high production cost.

The several reports investigated the production of CLAs by LAI from microorganisms. For instance, Alonso et al. (2003) proposed that *Lb. acidophilus* and *Lb. casei* are able to produce CLAs from LA in MRS broth. The maximum CLA production of 0.08 and 0.13 mg/mL was obtained at 24 h of the incubation in broth containing 0.02% of LA. In addition, the ability of *Lb. acidophilus* and *Lb. casei* to produce CLAs in skim milk supplemented with 0.02 % of LA was evaluated as well. The total amount of CLAs after 24 h of the incubation is ranged from 0.05 to 0.12 mg/mL. In addition, main isomer of CLAs was *cis*-9, *trans*-11-CLA and they contained more than 80% of the total CLAs. Van Nieuwenhove et al. (2007) studied the ability of LAB to produce CLAs in MRS broth and buffalo milk supplemented with LA. They found that the MRS broth supplemented with 0.20 mg/mL of LA gave the conversion rate of CLA production from *Lb. casei* CRL 431 and *Lb. rhammosus* C14 of 35.90% and 34.50%, respectively. In addition, the buffalo milk supplemented with 0.20 mg/mL of LA gave the maximum CLA production from *Lb. casei* CRL 431 and *Lb. rhammosus* C14 of 4.0 and 6.0 mg/mL of fat, respectively. Moreover, All the investigated strains of LAB were able to produce CLAs at the high LA concentration of 1.0 mg/mL. Hermandez-Mendoza et al. (2009) revealed the effect of different growth conditions on bioconversion of LA to CLAs by *Lb. reuteri* ATCC 55739. They found that the highest CLA production of 0.11 mg/mL was obtained in a MRS broth supplemented with 20.0 mg/mL of LA at initial pH of 6.50 and was grown in aerobically at 10 °C for 30 h. CLA production was decreased when the initial pH of the reaction medium was reduced. Moreover, micro-aerobic conditions reduced the CLA production. Gorissen et al. (2010)

investigated 36 different strains of *Bifidobacteria* from different sources for their capabilities to produce CLAs and/or Conjugated linolenic acids (CLNAs) from LA and α-linolenic acid as substrate. These results revealed that only 7 strains were able to convert fatty acids to conjugated fatty acids. These strains were identified as a *B. bifidum*, *B. pseudolongum*, *B. breve* LMG 11084, *B. breve* LMG 11613, *B. breve* LMG 13194, *B. bifidum* LMG 10645 and *B. pseudolongum* LMG 11595. The main CLAs and CLNAs have been identified as *cis*-9, *trans*-11-CLA and *trans*-9, *trans*-11-CLA and the putative *cis*-9, *trans*-11, *cis*-15-CLNA and *trans*-9, *trans*-11, *cis*-15-CLNA. Hou et al. (2011) studied the effects of the different concentrations of LA added to the medium reaction on CLA production by *Lb. plantarum* L12. These results suggested that the highest CLA production of 0.06 mg/mL was observed in a MRS broth with the LA concentration of 1.0 mg/mL and the incubation of 30 h. At the LA concentration more than 1.0 mg/mL, Growth of this bacteria was inhibited and the ability to produce CLAs from the strain was correlated with the ability to tolerate LA. However, micro-aerobic environment could promote the production of CLAs. Gorissen et al. (2011) investigated effects of pH and temperature on CLA production by *Lb. sakei* LMG 13558. These reports suggested that at 30 °C of fermentation temperature, the maximum CLA production of 0.08 mg/mL was obtained in a MRS broth supplement with 0.05 mg/mL of LA at the initial pH of 6.20 while the fermentation temperature of 30 °C or 37 °C could not detect CLA production in MRS broth supplement with 0.05 mg/mL of LA at the initial pH of 5.50 and 6.20. In addition, they concluded that the ability of LAI from LAB to produce CLAs depends on a unique characteristic of each strain and production of CLAs affected by pH and temperature.

2.3.5. CLA production from oil with high content of LA

Normally, LA is commonly the major substrate to produce CLAs. However, various reports attempted to use oil with the high content of LA as a source of fatty acids such as safflower and sunflower oil. The production of CLAs by microorganisms is unable to directly use triacylglycerols as a starting material. Therefore, bacteria must have the ability to hydrolyze the triacylglycerols and liberate LA for the further conversion. Only oil hydrolysate can offer the fatty acids and could be used as a substrate for CLA production (Van Nieuwenhove et al., 2012). Several reports show the production of CLAs from oil with the high content of LA. For example, Puniya et al., (2008) studied the production of CLAs from sunflower oil by *Lb. brevis* isolated from a cattle rumen. They found that *Lb. brevis* was able to produce CLAs of 10.53 mg/g of fat

when it was cultured in skim milk medium supplemented with 0.25% of sunflower oil at 37 °C for 12 h. Puniya et al. (2009) studied effects of sunflower oil concentration on CLA production by *Lb. acidophilus* and *Lb. casei*. These results revealed that *Lb. casei* was able to produce CLAs of 11.0 mg/g of fat at 1.0% of sunflower oil in skim milk medium whereas *Lb. acidophilus* produced CLAs of 2.73 mg /g of fat. Rodriguez-Alcala et al. (2011) proposed CLA formation by *Lb. acidophillus* and *Bifidobacterium* sp. using safflower oil as substrate. Safflower oil was added to skim milk at 1.0 mg/ml. They found that *Lb. acidophillus* and *Bifidobacterium* sp. were able to produce CLAs in the range of 0.04 to 0.05 mg/mL and they produced *cis*-9, *trans*-11 CLA as the main products. Dubey et al. (2012) investigated the ability of *Pediococcus* spp. GS4 to produce CLAs from sesame seed oil. They concluded that the sesame oil could use as a substrate for the CLA production with the aid of the lipase-catalyzed triacylglycerols hydrolysis. The amount of CLAs was 0.15 mg/mL at 1.0% of oil hydrolysate in the skim milk medium.

Table 2.7 CLA production from LA using several microorganisms

Microorganisms	Content of LA (mg/L)	Content of CLAs (mg/L)	Content of CLA isomers (%)		
			***cis*-9, *trans*-11**	***trans*-10, *cis*-12**	**Others**
B. adolescentis NCFB 2204	550	4	46	34	20
B. bifidum LMG 10645	500	207	82	-	12
B. breve LMC 520	2,200	>600	>90	-	-
B. brevis KCTC 10462	500	160	96	-	-
E. faecium AKU 1021	4,000	100	40	-	60
Lb. acidophillus ADH	1,000	630	-	-	-
Lb. acidophillus F0221	500	161	-	-	-
Lb. acidophillus Ki	1,000	9	-	-	-
Lb. curvatis LMG13553	500	8	-	-	-
Lb. plantarum L12	1,000	57	-	-	-
Lb. plantarum NCUL005	1,000	623	32	68	-
Lb. plantarum Ip5	600	142	77	23	-
Lb. reuteri ATCC 55739	200	119	-	-	-

(Andrade et al., 2012)

Table 2.7 CLA production from LA using several microorganisms (cont.)

Microorganism	Content of LA (mg/L)	Content of CLAs (mg/L)	Content of CLA isomers (%)		
			***cis*-9, *trans*-11**	***trans*-10, *cis*-12**	**Others**
Lc. lactis subsp. *lactis* CCRC10791	1,000	78	-	-	-
Lc. lactis subsp. *cremoris* CCRC12586	1,000	63	-	-	-
Lc. lactis LMGS 19870	1,000	7	-	-	-
P. freudenreichii ssp. *shermanii* CGMCC 1.2227	1,000	79	-	-	-
P. shermanii AKU1254	600	110	82	82	18
P. shermanii B6022	1,000	163	-	-	-
Pediococcus acidilactici AKU 1059	4,000	1,400	71	-	29
Streptococcus salivarius subsp. *thermophilus* CCRC12257	1,000	74	-	-	-

(Andrade et al., 2012)

2.4. Lipase

Lipase is widely used in enzyme technology because it is able to catalyze numerous reactions. Unique characteristics of lipase involve specificities and selectivity of the enzyme, purity of the final products, simplicity of the downstream process and reduction of energy consumption (Kapoor and Gupta, 2012; Reis et al., 2009). Application of lipase is widely found in several industries such as process of pulp, paper and food as well as production of biodiesel and oleochemicals (Hayes, 2004).

2.4.1. Characteristics of lipase

Lipase (EC 3.1.1.3) is the carboxyl esterase acting on long-chain acylglycerols. It liberates diacylglycerols and monoacylglycerols as intermediates and then coverts to free fatty acids and glycerol, respectively. In addition, Lipase is high stability in organic solvent and is able to catalyze several reactions such as hydrolysis, esterification and transesterification. Moreover, Lipase possesses ability to differentiate the positions and the types of fatty acids in triacylglycerols so called chemo-, regio- and stereoselectivities (Hayes, 2004; Kapoor and Gupta, 2012).

2.4.2. Mechanisms and structures of lipase

The catalytic activity of lipase acts on interface between aqueous and oil phase, which is recognized as interfacial activation (Reis et al., 2009). Generally, lipase has an amphiphilic peptide loop so called lid or flap. This feature covers the active site and substrate cannot access to this site. The lid is very small, simple and quite complex in its structures (Kapoor and Gupta, 2012). In the presence of oil drop, interaction of the hydrophobic force between oil drop molecules and surface of the active site changes the conformational of the lid. Then, the active site is exposed. Therefore, substrate gets to reach to the active site (Dominguez de Maria et al., 2006).

Lipase is a member of the α/β hydrolase fold family. Structure of the lipase consists of a specific sequence of α-helices and a core of predominantly parallel β-strands. Active site of the lipase composes of serine, histidine, arpartate and glutamate. Mechanism of catalytic reaction depicts in Figure 2.4. Initially, serine is stimulated by deprotonation. Then, histidine and aspartate are activated (Figure 2.4a). Consequently, nucleophilicity of the hydroxyl residue of serine is enhanced and attacked the carbonyl group of substrate forming an acyl-enzyme intermediate (Figure 2.4b). The presence of an oxianion hole contributes to stabilization of the charge distribution and reduction of ground state energy of the tetrahedral intermediate. The deacylation step is controlled

by electronegativity of molecules populating the interface (Figure 2.4c). In this process, a nucleophile (e.g. water or monoglycerides) attacks the acylated enzyme leading to form new products and regeneration of the catalytic site (Reis et al., 2009).

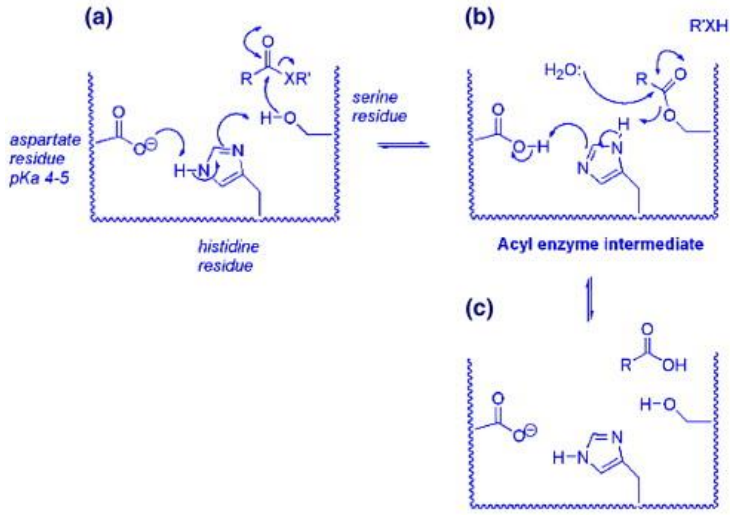


Figure 2.4 Mechanism of lipase-catalyzed hydrolysis (Reis et al., 2009)

2.4.3. Catalytic reactions of lipase

Normally, the catalytic reactions of lipase may be classified into two categories such as hydrolysis and synthesis (Hayes, 2004; Reis et al., 2009). These catalytic reactions are reversible. Therefore, lipase catalyzes the hydrolysis in an aqueous system and prefers to catalyze the synthesis in a microaqueous system such as esterification and transesterification (Figure 2.5). The last four reactions are known as the transesterification. This reaction is classified into four subclasses according to types of the reactant. It is alcoholysis, acidolysis, interesterification and aminolysis. Alcoholysis is the reaction of an ester and an alcohol while acidolysis is the reaction of an ester and an acid. Interesterification is the reaction between two different esters where acyl groups from the two esters are swapped. In aminolysis, the ester is reacted with an amine and the products are an amide and an alcohol.

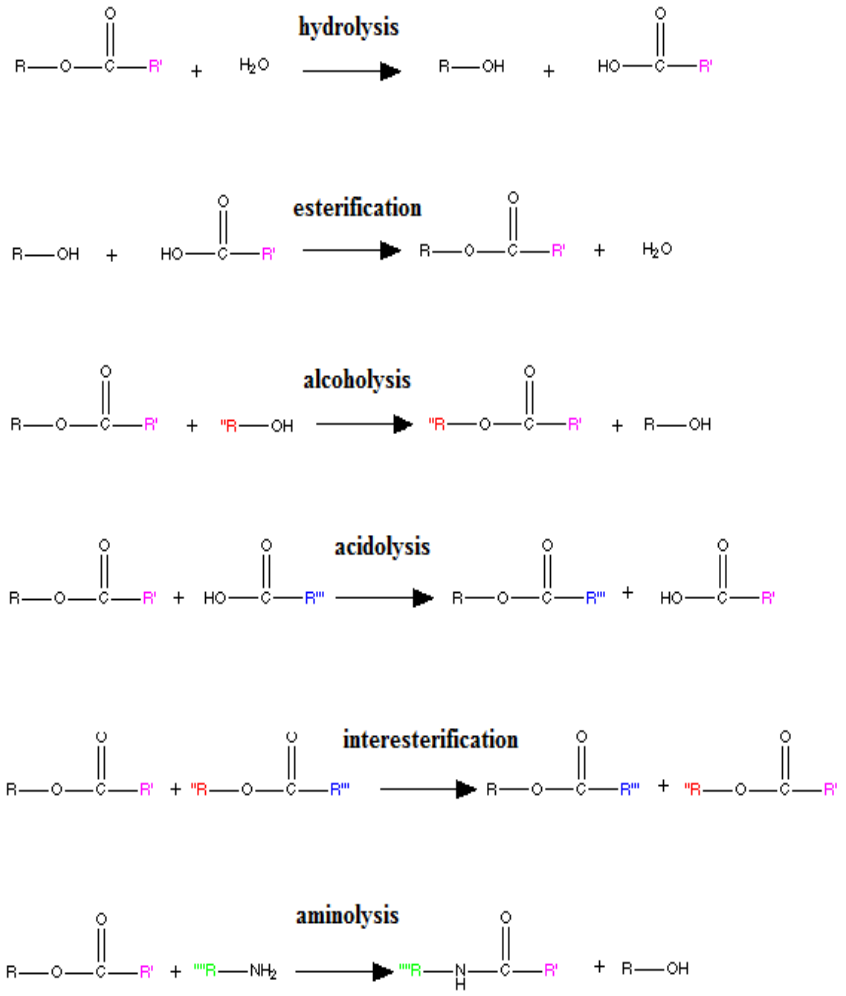


Figure 2.5 Catalytic reactions of lipase (Hayes, 2004; Reis et al., 2009)

2.4.4. Specificities of lipase

Lipase prefers to catalyze a broad range of substrate such as an ester of fatty acids, alcohol, glycerol and long chain of triacylglycerols. This enzyme exhibits various levels of specificity and selectivity toward substrate for example, regio-selectivity, fatty acids and acylglycerol-specificity (Diks and Bosley, 2000; Kapoor and Gupta, 2012). The main specificities of lipase are given in Table 2.8.

Table 2.8 The major specificities of the lipase toward oil hydrolysis

Specificity	**Lipase sources**
Regio-selectivity	
- Non-specific lipase	*Burkchoderia Glumae*
	Burkchoderia cepacia
	Candida rugose
	Chromobacterium visosum
	Pseudomonas fluorescens
-1,3-specific lipase	*Aspergillus niger*
	Mucor javanicus
	Rhizomucor miehei
	Rhizomucor delemar
	Rhizopus niveus
Fatty acids specificity	
- Saturated acids	*Fusarium oxysporum*
- *Cis*-Δ9 unsaturated acids	*Geotrichum candidum B*
Acylglycerol selectivity	
- Monoacylglycerols	*Potato acylhydrolase (patatin)*
- Mono- and diacylglycerols	*Penicillium camembertii*
	Penicillium cyclopium M1
	Fusarium sp.
-Triacylglycerols	*Penicillium roquefortii*
	Penicillium expansum

(Diks and Bosley, 2000)

2.4.4.1. Regio-selectivity

This selectivity of lipase can be divided into two groups according to their positions of the fatty acids on the triacylglycerol molecules. The first group is the non-specific lipase. This lipase randomly catalyzes the reaction on all positions of the fatty acid in triacylglycerols. During oil hydrolysis, it liberates triacylglycerols to fatty acids and glycerol. Di- and mono-acylglycerols are intermediates of the reaction. The other groups are 1, 3-specific lipase. It prefers to act on sn-1 or sn-3 position more than sn-2 position of fatty acid in triacylglycerols. This group of lipase releases different types of di-and mono acylglycerols (Kapoor and Gupta, 2012). Figure 2.6 shows the catalytic reaction of non- and 1, 3- specific lipases.

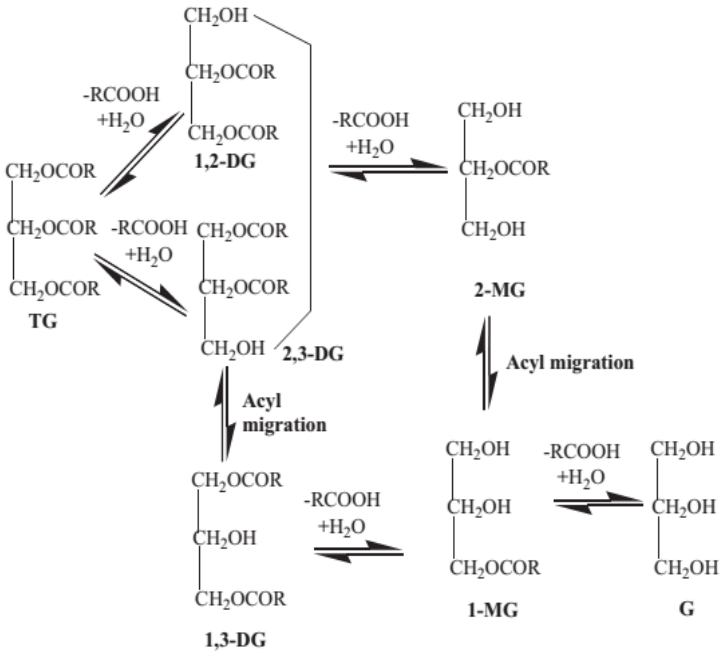


Figure 2.6 The catalytic reaction of non-specific and 1, 3-specific lipases (Kapoor and Gupta, 2012)

2.4.4.2. Fatty acid specificity

Numerous lipases are specific toward particularly fatty acids. For example, lipase from *Geotrichum candidum* prefers to hydrolyze long chain fatty acids with the double bond of *cis*-9 (Kapoor and Gupta, 2012). In addition, several lipases prefer to catalyze specific chain-length of the fatty acids. Some lipases are discriminated toward the long-chain fatty acids more than the medium- and the short-chain fatty acids. For instance, porcine pancreatic lipase is specific toward the short-chain fatty acids while fungal lipases prefer to catalyze toward the short- and the medium-chain fatty acids (Alejandro, 2005).

2.4.4.3. Acylglycerol selectivity

Enzymatic production of the di- and mono-acylglycerols usually depends on the selectivity of lipase on acylglycerols. Some lipases discriminate against the diacylglycerols during esterification such as lipase from *potato acylhydrolase*. In addition, several lipases from *penicillium* have strong selection against triacylglycerols and the less selection of the monoacylglycerols (Diks and Bosley, 2000).

2.4.5. Enzymatic production of fatty acids

Enzymatic preparation of the fatty acids usually depends on the specificities and the selectivity of lipase. Generally, selective hydrolysis of oil is used to produce the desired fatty acid. Then, the desired fatty acid is concentrated using enzymatic esterification or transesterification in order to eliminate the undesirable products. Moreover, additional downstream process such as crystallization or urea complex fractionation is required in order to obtain high purity of the fatty acids. There are several studies using the lipase-catalyzed reaction for preparation of the valuable fatty acids as summarized in Table 2.9

Shimada, Maruyama, Sugihara, Moriyama and Tominaga (1997) proposed the preparation of DHA from tuna oil by enzymatic hydrolysis and esterification. Among the tested enzymes, the lipases from *Pseudomonas* sp. and *Rhizopus delemar* were suitable for the hydrolysis and esterification of the tuna oil. The hydrolysis degree of 68.40 % was archived under the following conditions; the enzyme loading of 2,500 U/g, the ratio of the oil to water of 1.00 (w/w) and the reaction of the temperature of 40°C. After that, two stages of the esterification by *R. delemar* lipase were conducted at 30°C for 20 h by stirring a mixture of 4.00 g of tuna-FFA-Ps/lauryl alcohol (1:2 mol/mol), 1.00 g of water and 1,000 U of lipase. The overall content of DHA was raised to 91 % (w/w) with the recovery of 88%.

Vacek et al. (2000) developed the enrichment of α-LNA (α-linolenic acid) and GLA from blackcurrant oil. They found that the immobilized lipase from *Burkholderia cepacia* was specific towards to ALA and GLA. The higher hydrolysis rate of 80 % was obtained under the following conditions; the enzyme concentration of 18 U, the ratio of oil to water of 1.00 (v/w), the reaction temperature and time of 40°C and 24 h, respectively. The ALA and GLA were mainly found in the mono- and diacylglycerol fraction.

Tuter et al. (2003) proposed the enrichment of GLA from the borage oil by the *Nigella sativa* L. lipase. They revealed that the partial purification of the *N. sativa* L. lipase by the ammonium sulfate precipitation was more effective biocatalyst for the hydrolysis of the borage oil. The content of GLA was found in TAG and DAG fractions of 29.60 % and 41.80 %, respectively. They conclude that the ability of the *N. sativa* lipase could be used for the enrichment of GLA in the acylglycerol fractions.

Yamauchi et al. (2005) investigated the production of AA from the single-cell oil by the enzymatic hydrolysis and esterification. In order to prepare AA, the lipase from the *Alcaigenes* sp. was found to be the most effective biocatalyst. The degree of the hydrolysis was reached to 98 % using enzyme loading of 1,200 U/g and oil to the water ratio of 1:2 (w/w) at 40^{o}C for 48 h. The *B. cepacia* lipase was chosen as an efficient biocatalyst for the esterification. The first-esterification was performed by using 2 molar equivalents of the LauOH at 30°C for 16 h in a mixture with 20% water and 20 U/g-mixture of Lipase. The second esterification was carried out at the same condition. The overall of the content of AA was up to 97 % (w/w) with the recovery of 49 %.

Rupani et al. (2012) studied the enrichment of α-LNA from the flax seed oil by lipase catalyzed hydrolysis. They found that the commercial lipase from *Candida rugosa* was an efficient biocatalyst, and α-LNA was enriched in the free fatty acids. The yield of α-LNA was increased to 69.20 % and could be further purified to 80 % by urea complexation.

Table 2.9 Enzymatic preparation of the valuable fatty acids

Type of fatty acids	Production steps	Source of Lipase	Purity (%)	Recovery (%)	References
DHA+EPA	1) Selective hydrolysis	*Candida rugosa*	63.80	-	(Kahveci et al., 2010)
Dihomo-GLA	1) Hydrolysis	*Candida rugosa*	39.10	96.90	(Nagao et al., 2007)
	2) Urea adduct Fractionation	-	55.40	92.50	
	3) Three esterification With LauOH	*Pseudomonas aeroginosa*	94.80	51.30	
GLA	1) Selective hydrolysis	*Candida rugosa*	18.20	77.40	(LÓPez-MartÍNez et al., 2006)
	2) Two -step esterification with LauOH	*Candida rugose*	91.0	61.60	
Mead acid	1) Selective hydrolysis	*Pseudomonas aeroginosa*	6.30	50.30	(Shimada et al., 2003)
	2) Urea adduct Fractionation		27.20	47.20	
	3) Two-step esterification with LauOH	*Candida rugosa*	54.0	38.20	

Table 2.9 Enzymatic preparation of the valuable fatty acids (cont.)

Fatty acids	Production steps	Source of Lipase	Purity (%)	Recovery (%)	References
Petroselinic acid	1) Selective hydrolysis	*Burkhoderia cepacia*	70.0	-	(Foglia et al., 2000)
	2) Esterification with 1-Butanol	*Geotrichum candidum*	97.0	93.0	
DHA+EPA	1) Selective hydrolysis	*Candida rugosa*	27.81	-	(Okada and Morrissey, 2007)
DHA+EPA	1) Selective hydrolysis	*Burkhoderia cepacia*	27.06	-	(Gámez-Meza et al., 2003)
	2) Urea adduct Fractionation		86.58	78.0	

2.5. Optimization of process

Traditionally, optimization has been performed by monitoring the influence of one variable at a time on an experimental response. This means that only one variable is changed but others are kept at a constant level. The approach is called one factor at a time (OFAT). This design does not include the interactive effects among the variables studied and does not illustrate the complete effects of the parameter on the response. Moreover, number of the experiments is increased and excessive consumption of time, reagent, and materials are expected (Bezerra et al., 2008). Normally, the optimal response surface of the interaction between the two factors is a circular or ellipse oriented along the axes (Figure 2.7). In the case of OFAT, the response of this approach does not even reach the true optimum (Figure 2.7a). On the other hand, the space of the factor level is covered the optimal response surface using response surface design (Figure 2.7 b) (Hibbert, 2012).

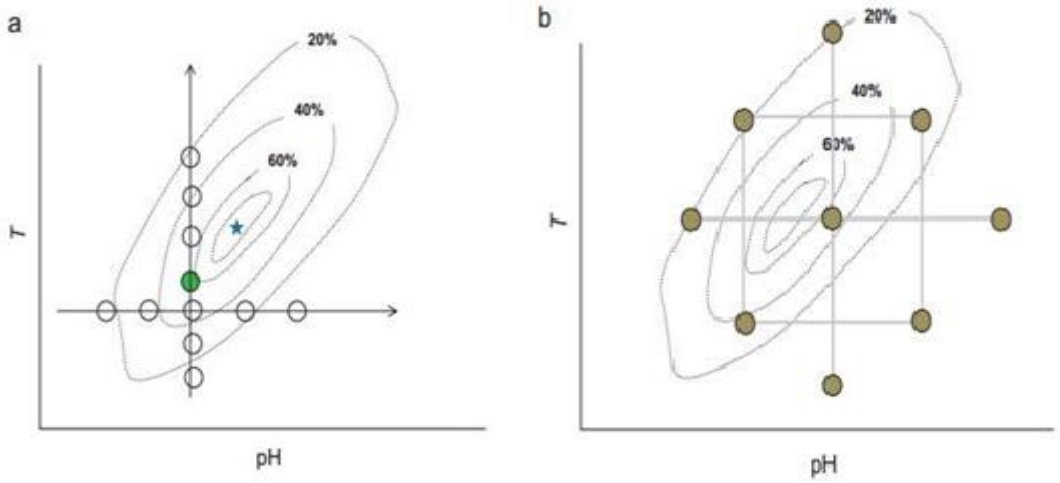


Figure 2.7 A hypothetical response surface with the best experiment point (closed circular) and the true optimum (star); (a) OFAT and (b) RSM (Hibbert, 2012)

Currently, the optimization has been carried out using the multivariate statistic technique. This technique is called response surface methodology (RSM). This method refers a collection of mathematical and statistical techniques and based on the fit of a polynomial equation to the experimental data obtaining in relation with the experimental design. The characteristic of RSM is to simultaneously optimize the levels of these variables to attain the best system performance (Montgomery, 2001).

2.5.1. Definitions of some terms in the experimental design

Since the term of experimental design does not use in general and complication, in order to convenience, the definitions of some terms are introduced and defined below.

- An experimental design is a specific set of experiments. This term refers to the matrix containing the combination levels of the variables.

- Factors are the experimental variables such as the pH, temperature, time and other variables. The experimental variables show their influence to the response. The synonyms of them are the independent variables or parameters.

- Levels of a variable mean the value of the variable. It is prescribed in an experimental design. For example, temperature can be investigated in four levels: 30, 40, 50 and 60 in the optimization of oil hydrolysis.

- Response or dependent variables refer to the measured values of the results from the experiments. The typical responses are yield of the fatty acids, degrees of hydrolysis and others responses.

- Response surfaces are the relationship between the responses and the values of one or more factors.

- A model is an equation related to a response to factors. Generally, model can be empirical and based on a theoretical understanding of the process that gives the response.

2.5.2. Steps of optimization using RSM

In order to attain the optimal condition, stages of the optimization techniques using RSM. It consists of 1) selection and identification of the influence variables to the response of the process, 2) choosing the experimental design and performing the experiment according to the experimental matrix, 3) model fitting using the mathematical or statistical method, 4) evaluation of the fitted model, 5) verification of the optimal conditions. Finally, the optimum values of the process is achieved (Bezerra et al., 2008).

2.5.2.1. Screening of the variables

The systems have numerous variables affecting the response. It is difficult to control the small contributions to the response from each variable. In order to eliminate these variables, finding influent variables is required. Generally, the full or fractional

factorial design typically uses for this study. This approach includes selection and identification of the major influent variables to the response. (Bezerra et al., 2008).

2.5.2.2. Selection of the experimental design

In RSM, choice of the experimental design is the first priority in this approach. It defines the experimental region of the variables. In general, the simplest model which can be used in RSM is based on a linear function. However, this response does not present any curvature. To evaluate the curvature, a second-order model must be used. Two-level factorial designs are used to the estimation of the first-order effects, but they fail when the additional effects occur. So, a central point in the two-level factorial designs can be used for evaluating the curvature. The next level of the polynomial model should contain with the additional terms since these additional terms describe the interaction between the different experimental variables. Finally, in order to determine a critical point (maximum, minimum, or saddle), it is necessary for the polynomial function to contain the quadratic terms according to the equation presented below:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{1 \le i \le j}^{k} \beta_{ij} x_i x_j + \varepsilon \quad \textbf{(2.1)}$$

where Y is the response; k is the number of variables; x_i and x_j represent the variables; β_0 is the constant term; β_i, β_{ij} and β_{ii} represent the coefficients of the linear, the interaction and the quadratic parameters, respectively and ε is the residual associated with the experiments.

To estimate the parameters in Equation 2.1, the experimental design has to assure that all studied variables are carried out at least three factor levels. The second order symmetrical designs are used for this study. This designs differ in the selection of the experimental points, the number of the levels for the variables and the number of the runs and the blocks (Bezerra et al., 2008).

Normally, the experimental design is written in the terms of the coded variables. These terms enable the investigation of the variables in the different orders of the magnitude without the greater influence on the evaluation of the lesser. The codification approach consists of transforming each studied real value into coordinates inside a scale with dimensionless values. It must be proportional at its localization in the experimental space. The following equation can be applied to transform a real value (z_i) into a coded value (x_i) according to the determinate experimental design:

$$x_i = \left(\frac{z_i - z_i^0}{\Delta z_i}\right)\beta_d \quad \textbf{(2.2)}$$

where x_i is a coded value; z_i is a real value; Δz_i is the distance between the real value in the central point and the real value in the superior or inferior level of a variable; β_d is the major coded limit value in the matrix for each variable and $z_i^0$is the real value in the central point.

2.5.2.3. Model fitting using mathematical or statistical method

After carrying out the experiments according to the chosen design, the multiple linear regression model is built by mathematical–statistical techniques. This approach is called model fitting. In general, the method of the least square (MLS) is typically used to estimate the regression coefficient of the multiple linear regression model (Equation 2.1). In the MLS, it is assumed that the errors could occur and show a random distribution profile with a zero mean and a common unknown variance. Therefore, these errors are independent from each other. After the regression coefficients were obtained by MLS, the estimated response could be calculated using model equation. This equation can be described the behavior of the response according to the level of the values studied by generating the response surfaces (Bezerra et al., 2008).

2.5.2.4. Evaluation of the fitted model

After the estimated response model was obtained. The quality of the mathematical model is evaluated by the analysis of variance (ANOVA) and the determination of coefficient (R^2). In general, ANOVA aims to evaluate the significance of the regression used to foresee responses considering the sources of the experimental variance. The significance of regression can be done by the *Fisher* distribution (*F* test). This method is the compare the ratios between the values of the square of the regression and the values of the square of the residuals. Thus, the statistically significant value for this ratio must be higher than the tabulated value for *F* test. This indicates that the mathematical model is well fitted to the experimental data (Bezerra et al., 2008). Moreover, capability of the model to explain the variability of the estimated response is commonly represented by the coefficient of determination (R^2). However, only R^2 is not enough to determine the adequacy of the regression model. In addition, the lack of the fit test is another way to evaluate the model adequacy. This test is assumed that the random errors inherent to the system and the estimation of these random errors are not statistically different.

Therefore, the precision and the accuracy of the model present a significant regression and a non-significant lack of the fit test (Bezerra et al., 2008).

2.5.2.5. Determination of the optimal conditions

In order to attain the optimal conditions, the predicted model equation is solved to find the values of variables that give the highest or lowest response by the first derivate of the mathematical function. The visualization of the predicted model equation by surface plots can be used to describe the relationship between the response and the independent variables. The quadratic response surface plots are generated from the quadratic model in the optimization of the two variables. However, this plots may not represent the true behavior of the system because they are estimated response of predicted model. Thus, the verification of the estimated model are required. For the quadratic models, the critical point can be characterized as the maximum, the minimum, or the saddle (Figure 2.8). The maximum point of the surface is located inside the experimental region as shown in Figure 2.8(a). The surface shown in Figure 2.8(b) presents the minimum point as shown in Figure 2.8(c). It presents the saddle point as the critical point. The saddle point is an inflexion point between a relative maximum and a relative minimum (Bezerra et al., 2008).

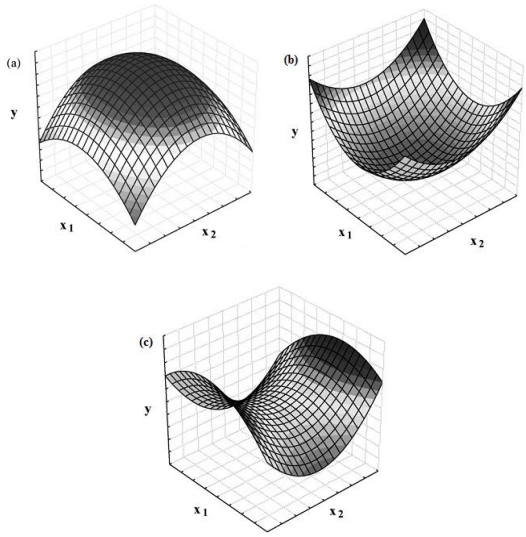


Figure 2.8 Some profiles of the surface response generated from the quadratic model in the optimization of two variables; (a) maximum, (b) minimum and (c) saddle surface (Bezerra et al., 2008)

2.5.3. Types of the experimental design

2.5.3.1. Box–Behnken design (BBD)

This design is the rotatable second-order design based on the three-level incomplete factorial design. The special characteristic of the BBD levels allows the number of design points to increase at the same rate as the number of polynomial coefficients. For example, for three factors, the design can be constructed as three blocks of four experiments consisting of the full two-factor factorial design with the level of the third factor set at the zero factors. BBD are more efficient and economical and mainly for a large number of variables. The number of the experiments (N) required for the development of the BBD is defined as $N=2k(k-1)+c_p$, where k is the number of factors and (c_p) is the number of the central point (Bezerra et al., 2008). The experimental points of this design are detailed in Figure 2.9. This design consists of the central point and the middle points of the edges.

2.5.3.2. Central composite design

Central composite design (CCD) is widely used as the experimental designs for the response surface methodology to estimate a second-order polynomial approximation to a response in the region. The design usually consists of a 2^n full factorial design, 2×n axial designs, and m central designs. The axial design is identical to the central design, except for one factor which will take on levels either above the high levels or below the low levels of the 2^n full factorial design. The number of the experiments (N) required for the development of CCD is defined as $N=k^2+2k+c_p$, where k is the factor number and (c_p) is the replicate number of the central point (Bezerra et al., 2008). The experimental points of this design are detailed in Figure 2.9.

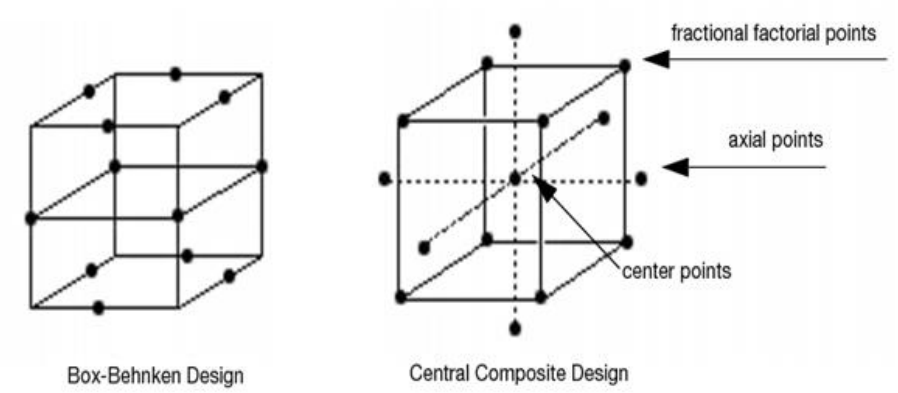


Figure 2.9 Graphic representations of BBD and CCD (Bezerra et al., 2008)

2.5.4. Application of RSM for optimization of bioprocess

Production of fatty acids by enzymatic hydrolysis has gained considerable attention because it can be carried out under the mild condition, the purity of product is high and the operation of downstream process is simple. For the enzymatic hydrolysis, the independent variables like buffer to oil ratio, enzyme to oil ratio, initial pH, reaction time, reaction temperature and shaking rate may effect to the efficient of oil hydrolysis. Currently, the production of fatty acids by enzymatic hydrolysis used RSM to optimize the variables process. This approach could explain the effects of the independent variables on the response and determine the optimal condition of the independent variables at the maximum of the response. Some of independent variables and optimal level affecting to enzymatic hydrolysis using RSM are represented in Table 2.10 and a previous studies have also been reported on the enzymatic hydrolysis for the preparation of the free fatty acids by RSM as well.

Table 2.10 The independent variables affecting the enzymatic oil hydrolysis

Type of fatty acids	Types of design	The investigated Variables	Optimal levels	Conversion (%)	References
Ricinoleic acid	CCD	Concentration of lipase Concentration of buffer Initial pH Reaction temperature	2.78 mg/g oil 1.27 g/g oil 8 40°C	65.50	Goswami et al. (2009)
Erucic acid	CCD	Concentration of lipase Concentration of buffer Initial pH Reaction temperature	2.71 mg/g oil 1.25 g /g oil 8.70 24.2 °C	95.50	Goswami et al. (2012)
DHA+ EPA	CCD	Concentration of lipase Reaction time, Reaction temperature	340 U/g oil 45 h 38 °C	54.40	Wanasundara and Shahidi (1998)

Teng et al. (2009) revealed that the optimal hydrolysis of OA and LA from chicken oil using CCD was obtained. Variables affecting to the degrees of hydrolysis and the yields of the free fatty acids were temperature, enzyme loading, and reaction time. Under the optimal condition, the yields of LA and OA were enhanced to 0.12 and 0.47 (w/w) with the hydrolysis degrees of 93.70 % and 94.60 %, respectively.

Avelar et al. (2013) investigated the optimization of the canola oil hydrolysis using the lipase from the dormant castor bean oil. The CCD design was carried out to attain the optimal conditions. The maximum degree of hydrolysis was observed using the enzyme loading of 2 % (wt), the oil to buffer ratio of 30 % (w/w), initial pH of 4.50 and reaction temperature of 37.50°C without $CaCl_2$ addition.

Chen et al. (2014) developed the optimization of linseed oil using RSM. The effects of the variables were also evaluated and optimized by BBD. This design was used to study the effects of five independent variables such as temperature, pH, oil to aqueous phase ratio, enzyme loading and reaction time on the oil hydrolysis. The optimal conditions were the temperature of 33°C, the pH of 5.80, the oil to the aqueous phase ratio of 0.90 (w/w), the enzyme loading of 1.20 % (w/w) and the reaction time of 3.33 h. Under these conditions, the hydrolysis rate of 93.92 % was obtained.

CHAPTER III
Materials and Methods

3.1. Materials

3.1.1. Chemicals

Sunflower oil was purchased from the local market (Thanakorn vegetable oil product Co., Ltd, Samutprakan, Thailand). Linoleic acid, heptadecanoic acid, 14 % (v/v) borontrifluoride solution and Methyl ester isomer of *cis*-9, *trans*-11 CLA and *trans*-10, *cis* -12 CLA were bought from Sigma-Aldrich Co., LLC. (St. Louis, MO, USA). Methyl ester (FAMEs) of palmitic acid, stearic acid, heptadecanoic acid, oleic acid and linoleic acid was obtained from Sigma-Aldrich Co., LLC (St. Louis, MO, USA) and Supelco Co. (Bellefonte, PA, USA). De Man-Rogosa-Sharpe (MRS) broth was purchased from Hi Media Laboratory Pvt., Ltd. (Mumbai, India). All solvents and reagents for experiments were analytical grade.

3.1.2. Enzyme for oil hydrolysis

Lipases from *Aspergillus niger* (ANL), *Burkholderia cepacia* (BCL), *Candida rugosa* (CRL) and *Pseudomonas fluorescens* (PFL) were kindly gift from the Amano Enzyme Inc. (Nogaya, Japan). While, Other lipases from *Mucor javanicus* (MJL) and *Rhizopus niveus* (RNL) were purchased from Sigma-Aldrich Co., LLC. (St. Louis, MO, USA). Catalytic activity of lipase was evaluated prior to do the experiments.

3.1.3. Microorganisms

According to several reports, 12 strains of potential LAB were selected and purchased from Thailand Culture Collection Centre, Thailand Institute of Scientific and Technological Research; TISTR (Pathumthani, Thailand). A list of LAB strains was shown in Table 3.1.

3.2. Enzyme screening for oil hydrolysis

Enzymatic hydrolysis of sunflower oil was modified from previous reports (Gámez-Meza et al., 2003; Rooney and Weatherley, 2001). 21 g of sunflower oil and 25 g of 100 mM potassium phosphate buffer pH 7.0 were mixed and incubated at 40 °C with stirring at 500 rpm. Then, 3 mL of enzyme solution was added at 500 U/g of oil. The reaction was carried out under nitrogen stream for 24 h. At a time interval, 0.50 g of sample was withdrawn from the reaction for determination of degree of hydrolysis. The

reaction was terminated by 50 mL of solvent solution containing ethanol and acetone at the ratio of 1:1 (v/v).

Table 3.1 The selected LAB strains for CLA production

LAB Strains	Source
Lactobacillus acidophilus TISTR 1034	Rut gut
Lactobacillus acidophilus TISTR 1338	Chicken gut
Lactobacillus brevis TISTR 868	Fermented bamboo shoot
Lactobacillus casei TISTR 390	Bagasses
Lactobacillus plantarum TISTR 1465	Swine dung
Lactobacillus plantarum TISTR 543	Fermented Pork
Lactobacillus reuteri	Unknown
Lactobacillus casei subsp. rhamnosus TISTR 047	Unknown
Lactococcus lactis TISTR 1401	Milk product
Lactococus lactis	Unknown
Pediococcus acidilactici TISTR 051	Unknown
Streptococcus thermophilus TISTR 894	Pasteurized milk

3.3. Optimization of enzymatic oil hydrolysis

3.3.1. Screening of independent variables

In this study, 2^5-1 FFD was applied to find out the significant variables affecting degree of oil hydrolysis. Levels of independent variables used in experimental design were shown in Table 3.2 and the degree of hydrolysis was selected as response. The variables were coded according to the Equation 3.1.

$$x_i = \frac{(X_i - X_o)}{\Delta X_i} \quad \textbf{(3.1)}$$

where x_i is the coded value of the independent variable; X_i is the real value of the independent variable; X_o is the real value of the independent variable at the centre point and ΔX_i is the step change value.

The response of FFD experiment is based on the first order model as illustrated in Equation 3.2. The significant variables were further optimized by central composite design (CCD).

$$Y = \beta_0 + \sum \beta_i X_i \quad \textbf{(3.2)}$$

where Y is the response; β_0 is the model intercept; β_i is the linear coefficient and X_i is the coded value of the independent variables.

Table 3.2 Levels of the independent variables for FFD

Independent Variables	Symbol	Levels		
		-1	**0**	**1**
Enzyme to oil ratio (U/g)	X_1	200	500	800
Reaction temperature (°C)	X_2	35	40	45
Initial pH	X_3	6	7	8
Buffer to oil ratio (w/w)	X_4	1	2	3
Reaction time (h)	X_5	12	24	36

3.3.2. Optimization of enzymatic process

CCD was employed to evaluate the optimal conditions for enzymatic hydrolysis. The significant variables ($p<0.05$) were enzyme to oil ratio, initial pH, buffer to oil ratio and reaction time. Levels of the variables in CCD were shown in Table 3.3. The experimental design consists of 30 treatments with 2^4 full factorial design, 12 of axial points and 6 replicates of the central points. The degree of hydrolysis was selected as response. The model was fitted to a quadratic polynomial equation according to Equation 3.3.

$$Y = \beta_o + \sum \beta_j X_j + \sum \beta_{jj} {X_j}^2 + \sum \beta_{jk} X_j X_k \quad \textbf{(3.3)}$$

where Y is the response; $\beta_o$is the intercept; β_j, β_{jj}, β_{jk} are linear, quadratic and interaction coefficients, respectively.; X_j and X_k are the coded value of the independent variables.

Table 3.3 Levels of the significant variables for CCD

Independent Variables	Symbol	Levels				
		-2	**-1**	**0**	**+1**	**+2**
Enzyme to oil ratio (U/g)	X_1	50	350	650	950	1250
Initial pH	X_3	6.0	6.5	7.0	7.5	8.0
Buffer to oil ratio (w/w)	X_4	1	2	3	4	5
Reaction time (h)	X_5	8	16	24	32	40

3.4. Enrichment of LA using urea complex fractionation

The fatty acids were enriched by urea complex fractionation according to previous study with modification (Gámez-Meza et al., 2003). 15 g of the fatty acids extracted was mixed with 100 mL of 25 % (w/v) urea in ethanol at 60^{o}C with stirring until the mixture was turned into a clear homogeneous solution. The mixture was allowed to crystallize at 4^{o}C for 10 h with stirring. The crystals were removed by filtration. Then, the composition of total fatty acids in crystals and filtrate was determined by gas chromatography.

3.5. Starter preparation

The lyophilize LAB were activated in submerge culture with 0.50 mL of MRS broth and incubated at 37^{o}C for 24 h. Then, the single colony of the LAB was obtained by streaking on MRS agar plate. The starter culture was prepared by a consecutive growth in MRS broth for 18 h until the cell concentration of 1.0 $\times 10^{8}$ CFU/mL was obtained.

3.6. Screening of the selected LAB for CLA production

The ability of selected LAB to convert LA to CLA was determined according to previous reports (Rodríguez-Alcalá et al., 2011; Xu et al., 2008). The LAB starter of 1.0 $\times 10^{8}$ CFU/mL was cultured in MRS broth supplemented with LA concentration of 0.20 mg/mL at 37^{o}C for 48 h. Then, the viable cell concentration was evaluated by spreading MRS agar plate. The culture was centrifuged at 10,000 rpm for 10 min at 4^{o}C and the supernatants were recovered for pH measurement and CLA analysis.

3.7. Effect of LA concentration on growth of the positive LAB

This study was performed by a modified method according to previous report (Xu et al., 2008). The positive LAB was grown in MRS broth supplemented with LA at 37^{o}C for 48 h. The effect of LA concentration at 0.10, 0.50, 1.00 and 2.00 mg/mL on cell growth was examined. At specific time interval of 12 h, the samples were withdrawn and determined cell concentration and pH measurement.

3.8. Preparation of CLA from sunflower oil

The preparation of CLA from sunflower oil in the presence of lipase and positive LAB as biocatalysts was carried out according to reports (Ando et al., 2004; Rodríguez-Alcalá et al., 2011). Initially, enzymatic hydrolysis was performed at the optimal condition in MRS broth supplemented with oil at the concentration of 2.00 mg/mL and enzyme was added at the concentration of 750 U/g oil. Then, the positive LAB was added in the mixture and grown at 37^{o}C for 48 h. At the time interval of 12 h. the samples were withdrawn and determined the concentration of CLA by gas chromatography.

3.9. Analytical methods

3.9.1. Determination of Oil hydrolysis

The degree of oil hydrolysis was determined by titration of the released fatty acid against 25 mM NaOH solution and defined as the weight percent of the free fatty acid in the sample to the maximum theoretical amount according to Equation 3.4 (Rooney and Weatherley, 2001).

$$\text{Degree of hydrolysis (\%)} = \frac{V_{NaOH} \times M_{NaOH} \times MM}{W_t \times f_o} \times 100 \qquad \textbf{(3.4)}$$

where V is the required volume of NaOH solution during titration (L); M is the molar concentration of NaOH (M); MM is the average molecular mass of fatty acids in sunflower oil; W_t is the weight of oil in the sample (g); f_o is the fraction of oil reaction mixture at the beginning of mixture.

3.9.2. Determinations of fatty acids composition

- Extraction of fatty acids

In case of oil hydrolysis, fatty acids were extracted according to modified method of previous report (Gámez-Meza et al., 2003). 0.50 g of reaction mixture were extracted by added 3.50 mL of 0.50 N ethanolic KOH and vigorously mixed for 60s. Then, 10 mL of *n*-hexane was added and mixed for 60s. The lower layer was collected. Then, the upper layer was acidified to pH 2.00 with 6 N HCl and 10 mL of the *n*-hexane was added. The *n*-hexane layer was collected and removed under a stream of nitrogen. All extracted fatty acids were stored at -20^{o}C prior to methylation.

In case of urea complex fractionation, the extraction of fatty acid in filtrate and crystals was similar according to extraction of fatty acid in oil hydrolysis with a modification. The crystals mixed with 100 mL of distilled water. This solution was acidified to pH 2.00 by 6 N HCl. Then, 100 mL of *n*-hexane were added and separated in funnel. The *n*-hexane layer was collected and removed by evaporator, while, a filtrate was acidified to pH 2.00 by 6 N HCl. After that, equal volume of distilled water and *n*-hexane were added. The separation phase of *n*-hexane was done and removed by evaporator.

In case of CLA production, free fatty acids in supernatants were recovered by using isopropanol/hexane extraction according to the method of Hernandez-Mendoza et al. (2009). 4 mL of supernatants and 2 mL of 0.20 mg/mL of heptadecanoic acid were vigorously mixed with 2 mL of *n*-hexane for 60s. Then, the upper layer was collected and the lower layer was extracted again with 4 mL of *n*-hexane. The upper layer was pooled with the previous hexane layer in a glass tube. The *n*-hexane layer was removed under a stream of nitrogen. All extracted fatty acids were stored at -20^{o}C prior to methylation.

- Composition of fatty acid methylation and analysis

All extracted fatty acids were methylated to fatty acids methyl ester (FAMEs) and analyzed with gas chromatography. In case of enzymatic production of fatty acids from sunflower oil, free fatty acids were methylated according to standard protocol (AOCS, 1998). The free fatty acids were transformed to methyl ester with 3.50 mL of 14% (v/v) borontrifluoride in methanol solution at 55^{o}C for 5 min. The FAMEs were extracted by added 1 mL of distilled water and 1 mL of *n*-hexane. The hexane layer was collected and analyzed by gas chromatography (Shimadzu GC 2014, Kyoto, Japan) equipped with a flame ionization detector. A capillary column (30 m x 0.25 µm x 0.25 µm; DB-5) was used. The injector and flame ionization detector temperature were 250^{o}C and 260^{o}C, respectively. The initial oven temperature was raised from 150^{o}C to 220^{o}C at 4^{o}C/ min

and holding at 220^{o}C for 8 min. Helium was used as a carrier gas at 87.50 kPa. FAMEs were identified by comparison with standards and quantified as the percent by weight of each FAME.

In case of CLA production, the free fatty acids extracted were transformed to FAMEs according to the previous report (Hernandez-Mendoza et al., 2009). The extract was methylated with 2 mL of 5% hydrochloric acid in anhydrous methanol at 60^{o}C for 20 min and stand for 5 min at room temperature. The methylation reaction was stopped by addition of 1 mL of distilled water. Then, the FAMEs were extracted with 1 mL of *n*-hexane and mixed for 30s. The upper hexane layer was transferred to a vial. The lower layer was re-extracted with 0.50 mL of hexane and the upper layer was pooled with the previous upper layer. A 1 µL of FAME sample was injected into the gas chromatograph. The FAMEs were analyzed using gas chromatography (Shimadzu GC 2010, Kyoto, Japan) equipped with flame ionization detector. A polyethylene glycol capillary column (30 m x 0.25 µm x 0.25 µm; HP-Innowax) was used. The injector and flame ionization detector temperature were 250^{o}C and 260^{o}C, respectively. The initial oven temperature was started at 150^{o}C and raised to 230^{o}C at 20^{o}C/ min and holding at 230^{o}C for 15 min. Helium was used as a carrier gas at 10 mL/min. The FAMEs of CLA peak were identified and quantified by comparison with the retention time and peak area of high purity standards. The concentration of total CLAs (*cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLAs) in each sample was expressed in mg/mL.

3.10. Statistical analysis

All experiments were carried out in triplicate and average values were given. Design expert version 7.00 (Stat-Ease, Minneapolis, MN, USA) was used for the statistical and graphical analysis of the experimental data. The statistical significance of the independent variables and model were determined by *F*-test. The quality of the quadratic model was judged statistically by the coefficient of determination (R^2), the lack of fit and the coefficient of variation.

CHAPTER IV
Results and Discussion

4.1. Enzyme screening for oil hydrolysis

In this study, the several types of lipase were used to hydrolyze sunflower oil for preparation of linoleic acid (LA). All lipases used in this study were selected based on source of lipases. Filamentous fungal lipases were *Aspergillus niger* lipase (ANL), *Mucor javanicus* lipase (MJL) and *Rhizopus niveus* lipase (RNL). Bacterial lipases were *Burkholderia cepacia* lipase (BCL), *Pseudomonas fluorescens* lipase (PFL) and yeast lipase was *Candida rugosa* lipase (CRL). The results suggested that the degree of oil hydrolysis was extensively increased within 12 h. Then, it was nearly constant (Figure 4.1-4.2). Moreover, the use of lipases from bacteria and yeast gave higher degree of hydrolysis than using the filamentous fungi lipases (Figure 4.2). This observation could be related to substrate specificity and the structure of active center. Typically, bacterial and yeast lipases are recognized as non-specific lipase. This means that these lipases randomly liberate fatty acids on triglyceride. Therefore, high degree of oil hydrolysis is usually observed. However, filamentous fungi lipases mostly are recognized as 1,3-specific lipase. They prefer to release fatty acids in the position of 1 and 3 on triglyceride. Thus, to obtain high degree of oil hydrolysis, long reaction time is required. In addition, the active centre of filamentous fungi lipases exhibits a quite complex structure more than the active centre of lipases from bacteria and yeast. Hence, the substrate is gradually accessible to the active centre of filamentous fungi lipases (Kapoor et al., 2012; Rodrigues and Fernandez-Lafuente, 2010).

In case of using filamentous fungal lipases as biocatalyst, conversion rate of the reaction was obtained in the range of 27-43 %. Ability of ANL to hydrolyze oil was less than ability of other filamentous fungi lipases. The higher conversion rate of 42.75 % was obtained using MJL as a catalyst (Figure 4.1). The results indicated that sunflower oil was steadily hydrolyzed by filamentous fungi lipases. The results could be associated with composition of fatty acids in this oil and their substrate specificity. Normally, long chain fatty acids are major composition in sunflower oil such as oleic acid (OA) and LA (Table 4.1). While, the fatty acid specificity and structure of filamentous fungi lipases favor hydrolyze short- and medium chain fatty acids (Hayes, 2004; Pleiss et al., 1998). In this study, MJL gave the conversion rate more than other

fungal lipases. These results depend on the conformation of active site and reaction conditions. For example, the active site of *Rhizomucor miehei* is a shallow bowl with a long axis of 18°A and width of 4.5°A at its base and 6°A at the protein surface. The binding site is located at the bottom of active site. Thus, the substrates are accessible to binding pocket from the top (Pleiss et al., 1998). The capability of lipases to hydrolyze oil also depends on optimal catalytic conditions. The reaction conditions of this study are suitable for MJL to hydrolyze sunflower oil more than other fungal lipases (Murty, Bhat and Muniswaran, 2000).

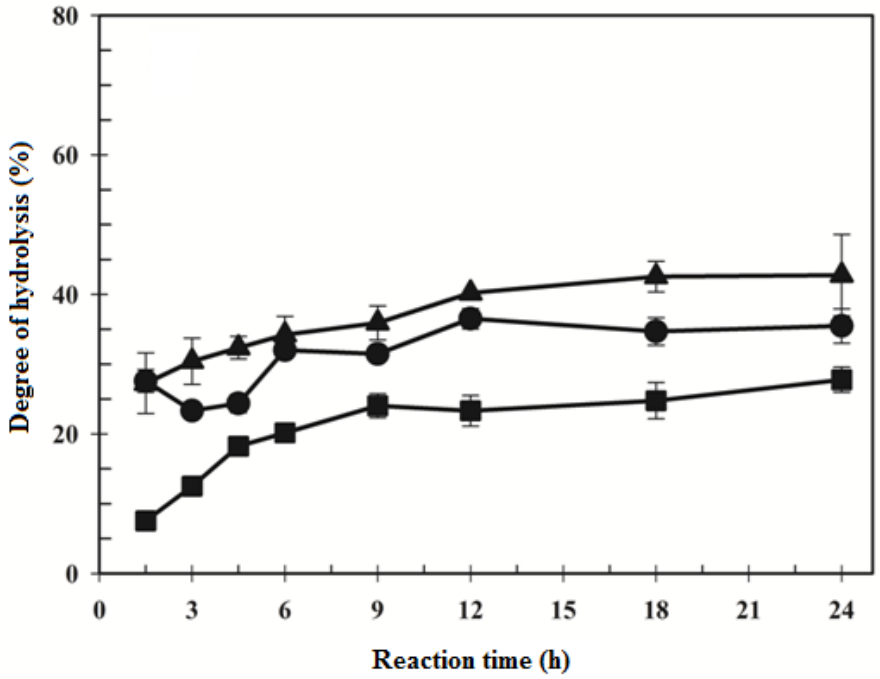


Figure 4.1 Time course of enzymatic hydrolysis using lipases from filamentous fungi as a biocatalyst; ANL (■), MJL (▲) and RNL (●) at 500 U/g oil, buffer to oil ratio of 1.0 (w/w), 40°C and 500 rpm

In case of using non-specific lipase form bacteria and yeast, the degree of hydrolysis was in the range of 42-68 %. The conversion rate by BCL and PFL was gradually increased within 12 h and almost constant to 54.46 and 42.43 %, respectively. However, the degree of hydrolysis by CRL was rapidly increased within 6 h and steadily improved to 67.12 %. The higher degree of oil hydrolysis was obtained using CRL (Figure 4.2). In this study, sunflower oil was extensively hydrolyzed by bacteria and yeast lipases more than the filamentous fungi lipases. This observation could be related to their positional specificity and preference of the fatty acids. Since bacteria and

yeast lipases are recognized as non-specific lipase and prefer to hydrolyze medium to long chain fatty acids (C_{10}-C_{21}), they could rapidly hydrolyze sunflower oil. According to the results, the high conversion rate of sunflower oil was obtained using CRL as catalyst. This observation depends on its conformation and location of the active site as well as preference of fatty acids. CRL exhibits the conformation of active site like a tunnel. This site is a long of 22°A with a diameter of 4°A and at total size of 25°A. The position of binding site is located inside a tunnel with a wide of entrance for substrate. Moreover, the catalytic site is located near the entrance to the tunnel. Thus, the substrate is easily access to the active site of CRL (Pleiss et al., 1998). In addition, CRL prefers to hydrolyze the long chain fatty acids, especially OA and LA (Domínguez de María et. al., 2006). On the other hand, the shape of active site of PFL and BCL exhibits an elliptical funnels with a length of 17°A. The catalytic site is located at bottom of the funnel. Therefore, the substrate is difficult to access the active site. Moreover, PFL and MJL are perfer to hydrolyze the short- and medium chain fatty acids (Hayes, 2004; Pleiss et al., 1998).

The fatty acid profiles of enzymatic hydrolysate form sunflower oil were investigated. The main fatty acid composition of the examined sunflower oil was OA with a content of 48.07 % and LA with a content of 42.61 % (Table 4.1). Similarly, the content of OA and LA in the enzymatic hydrolysate was in the range of 47.76 to 46.71 and 43.30 to 42.45 %(w/w), respectively (Figure 4.3). The results showed that content of fatty acid composition in enzymatic hydrolysate using non-specific and 1,3 -specific lipases was almost equivalent. This observation may be related to positional distribution of fatty acids in sunflower oil. Normally, sunflower oil contains the LA content at each position in triacylglycerols almost the same (Gunstone and Harwood, 2007). Thus, in this study, the positional specificity of lipases is not affecting to the content of fatty acid composition in the hydrolysate.

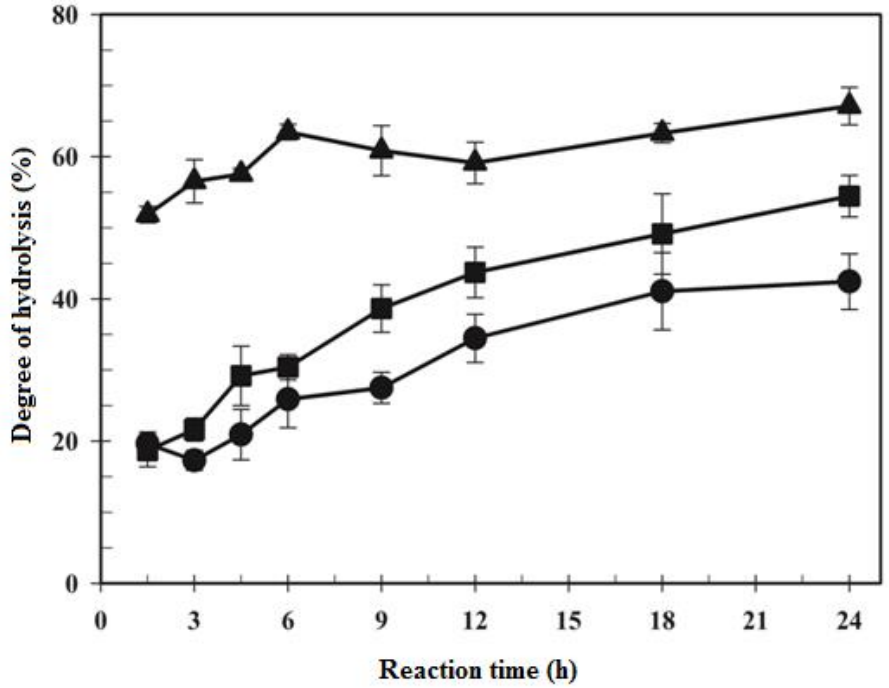


Figure 4.2 Time course of enzymatic hydrolysis using bacteria and yeast lipase as a biocatalyst; BCL (■), PFL (●) and CRL (▲) at 500 U/g oil, buffer to oil ratio of 1.0 (w/w), 40°C and 500 rpm

Table 4.1 The fatty acids composition of the sunflower oil

Type of fatty acids	**Content of fatty acids [% (w/w)]**
Palmitic acid (C16:0)	5.22
Stearic acid (C18:0)	2.83
Oleic acid (C18:1)	48.07
Linoleic acid (C18:2)	42.61
Linolenic acid (C18:3)	0.22

According to the results, CRL was found to be a suitable biocatalyst for sunflower oil hydrolysis. This finding was analogues to the previous reports. Freitas, Bueno, Perez, Santos and Castro (2007) observed that CRL exhibited the hydrolysis rate of soybean oil by several lipases. A similar trend of hydrolysis degree (up to 70%) was occurred by CRL following conditions; enzyme loading of 1.00 % (w/w), oil to water ratio of 1:4 (w/w) and 25 % (w/v) of arabic gum at 40°C. For ricinoleic acid production, the high conversion rate of castor oil was obtained using CRL as biocatalyst (Foglia et.

al, 2000). Moverover, CRL was found to be a suitable biocatalyst for enrichment of α-LNA from flax seed oil through hydrolysis (Rupani et.al, 2012). Preparation of ɣ-GLA from seed oil of two plant species, *Borago officinalis* and *Echium fastuosum* was successful by using CRL (LÓPez-MartÍNez
et al., 2006).

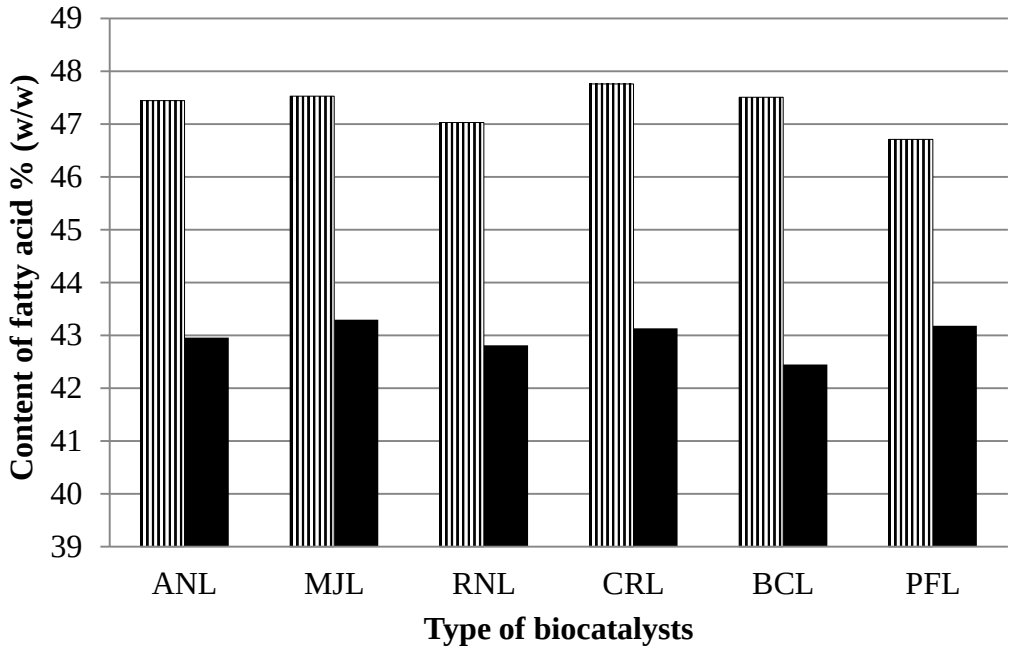


Figure 4.3 The main fatty acids of the oil hydrolysate using several lipases as biocatalysts at 500 U/g oil, buffer to oil ratio of 1.0 (w/w), 40°C and 500 rpm for 24 h; OA (▒) and LA (■)

4.2. Optimization of enzymatic hydrolysis

4.2.1. Screening of the independent variables

The independent variables were selected depending on process parameters of hydrolysis such as buffer to oil ratio, enzyme to oil ratio, initial pH, reaction time and reaction temperature. Based on previous studies, Teng et al. (2009) studied effect of independent variable on enzymatic hydrolysis from chicken oil. The investigated parameters were reaction temperature, loading of enzyme, shaking rate and reaction time. In case of sunflower oil hydrolysis, the lipase concentration and ratio of oil to water also affect to degree of hydrolysis (Rooney and Weatherley, 2001). Therefore, in this study, these parameters were used for optimization of oil hydrolysis by CRL as biocatalyst. Firstly, the 2^{5-1} FFD was applied to screen and identify the significant

variables affecting to the degree of hydrolysis. The experimental design and the results of the response were shown in Table 4.2. The results revealed that the hydrolysis degree ranging from 35 to 94 % was obtained. In order to identify significant variables, statistical analysis was performed by Design-Expert program (State Ease Inc., Minneapolis, MN, USA). Effect and interaction of independent variables were evaluated using *F*isher-test and analysis of variance (ANOVA) was performed as shown in Table 4.3. The results indicated that the significant variables affecting to degree of oil hydrolysis were enzyme to oil ratio, initial pH, buffer to oil ratio and reaction time ($p<0.05$) while, reaction temperature was found to be insignificant variable to the degree of hydrolysis ($p>0.05$). This may be related to the selected level of this variable. Range of reaction temperature used in experimental design may not significant affecting to the response. In addition, interactions of the significant variables were the interactions of enzyme to oil and reaction temperature, enzyme to oil ratio and initial pH, reaction temperature and reaction time, initial pH and buffer to oil ratio, initial pH and reaction time, buffer to oil ratio and reaction time ($p<0.05$). Moreover, the calculated *F*-values and *p*-values of the model were 112.76 and <0.0001. These results implied that the model was adequate precision ($p<0.01$). The coefficient of determination (R^2) of the model was 0.994. Thus, the model was fitted with the first order linear equation as detailed in Equation 4.1. These 4 variables including enzyme to oil ratio, initial pH, buffer to oil ratio and reaction time were selected for further experiments**.**

$$\begin{aligned}\text{Hydrolysis (\%)} = {} & + 58.65 + 3.04\,X_1 + 0.56\,X_2 - 4.09\,X_3 + 15.05\,X_4 + 4.32\,X_5 \\ & + 3.23\,X_1X_2 + 4.15\,X_1X_3 + 2.57\,X_2X_5 - 1.85\,X_3X_4 + 3.86\,X_3X_5 \\ & + 3.22\,X_4X_5 \end{aligned} \quad \textbf{(4.1)}$$

where, X_1, X_2, X_3, X_4 and X_5 are the coded values of enzyme to oil ratio, reaction temperature, initial pH, buffer to oil ratio and reaction time, respectively.

Table 4.2 Experimental design of FFD and results of the response

Run No.	Coded (Actual) variables					Degree of hydrolysis (%)
	X_1	X_2	X_3	X_4	X_5	
1	0 (500)	0 (40)	0 (7)	0 (2)	0 (24)	69.34
2	-1 (200)	-1 (35)	+1 (8)	+1 (3)	+1 (36)	70.32
3	+1 (800)	-1 (35)	+1 (8)	-1 (1)	+1 (36)	46.08
4	-1 (200)	+1 (45)	+1 (8)	+1 (3)	-1 (12)	43.93
5	-1 (200)	-1 (35)	+1 (8)	-1 (1)	-1 (12)	35.13
6	0 (500)	0 (40)	0 (7)	0 (2)	0 (24)	68.62
7	+1(800)	+1 (45)	-1 (6)	-1 (1)	+1 (36)	47.38
8	0 (500)	0 (40)	0 (7)	0 (2)	0 (24)	68.13
9	+1 (800)	-1 (35)	+1 (8)	+1 (3)	-1 (12)	62.32
10	+1 (800)	+1 (45)	+1 (8)	-1 (1)	-1 (12)	44.14
11	0 (500)	0 (40)	0 (7)	0 (2)	0 (24)	65.03
12	-1 (200)	+1 (45)	-1 (6)	+1 (3)	+1 (36)	82.46
13	+1 (800)	+1 (45)	+1 (8)	+1 (3)	+1 (36)	94.46
14	+1 (800)	+1 (45)	+1 (8)	+1 (3)	+1 (36)	75.95
15	+1 (800)	-1 (35)	-1 (6)	+1 (3)	+1 (36)	77.72
16	+1 (800)	-1 (35)	-1 (6)	-1 (1)	-1 (12)	45.51
17	-1 (200)	+1 (45)	-1 (6)	-1 (1)	-1 (12)	45.25
18	-1 (200)	-1 (35)	-1 (6)	-1 (1)	+1 (36)	45.25
19	-1 (200)	+1 (45)	+1 (8)	-1 (1)	+1 (36)	40.11
20	-1 (200)	-1 (45)	-1 (6)	+1 (3)	-1 (12)	82.44

4.2.2. Optimization of enzymatic hydrolysis

This experiment was designed to optimize the conditions of the significant variables in order to maximize degree of oil hydrolysis using CCD. The significant variables including enzyme to oil ratio, initial pH, buffer to oil ratio, reaction time were examined. The experimental design and the results of the response were shown in Table 4.4. The results suggested that the degree of hydrolysis was fluctuated from 38.67 to 77.85%. Low degree of oil hydrolysis was obtained in experiment no. 14 while, high degree of oil hydrolysis was obtained in experiment no 9. Moreover, the higher degree of hydrolysis was achevied when the indepedent variables were set to center point. Statistical analysis was performed by Design-Expert program (State Ease Inc., Minneapolis, MN, USA).

Table 4.3 Analysis of variance for FFD

Source	df	Mean Squares	*F*-values	*p*-value
Model	11	486.36	112.76	< 0.0001*
X_1	1	147.93	34.30	0.0006*
X_2	1	4.96	1.15	0.3189
X_3	1	267.72	62.07	0.0001*
X_4	1	3623.07	839.99	< 0.0001*
X_5	1	298.60	69.23	< 0.0001*
$X_1 X_2$	1	167.00	38.72	0.0004*
X_1X_3	1	275.12	63.79	< 0.0001*
X_2X_5	1	106.01	24.58	0.0016*
X_3X_4	1	54.77	12.70	0.0092*
X_3X_5	1	238.64	55.33	0.0001*
X_4X_5	1	166.09	38.51	0.0004*

* Statically significant ($p<0.05$); df = degree of freedom

Effect and interaction of independent variables were evaluated using *Fisher*-test and ANOVA was performed as shown in Table 4.5. The results indicated that enzyme to oil ratio, initial pH and buffer to oil ratio were statistically significant ($p<0.05$). However, reaction time was found to be insignificant variable to degree of oil hydrolysis ($p>0.05$). These results may be related to the selected level of this variable. Range of reaction time used in experimental design may not significant affecting to the response. The calculated *F*-values of 9.16 and the model *p*-values was very low implies that the model is statistical significant with high level of confidence ($p<0.01$). The goodness, degree of precision and reliability of the model were examined by R^2, lack of fit and the coefficient of variation (C.V.). R^2 value of 0.8953 revealed that the model could explain 89.53% of the experimental variability. The lack of fit was not significant and low values of the C.V. was obtained (5.92 %). The model was fitted to a quadratic polynomial model in Equation 4.2. The positive coefficients in each term of equation suggested that independent variables such as enzyme to oil ratio, buffer to oil ratio and reaction time had a direct relationship with the degree of oil hydrolysis. These results could be implied that the predicted model was suitable for enzymatic hydrolysis of sunflower oil.

$$Y = 74.99+2.37X_1-1.80X_3+6.91X_4-2.81X_1^2-4.36X_5^2 \quad \textbf{(4.2)}$$

where Y is the degree of hydrolysis, X_1 is the enzyme to oil ratio, X_3 is initial pH, X_4 is the buffer to oil ratio and X_5 is reaction time.

Table 4.4 Experimental design of CCD and results of the response

Run	Coded (Actual) Variables				Degree of hydrolysis (%)	
	X_1	X_3	X_4	X_5	Predicted values	Actual values
1	-1 (350)	+1 (7.5)	+1 (4)	-1 (16)	64.97	60.70
2	0 (650)	0 (7.0)	0 (3)	0 (24)	74.99	69.14
3	0 (650)	0 (7.0)	+2 (5)	0 (24)	71.38	74.90
4	0 (650)	0 (7.0)	0 (3)	-2 (8)	72.09	72.20
5	+1 (950)	+1(7.5)	-1 (2)	- 1 (16)	62.15	62.14
6	0 (650)	0 (7.0)	0 (3)	+2 (40)	74.21	72.52
7	-1 (350)	-1 (6.5)	-1 (2)	+1 (32)	59.41	60.95
8	0 (650)	0 (7.0)	0 (3)	0 (24)	74.99	76.98
9	+1 (950)	-1 (6.5)	+1 (4)	-1 (16)	80.36	77.85
10	-1 (350)	-1 (6.5)	+1 (4)	-1 (16)	73.01	75.52
11	+1 (950)	-1 (6.5)	+1 (4)	+1 (32)	79.32	76.87
12	0 (650)	-2 (6.0)	0 (3)	0 (24)	75.57	75.09
13	+1(950)	-1 (6.5)	-1 (2)	-1 (16)	61.89	59.90
14	0 (650)	0 (7)	-2 (1)	0 (24)	43.76	38.67
15	+1(950)	+1 (7.5)	+1 (4)	+1 (32)	72.84	69.87
16	-1 (350)	+1 (7.5)	-1 (2)	-1 (16)	56.12	60.75

Table 4.4 Experimental design of CCD and results of the response (cont.)

Run	Coded (Actual) Variables				Degree of hydrolysis (%)	
	X_1	X_3	X_4	X_5	Predicted values	Actual values
17	0 (650)	+2 (8)	0 (3)	0 (24)	68.38	67.29
18	-2 (50)	0 (7)	0 (3)	0 (24)	59.01	53.68
19	0 (650)	0 (7.0)	0 (3)	0 (24)	74.99	74.65
20	-1 (350)	+1(7.5)	+1 (4)	+1 (32)	68.43	72.60
21	0 (650)	0 (7.0)	0 (3)	0 (24)	74.99	75.34
22	+2 (1250)	0 (7.0)	0 (3)	0 (24)	68.48	72.24
23	0 (650)	0 (7.0)	0 (3)	0 (24)	74.99	77.28
24	-1 (350)	+1 (7.5)	-1 (2)	+1 (32)	59.29	61.19
25	0 (650)	0 (7.0)	0 (3)	0 (24)	74.99	76.55
26	-1 (350)	-1 (6.5)	+1 (4)	+1 (32)	75.89	75.29
27	+1 (950)	-1 (6.5)	-1 (2)	+1 (32)	60.41	64.20
28	-1 (350)	-1 (6.5)	-1 (2)	-1 (16)	56.83	59.19
29	+1 (950)	+1 (7.0)	-1 (2)	+1 (32)	61.40	61.06
30	+1 (950)	+1 (7.0)	+1 (4)	-1 (16)	73.30	73.93

Table 4.5 Analysis of variance for CCD

Source	df	Mean Squares	F-values	*p*-value
Model	14	149.91	9.16	< 0.0001*
X_1	1	134.26	8.20	0.0118*
X_3	1	77.47	4.73	0.0460*
X_4	1	1144.35	69.92	< 0.0001*
X_5	1	6.72	0.41	0.5314
Lack of fit	10	19.94	2.16	0.2040
Residual	15	16.37		
Pure error	5	9.22		
Cor. total	29			

* Statically significant ($p<0.05$), df = degree of freedom, R^2= 0.8953, CV % = 5.92

The relationship between independent variables on the degree of oil hydrolysis was displayed in three dimension response surfaces (Figure 4.4-4.5). Figure 4.4 showed the combined effect of enzyme to oil ratio and buffer to oil ratio on the degree of hydrolysis. At the low and high level of buffer to oil ratio, the degree of hydrolysis initially improved with the increase in enzyme to oil ratio from 350 to 650 U/g oil. Then, it was nearly constant with enzyme to oil ratio higher than 650 U/g oil. In case of enzyme to oil ratio, at low and high level of buffer to oil ratio the hydrolysis degree was extensively enhanced with increase buffer to oil ratio of 3.0 and gradually constant at the high level of buffer to oil ratio. These results could be related to a catalytic activity of lipase. Normally, lipases initially hydrolyze oil when the interfacial activation occurs between the heterologous phase. Degree of hydrolysis is usually increased when the amount of enzyme is enhanced. However, at very high content of enzyme, the degree of hydrolysis does not depending on enzyme concentration because the resulting products could decrease the catalytic activity of the enzyme. In addition, increment of buffer to oil ratio raise the interfacial area of catalytic hydrolysis and leads to increase the degree of oil hydrolysis. (Teng et al., 2009; Rooney and Weatherley, 2001; Goswami et al., 2012; Noor, Hasan, and Ramachandran, 2003). Hence, the higher degree of hydrolysis was obtained at the enzyme to oil ratio more than 650 U/g oil. In addition, the high hydrolysis rate was found at buffer to oil ratio of 4 (w/w). This phenomenon was similar to the previous reports, Goswami et al. (2009) optimized the production of ricinoleic acid from castor oil using RSM. They found that, enzyme and buffer concentrations were the significant variables to the conversion rate. Likewise, Teng et al. (2009) studied the optimization of enzymatic hydrolysis from chicken oil. They found that, loading of enzyme and was the main factor affecting to degree of hydrolysis and content of fatty acids.

Figure 4.5 showed the effect of initial pH and the buffer to oil ratio on the degree of hydrolysis, At a low level of buffer to oil ratio, the increase of initial pH from 6.50 to 7.50 was not improved the hydrolysis degree. In contrast, at a high level of buffer to oil ratio the conversion rate was gradually decrease when initial pH was increased. In addition, conversion rate was increased when buffer to oil ratio increased.

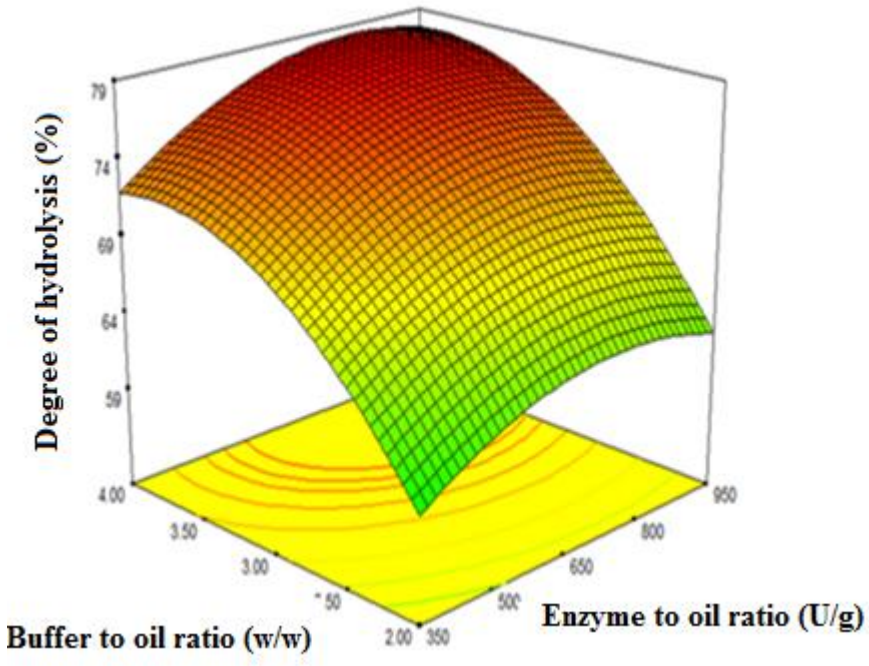


Figure 4.4 Three dimensional surface plot showing the combined effect of enzyme to oil ratio and buffer to oil ratio on the degree of hydrolysis (%) at initial pH of 7.0 and reaction time of 24 h

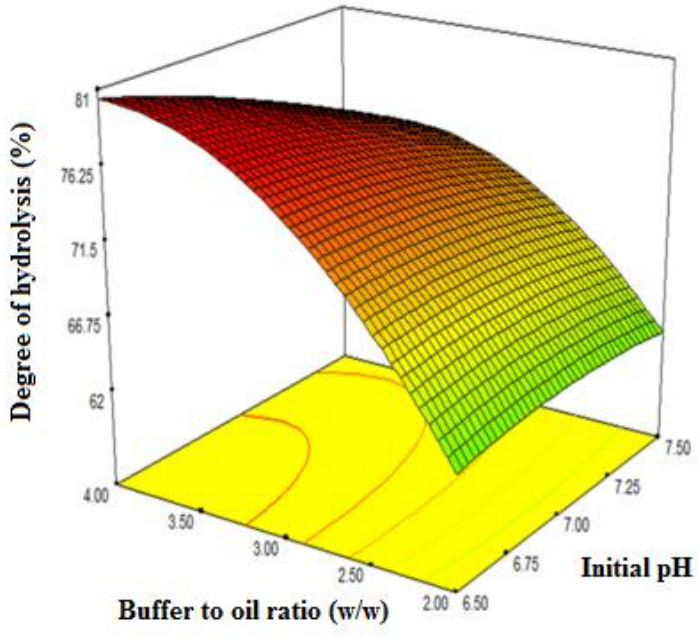


Figure 4.5 Three dimensional surface plot showing the combined effect of the initial pH and the buffer to oil ratio on the degree of hydrolysis (%) at enzyme to oil ratio of 650 U/g oil and reaction time of 24 h

This observation could be related to catalytic capacity of lipase. Generally, active site consists of catalytic amino acids. The shifting pH values of the environment lead to the change of ionization in the catalytic amino acids. Thus, low or high values of the pH values in the reaction could change the conformation of active site. Then, most the enzyme is denatured. The results in this study were analogous to the previous reports. Goswami et al. (2012) observed that initial pH of mustard oil hydrolysis was one of the major factors affecting to conversion rate.

4.2.3. Validation of the model

In order to confirm the adequacy of the model, the several levels of the independent variables were examined. Verification of the model was performed according to Table 4.6. The results of experiments indicated that this model was suitable because the difference between the predicted and actual values of the response was less than 5 %. The optimal conditions for oil hydrolysis were enzyme to oil ratio of 750.28 U/g, initial pH of 6.70, buffer to oil ratio of 4.0 (w/w) and reaction time of 19 h. Under these conditions, the hydrolysis degree of 76.07 % was obtained and it could improve more than 50 % when compared with the lowest degree of hydrolysis in CCD. In this study, enzyme to oil ratio was used less than 800 U/g without addition of the emulsifier While, some of the previous reports showed that high level of enzyme concentation was reqiured to prepare fatty acids. For example, Nagao et al. (2007) reported that prepartion of fatty acid from single-cell oil without addition of emulsifer by CRL was achieved by using enzyme to oil ratio of 1,200 U/g and reaction time of 72 h at 40^{o}C. Likewise, enzymatic hydrolysis of stearidonic acid from modified soybean oil was carried out by enzyme loading of 3,218 U/g and reaction time of 12 h. This conditions gave the hydrolysis degree of 73 % (Kleiner, Vazuez and Akon, 2012).

4.2.4. Enrichment of LA using urea complex fractionation

In this study, the urea complex fractionation was used to purify LA from the resulting fatty acids. The results indicated that the purity of LA was improved from 47% to 70%. In addition, the palmitic acid (PA) and stearic acid (SA) and OA mainly found in UCF (71.89%) While, LA and OA were remained in NUCF (Figure 4.6). Therefore, the saturated fatty acids are mostly found in UCF While, monounsaturated fatty acids and PUFAs are mainly observed in the NUCF. Separation of fatty acids using urea complex fractionation based on the presence of the multiple double bonds in the configuration of the fatty acid molecule (Schlenk, 1953). Normally, urea prefers to form a complex with saturated fatty acids because its chemical structure fits to conformation

of the saturated fatty acids. Thus, separation of PUFAs from mix fatty acids is achieved when low temperature is applied in order to crystallize this complex and separation is done by filtration.

Table 4.6 Verification of the predicted model

Goal of Response	Level of variables				Degree of hydrolysis (%)		Error (%)
	X_1	X_3	X_4	X_5	Predicted value	Actual value	
Low	350	7.5	2.0	16	55.12	55.34	0.41
In range	490	6.5	2.0	21	60.77	60.86	0.15
In range	350	7.5	4.0	16	64.97	64.45	0.08
In range	950	6.5	2.0	32	60.54	60.33	0.03
High	350	6.5	4.0	32	75.93	76.26	0.49
Optimum	750	6.7	4.0	19	75.93	76.07	4.83

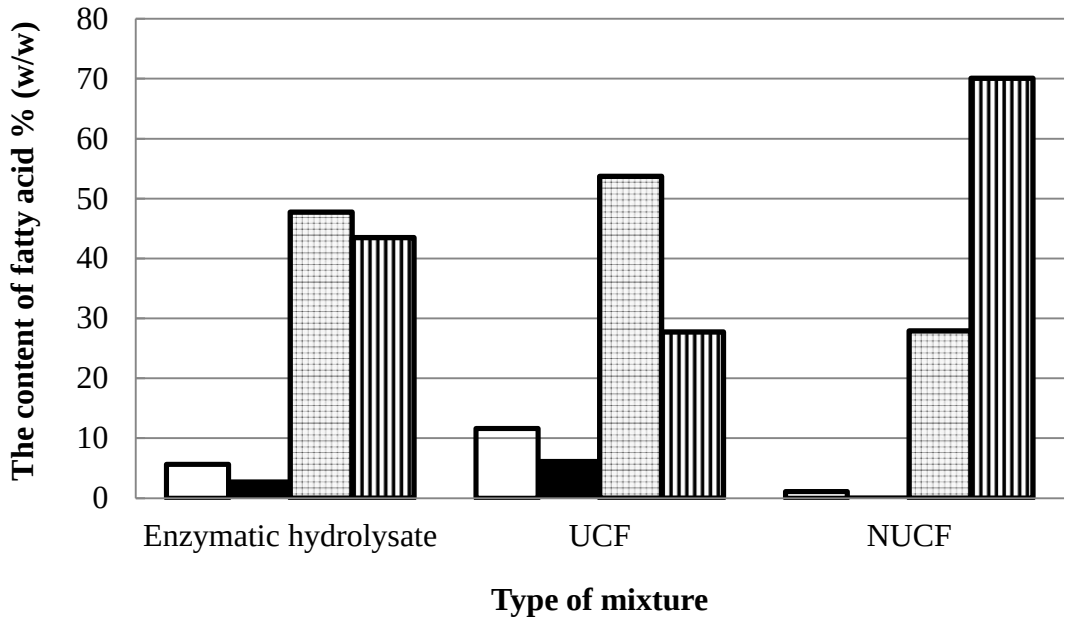


Figure 4.6 Change in composition during the enrichment of fatty acid using urea complex fractionation; PA (□), SA (■), OA (◙) and LA (◘)

4.3. Screening of the selected LAB for CLA production

In this study, the ability of the selected LAB to produce CLAs was investigated. 12 strains of the selected LAB were chosen based on the results of the previous reports. To screen the potential LAB, these bacterial strains were cultured in MRS broth supplemented with 0.20 mg/mL of LA. Growth of the selected LAB was evaluated from

viable cell count, optical density and pH values. The results of this study showed that all selected LAB grew well in the assay medium. However, only 7 strains were found to produce CLAs in the medium (Table 4.7). The higher concentration of total CLAs was produced by *Lb. plantarum* TISTR 543 and *Lb. acidophillus* TISTR 1338. They could produce CLAs of 0.04 mg/mL and 0.02 mg/mL, respectively. Moreover, *Lb. plantarum* TISTR 543 gave the highest productivity (Table 4.7). The results could be associated with a detoxifying mechanism and cultivation conditions of the LAB. Normally, the conversion of LA to CLA has been proposed as a detoxifying mechanism through the biohydrogenation pathway by linoleate isomerase (LAI) of LAB (Jiang, Birock and Fondon, 1998; Coakley, Ross, Nordgren, Fitzgerald, Devery and Stanton, 2003). This enzyme exists to specific strains of LAB (Dubey et al.,2012). In addition, LAI activity depends on optimal condition in the cultivation of LAB (Rodríguez-Alcalá et al., 2011). Therefore, the ability of LAB to produce CLAs is rarely observed in LAB. Productivity of CLA production from *Lb. plantarum* TISTR 543 is comparable to other reports. However, lower concentration of LA was used (Table 4.8). In addition, the potential LAB of this study was originated from the fermented meat. Commonly, source of potential LAB to produce CLAs was isolated from diary product, gut of animal and babies or adults human fecal, However, they may be found in other source such as silage or other fermented meat (Coakley et al., 2003; Barrett, Ross, Fitzgerald and Stanton, 2007; Andrade et al., 2012; Van Nieuwenhove et al., 2012). The results was similar previous studies. Ogawa et al.(2005) screened 250 strains of LAB from the culture collection. They found that some genera of LAB was found to be potential strains for CLAs production such as *Enterococcus, Pediococcus, Propionibacterium* and *Lactobacillus*. All of investigated *lactobaciilus* sp. produced CLA more than 0.07 mg/mL of reaction mixture. Highest productivity of total CLAs was 3.41 mg/mL by *Lb. plantarum* AKU 1006. Similarly, Zeng, Lin and Gong (2009) identified *Lb. plantarum* NCUL005 as potential CLA producer from natural seuerkraut fermentation. The concentration of CLAs production was 0.62 mg/mL at 0.10 mg/mL of LA at 37^{o}C for 24 h. Ability of 43 isolated LAB from naturally fermented pickle brines were investigated by Liu et al. (2011). *Lb. plantarum* IP 5 was identified as a potential CLAs producer. The lp15 strain gave the maximum CLAs of 0.05 mg/mL in the MRS broth containing 0.20 mg/ml of LA at 48 h. Additional, Gurovic, Gentili, Olivera and Rodríguez (2014) selected the LAB from fish gut to produce CLAs. They found that, *Leuconostoc mesenteroides* H20, *Leuconostoc mesenteroides* H22, *Leuconostoc lactis*

H24 and *Lb. pentosus* H16 were able to produced CLAs. The content of LA and percentage of CLAs were detected in fatty acid profile of the H16 strain of 18.30 % (w/w) and 5.86 % (w/w) when cultured in agar medium with the addition of LA for 24 h.

Table 4.7 CLA production by the selected LAB strains in MRS broth supplemented with LA concentration of 0.20 mg/mL at 37°C for 48 h

The selected LAB strains	**pH**	**OD_{600}**	**Viable cell (log CFU/mL)**	**Content of CLAs (mg/mL)**	**Productivity (mg/mg of LA)**
Lb. acidophilus TISTR 1034	3.94	5.13	3.07	0.001	<0.01
Lb.acidophilus TISTR 1338	4.25	7.56	2.84	0.021	0.11
Lb. lactis TISTR 1401	4.81	2.49	1.94	0.002	0.01
Lb. plantarum TISTR 543	3.97	7.58	3.54	0.044	0.22
Lc. lactis	4.55	2.61	ND	0.001	<0.01
Lb. reuteri	3.90	8.61	3.26	0.001	<0.01
Lb. plantarum TISTR 1465	3.94	8.49	3.10	0.001	<0.01
S. thermophilus TISTR 894	4.00	7.67	2.63	0.001	< 0.01

Table 4.8 Comparison of CLA production by the potential LAB strains

Strains of LAB	Content of LA (mg/mL)	Content of CLAs (mg/mL)	Productivity (mg/mg of LA)	References
Lb. cuvatus LMG13555	0.50	0.01	0.02	Gorissen et al., 2011
Lb. acidophilus F0221	0.50	0.16	0.32	Li et al, 2011
Lb. plantarum	1.00	0.05	0.05	Rodríguez-Alcalá et al., 2011
Lb. plantarum ATCC 8014	0.50	0.02	0.05	Gorissen et al., 2011
Lb. plantarun TISTR 543	0.20	0.04	0.22	This study

4.3.1. Effect of LA concentration on bacterial growth

The effect of LA concentration on growth of the *Lb. plantarun* TISTR 543 was evaluated in MRS broth supplemented with different LA concentrations. The results showed that the content of the LAB cell was apparently inhibited when LA was supplemented in MRS broth (Figure 4.7). The growth patterns of *Lb. plantarum* TISTR 543 were divided into two groups depending on the concentration of LA. The first group was the cultivation of the LAB in MRS broth supplement with LA concentration in the range of 0.10-0.50 mg/mL. The results showed that two growth patterns were found such as log phase and stationary phase. Log phase existed within 12 h and stationary phase exhibited from 12 to 48 h. Growth of the LAB during log phase in this group was analogous to growth of the LAB cultivated in the enriched medium. On the other hand, the other group was the cultivation of *Lb. plantarum* TISTR 543 in MRS broth supplement with LA concentration in the range of 1.00-2.00 mg/mL. This resulted revealed that lag, log and stationary phases were observed. In case of LA concentration of 1.00 mg/mL, two stages of growth were obtained. Initially, log phase revealed within 24 h and stationary phase showed form 24-48 h. In case of LA concentration of 2.00 mg/mL, lag phase was found within 12 h and log phase existed within 12-36 h. Then, stationary phase was found from 36 to 48 h. Growth of this group during log phase was diminished when it was compared with the enrich medium and the first group of LA concentration (Figure 4.7).

According to the results, this observation associated with the response of LAB to LA concentration. Generally, LA has inhibitory effects on growth of gram positive bacteria. LAB used biohydrogenation pathway to reduce the antibacterial effect of LA (Jiang et al.,1998; Coakley et al., 2003). The bacterial growth is seriously inhibited at the high LA concentration. However, the tolerance of LAB on specific concentration of LA depends on intrinsic detoxification of each LAB strain (Ye et al., 2013). Moreover, addition of Tween 80 could reduce the toxic of LA on growth. Normally, Tween 80 could extract the fatty acid, which is adsorbed on the surface of cell bacteria. Therefore, it is used to reduce toxicification of LA and to improve solubility of LA in the medium (Hernandez-Mendoza et al., 2009; Li et al, 2011).

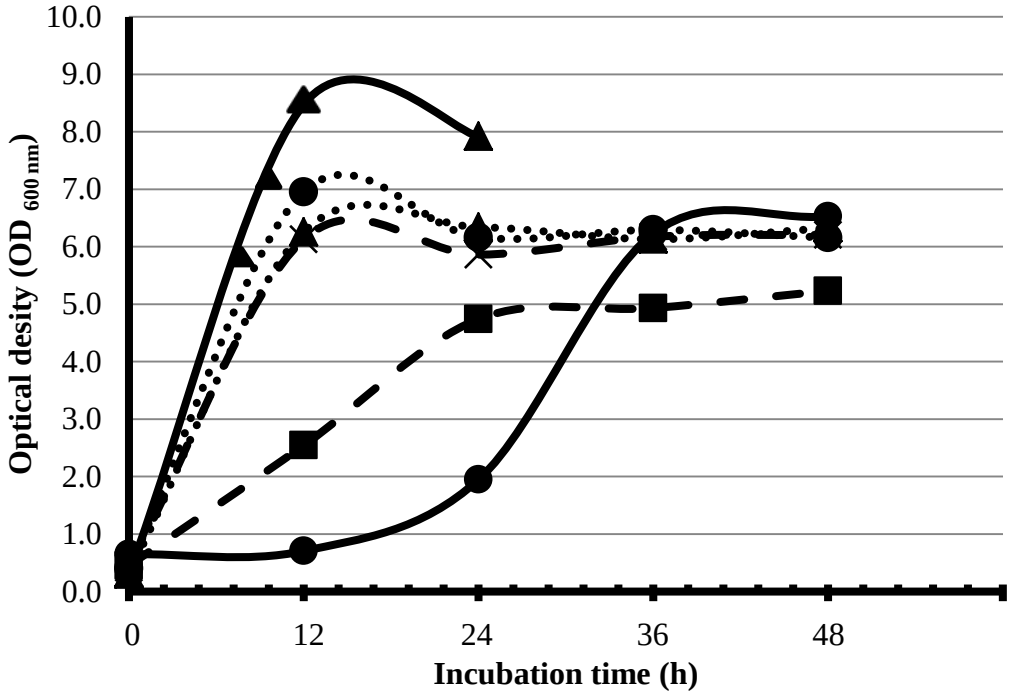


Figure 4.7 Optical density at 600 nm of *Lb. plantarum* TISTR 543 in MRS supplemented with 0.0 (—▲—), 0.10 (┈●┈), 0.20 (┈▲┈), 0.50 (--- × ---), 1.0 (--- ■---) and 2.0 (—●—) mg/mL of LA

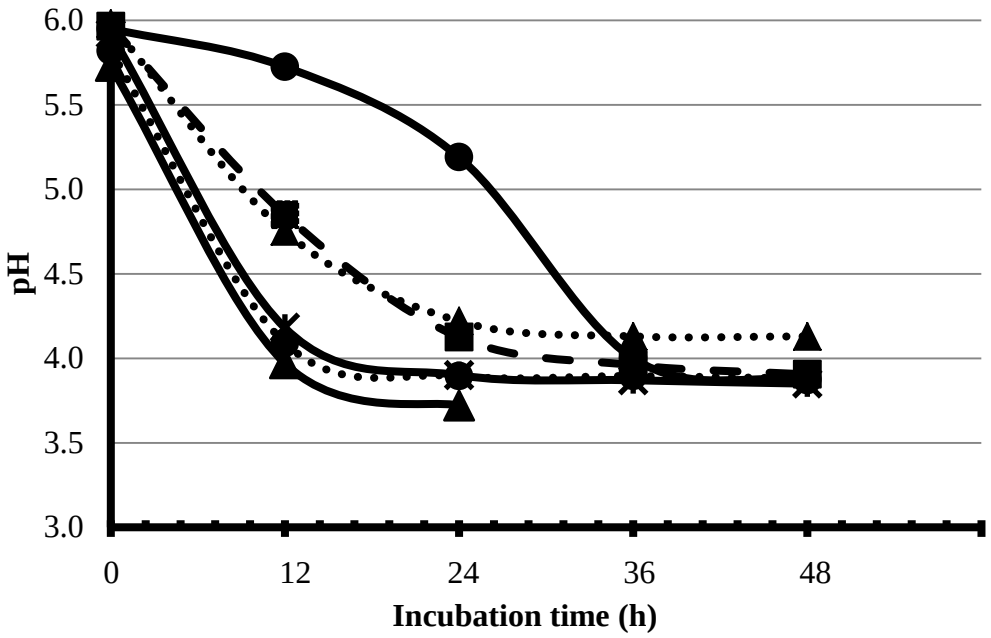


Figure 4.8 pH of the culture broth during growth of *Lb. plantarum* TISTR 543 in MRS supplemented with 0.0 (—▲—), 0.10 (┅●┅), 0.20 (┅▲┅), 0.50 (--- × ---), 1.0 (--- ■---) and 2.0 (—●—) mg/mL of LA

pH values in the medium after cultivation were found to increase when the concentration of LA increased (Figure 4.8). pH profiles of the medium supplement with 0.10 and 0.50 mg/mL were similar to the enriched medium. During the log phase, pH value was extensively decreased. Then, it was steadily when the growth reached to stationary phase. However, pH profile of the medium supplement with 0.20 mg/mL was gradually decreased when the growth reached to stationary phase. Then, it was steadily through the end of cultivation. At the medium supplement with the second group of LA concentration, pH profiles were gradually decreased during log phase. Then, they steadily reached to stationary phase (Figure 4.8). pH value is primarily related to the content of cell and stage of growth profile. In general, the content of cell is extensively increased during the log phase. High content of cell is achieved when the growth reached to steady-state phase. After that, the content of cell is steadily until the end of cultivation. Therefore, pH value is extensively decreased until the steady- state phase is reached. Then, pH value is stability through the end of cultivation.

4.4. Preparation of CLAs from sunflower oil

In this study, bioprocess for CLA production from sunflower oil was established. Enzymatic hydrolysis was performed in MRS broth supplemented with 2.0 mg/mL of sunflower oil by using CRL as a biocatalyst. Hydrolysis conditions were 750 U/g oil of CRL, reaction temperature of 40°C and reaction time of 19 h. Then, *Lb. plantarum* TISTR 543 was added to the mixture for CLA production. The productivity of CLAs was 85 mg/g oil when it was cultured in 0.10 mg/mL of LA at 37^{o}C for 48 h. This productivity was corresponded to the productivity of CLA production from other studies (Table 4.9). However, the obtained productivity of CLA production was not high enough for commercial production. Normally, yield of CLA production depends on ability of the potential LAB, optimal concentration of oil and optimal process operation (Rodríguez-Alcalá et al., 2011; Dubey et al., 2012). Thus, enzymatic oil hydrolysis should be improved using more suitable catalysts and optimization of the process is required in order to improve the productivity of CLA production.

Table 4.9 Comparison of CLA production from oil with high content of LA by The potential LAB strains

Plant Oil	Content of oil (g/L)	Type of catalyst	LAB strains	Content of CLAs (mg/g oil)	References
Sesame Oil	1.0	Microbial lipase	*Pediococcus* spp. G4S	150.0	Dubey et al., 2012
Sesame Oil	2.0	Microbial lipase	*Lc. lastic*, *Lb.acidophillus*, *E. faesium*	205.0 9.30 10.60	Abd E-Salam et al., 2010
Sunflower oil	2.50	Alkaline	*Lb. brevis*	110.0	Puniya et al., 2008
Safflower Oil	5.0	Alkaline	*Lb. acidophillus* + *Lb.plantarum*	72.30	Ye et al., 2013
Sunflower oil	2.0	CRL	*Lb. plantarum* TISTR 543	85.0	This study

CHAPTER V
Conclusion

5.1. Conclusion

This study aimed to investigate biological production of CLAs from sunflower oil. Initially, preparation of concentrated LA from sunflower oil was developed. This approach contained optimal conditions for enzymatic oil hydrolysis using a suitable lipase and the resulting fatty acids were enriched by urea complex fractionation. According to this method, degree of oil hydrolysis could be enhanced to 76% and the resulting LA could be enriched up to 70%. Therefore, the obtained LA can be directly used as a feed stock for production of CLAs as well as an ingredient for nutraceutical and cosmetic products. Subsequently, the capability of *Lb. plantarum* TISTR 543 to produce CLAs from LA was demonstrated. Finally, a bioprocess for the preparation of CLAs from sunflower oil using CRL combined with *Lb. plantarum* TISTR 543 was established. Therefore, preparation of bioactive CLAs could be used sunflower oil as a raw material with reduced cost of production.

5.2. The suggestion of this study

5.2.1. Enzymatic oil hydrolysis should be improved using more suitable catalysts.

5.2.2. The optimal conditions for biological preparation of CLAs should be examined.

5.2.3. Approach to purify the resulting CLAs should be investigated.

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Output of the research project

1. Academic Publication (the international journal)

1.1. Pongket, U., Piyatheerawong, W., Thapphasaraphong, S. and H-Kittikun, A. (Submitted). Enzymatic preparation of linoleic acid from sunflower oil: an experimental design approach. Biotechnology and Biotechnological Equipment [Impact Factor (2013) = 0.379].

2. Utilization of the research project

2.1. Concept of the research project was applied to incubate the new researcher in term of the academic achievement (Miss Urailuck Pongket, Ph.D. candidate).

3. Other outcomes of the research project

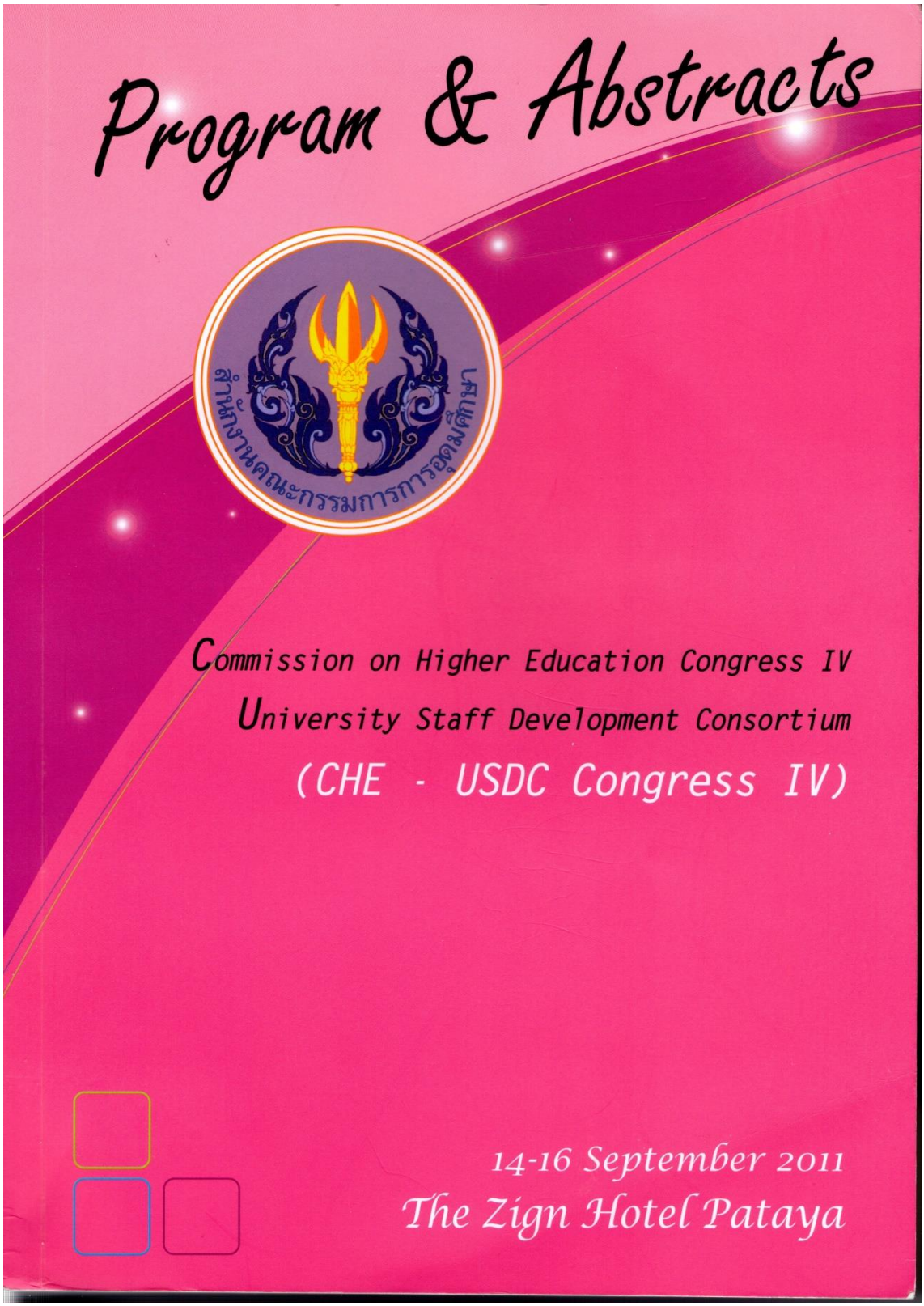
3.1. Presentation (the academic conferences)

3.1.1. Urailuck Pongket and Weera Piyatheerawong. 2011. Utilization of experimental design to enhance enzymatic hydrolysis of sunflower oil for linoleic acid production. Poster presentation at Commission for Higher Education Congress IV: University Staff Development Consortium, 14 – 16 September, 2011, The Zign Hotel Pataya, Chonburi.

3.1.2. Urailuck Pongket, Suthasinee Thapphasaraphong, Aran H-Kittikun and Weera Piyathreerawong. 2012. Enzymatic Preparation of Linoleic Acid from Sunflower Oil: An Experimental Design Approach. Poster Presentation at 13th International Biotechnology Symposium and Exhibition 16 – 21 September, 2012, Daegu, Korea.

APPENDIX

Output of the research project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The Zign Hotel Pataya

Utilizing experimental design to enhance enzymatic hydrolysis of sunflower oil for linoleic acid preparation

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Objective

Linoleic acid (LA) is an essential fatty acid and the parent fatty acids for the production of eicosanoids, prostaglandins. LA can be produced conjugated linoleic acid (CLA), which had anti-carcinogenesis properties and reducing body fat. In this study, effect of various conditions on enzymatic hydrolysis of sunflower oil was investigated by a fraction factorial design.

Methods

A two-level five-factor fractional factorial design was employed in this study. The variables and their levels selected for enzymatic hydrolysis were enzyme concentration (X_1), 200-800 U/g ; reaction temperature (X_2), 35-45 ^{o} C ; pH (X_3), 6-8 ; oil/water ratio (w/w) (X_4), 1:1 to 1:3 ; reaction time (X_5),12-36 hour. The percentage of hydrolysis was taken as the response of design experiment and was determined by titration the reaction mixture with potassium hydroxide.

Results and Discussion

The results of a fractional factorial design indicated that the percentage of hydrolysis varied from 35.43-94.46% with the different levels of variables. The highest percentage of hydrolysis (94.46 %) was obtained by using, enzyme concentration 800 (U/g), reaction temperature 45 ^{o} C,
pH 8, oil/water ratio: 1:3 (w/w) and reaction time 24 hours. The statistical analysis of variance suggested that enzyme concentration, pH, oil/water ratio and reaction time significantly affected to percentage of hydrolysis ($p < 0.05$). In addition, the interaction between enzyme concentration and reaction temperature (X_1X_2), enzyme concentration and pH (X_1X_3), reaction temperature and reaction time (X_2X_5), pH and oil/water ratio (X_3X_4), pH and reaction time (X_3X_5), oil/water ratio and reaction time (X_4X_5) were also found to significantly affect to the percentage of hydrolysis. The model was also significant ($p < 0.05$). The values of the coefficients were calculated and an equation of the linear model could be written from the coefficients:

Hydrolysis (%) = $+58.65+3.04\ X_1- 4.09\ X_3+15.05\ X_4 +4.32\ X_5+3.23\ X_1X_2 +4.15\ X_1X_3 +2.57\ X_2X_5 -1.85\ X_3X_4 +3.86\ X_3X_5+3.22\ X_4X_5$

The coefficient of determination (R^2) of the model was calculated to be 0.9935.This revealed that the model had a goodness adjustment to the experimental design and the model equation is a satisfactory to represent the correlation between the experimental results and the predict values.

Conclusion

According to the analysis of variance, the main effects of enzymatic hydrolysis were: enzyme concentration, reaction temperature, pH, oil/water ratio and reaction time. These factors were further optimized through response surface methodology.

Keywords: A fractional factorial design, enzymatic hydrolysis, Sunflower oil, Linoleic acid

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Enzymatic Preparation of Linoleic Acid from Sunflower Oil: An Experimental Design Approach

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Linoleic acid, a precursor for γ-linolenic acid and arachidonic acid, is recognized as an essential fatty acid. It widely uses in cosmetic industry and demonstrates beneficial properties such as anti-inflammatory and moisture retention. Furthermore, conjugated linoleic acids (CLAs) reveal several physiological activities, for example, anti-cancer, anti-atherogenic, anti-adipogenic, anti-diabetogenic, anti-inflammatory and reducing body fat. Industrial CLAs are mainly produced by isomerization and saponification of linoleic acid rich oil such as sunflower oil and safflower oil. In the present study, enzymatic preparation of linoleic acid was examined as a part of biological process for the production of food-grade CLAs. Initially, enzymatic hydrolysis of sunflower oil using 1,3-specific and non-specific lipases was investigated. The results indicated that using non-specific lipases as biocatalyst exhibited the higher rate of hydrolysis than the other lipases. Particularly, utilizing *Candida rugasa* lipase as biocatalyst gave the highest hydrolysis rate of 67.12%. Subsequently, optimization of enzymatic hydrolysis was studied by mean of experimental design approach. The effect and the interaction of variables for enzymatic process were examined with fractional factorial design. The variables comprised with ratio of enzyme concentration to oil, reaction temperature, initial pH, ratio of oil to water and reaction time. The results displayed that the main variables for enzymatic process were ratio of enzyme concentration to oil, initial pH, ratio of oil to water and reaction time as well as were statically significant ($p < 0.05$). Then, response surface methodology was employed in order to optimize the enzymatic process. A central composite design was used to expand the quadratic model and optimization. The predictive model was adequate due to no significant lack of fit and satisfactory level of coefficient of determination. The results indicated that the higher rate of hydrolysis was achieved as the following conditions, ratio of enzyme concentration to oil of 750 U/g, initial pH of 6.7, ratio of oil to water of 4 g/g and reaction time of 18.4 h. Under these conditions, the model predicted hydrolysis rate of 79.90%. Verification of the optimized conditions suggested that hydrolysis rate of 76.07% was observed.

keywords : Linoleic acid, Sunflower oil, Experimental design, *Candida rugosa* lipase, Response surface methodology

Enzymatic preparation of linoleic acid from sunflower oil: an experimental design approach

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Keywords:	Linoleic acid, enzymatic hydrolysis, sunflower oil, experimental design, Candida rugosa lipase, response surface methodology, central composite design

Abstract:	Enzymatic preparation of linoleic acid from sunflower oil was examined through statistical approach. Initially, enzyme screening for oil hydrolysis was investigated. The results indicated that using lipases from yeast and bacteria provided higher rates of hydrolysis than did using lipases from fungi. In particular, utilising *Candida rugasa* lipase offered the highest hydrolysis rate of 67.12%. Once the optimal lipase for oil hydrolysis was identified, an optimisation of the enzymatic process was further investigated. Response surface methodology, based on a central composite design, was employed. In this experiment, the independent variables affecting degree of hydrolysis were found to be buffer to oil and enzyme to oil ratios and initial pH ($p < 0.05$). Statistical analysis suggested that the predictive model was adequate precision due to the low p-values ($p < 0.01$) and satisfactory levels of the coefficient of determination ($R^2 = 0.89$). In addition, verification of the statistical model suggested that difference between the predicted and the actual degree of hydrolysis were less than 5%. To achieve the hydrolysis degree of 76.06%, the optimal conditions were as follow: buffer to oil ratio of 4.0 (w/w), enzyme to oil ratio of 750.28 U/g, and initial pH of 6.7. Subsequently, urea complex fractionation was performed to enrich polyunsaturated fatty acids in the enzymatic hydrolysates. The results suggested that the purity of linoleic acid could be enriched to 70% (w/w). Thus, this process could be used to effectively prepare linoleic acid as a bio-based ingredient for nutraceutical and cosmetic products.

Enzymatic preparation of linoleic acid from sunflower oil: an experimental design approach

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Introduction

Linoleic acid (LA), an essential fatty acid, is used as a precursor for biosynthesis of valuable fatty acids, such as ɤ-linolenic acid, arachidonic acid and prostaglandins.[1,2] Deficiency of LA causes numerous health abnormalities, for instance diabetic neuropathy, rheumatoid arthritis, cardiovascular diseases and autoimmune disorders.[3] LA is widely applied in nutraceutical and cosmetic products due to its skin-enhancement effects, including anti-inflammatory, acne-reducing and moisture retention.[4,5] Moreover, LA can be converted to conjugated linoleic acids (CLAs) through chemical and biological processes.[6,7] CLAs have several health benefits, such as anti-carcinogenesis, anti-atherosclerotic, anti-inflammatory and body fat reduction.[8,9]

Conventionally, industrial production of fatty acids is usually conducted via hydrolysis of triglycerides through the Colgate-Emery process and must be carried out at high temperatures and pressures (250 °C and 5,000 kPa, respectively). Then, the desired fatty acid is separated and purified. However, this procedure leads to formation of the undesirable reactions and low purity of products and high energy consumption. Alternatively, enzymatic production of fatty acids has gained considerable attention because it can be done under mild conditions. This process usually requires low energy consumption and achieves high purity of products.[10,11] Typically, lipase hydrolyses oil to fatty acids at the interphase between oil and aqueous phases. Moreover, this enzyme can catalyse various types of reactions, such as esterification and transesterification. The catalytic ability of lipase comprises several levels of specificities. For example, it is specific to the acyl group, the position and the stereoisomer of the substrate.[12-14] To concentrate the desired fatty acid, a

downstream process is usually required. Urea complex fractionation is frequently employed due to easily scale up and simple recovery and relatively cheap process. This procedure can be applied to eliminate saturated and monounsaturated fatty acids.[15,16] Enzymatic preparation of valuable fatty acids has been widely investigated.[17] For example, the concentration of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) from sardine oil using enzymatic hydrolysis and urea complex fractionation has been studied. Free and immobilised lipases from *Burkholderia cepacia* and *Pseudomonas* sp. were used to hydrolyse sardine oil. Utilising lipase from *B. cepacia* as a biocatalyst, 81.5% of EPA and 72.3% of DHA were converted to free fatty acids. Then, urea complex fractionation was employed to enhance concentrations of EPA and DHA to 46.2% (w/w) and 40.3% (w/w), respectively.[18] The production of arachidonic acid from single-cell oil through enzymatic hydrolysis and selective esterification was also demonstrated. Oil hydrolysis, using various types of lipases as biocatalysts, was investigated. Lipase from *Alcaigenes* sp. was found to be the most effective biocatalyst. The degree of hydrolysis was achieved to 98% using enzyme loading of 1,200 U/g and oil to water ratio of 1:2 (w/w) at 40 °C for 48 h. To obtain high purity of fatty acid [97% (w/w)], selective esterification was performed.[19] the preparation of α-linolenic acid from flax seed oil was also examined. Lipases from *Candida rugosa, B. cepacia, P. fluorescens* and *Rhizomucor miehei* were used to hydrolyse oil. Based on high degree of hydrolysis, lipase from *C. rugosa* was selected for preparation of the fatty acid. Conversion rate of 89.6% was obtained and purity of the fatty acid was improved to 80% (w/w) using urea complex fractionation.[20]

Traditionally, optimisation of any process requires altering the levels of one variable at a time to evaluate the process response. Therefore, large numbers of experimental trials

are typically required. Unfortunately, this approach does not describe interaction of the variables on the response and it is not assured to reach optimal conditions for the process. To overcome these limitations, response surface methodology (RSM) is generally applied. RSM is a collection of statistical and mathematical techniques useful for the development, improvement and optimisation of a process. This statistical approach is an effective method to investigate effects and interactions of the independent variables on response and optimal levels of the independent variables is usually obtained.[21] Among several experimental designs, a central composite design (CCD) is commonly used to optimise the production of valuable fatty acids.[22-24] For instance, the optimal conditions for enzymatic hydrolysis of chicken oil have been investigated. Variables affecting degree of hydrolysis and yield of fatty acids, such as temperature, enzyme loading, stirring rate and reaction time, were examined using CCD. Other than stirring rate, all examined variables were found to have significant influence on the responses ($p < 0.05$). Under optimal conditions, 93.7% of LA and 94.6% of oleic acid (OA) in oil were converted to the fatty acids. The contents of LA and OA were enhanced to 11.8% and 47% (w/w), respectively.[22] Enzymatic production of ricinoleic acid from castor oil was also demonstrated. Temperature, initial pH, enzyme concentration and buffer to oil ratio were investigated using CCD. Enzyme concentration and buffer to oil ratio were found to significantly affect content of ricinoleic acid ($p < 0.01$). Under optimised conditions, a conversion rate of 65.50% was obtained.[23] Likewise, the optimal preparation of erucic acid from mustard oil using porcine pancreas lipase as a biocatalyst was performed. Effects of temperature, initial pH, enzyme and buffer concentrations on the conversion rate were investigated using CCD. Among these variables, initial pH and buffer concentration were found to significantly affect the yield of fatty acids ($p < 0.01$). Conversion rate was improved to

95.5% under the optimised conditions.[25] In this study, enzymatic preparation of LA from sunflower oil was investigated through statistical approach. Initially, suitable types of lipases were investigated. Then, the optimal conditions for oil hydrolysis were determined. Finally, urea complex fractionation was used to enrich the desirable fatty acid.

Materials and methods

Materials

Sunflower oil was purchased from the local market (Thanakorn Vegetable Oil Product Co., Ltd, Samutprakan, Thailand). *Aspergillus niger* lipase (ANL), *B. cepacia* lipase (BCL), *C. rugosa* lipase (CRL) and *P. fluorescens* lipase (PFL) were kindly provided by the Amano Enzyme, Inc. (Nogaya, Japan). *Mucor javanicus* lipase (MJL) and *Rhizopus niveus* lipase (RNL) were purchased from Sigma-Aldrich Co., LLC. (St. Louis, MO, USA). Methyl esters of mixed fatty acids were obtained from Supelco Co. (Bellefonte, PA, USA). 14% (v/v) borontrifluoride in methanol solution was purchased from Sigma-Aldrich Co., LLC. (St. Louis, MO, USA). All solvents and reagents for experiments were analytical grade.

Enzyme screening for oil hydrolysis

Enzymatic hydrolysis of sunflower oil was performed according to the previous reports.[10,18] The reaction conditions were as follows: enzyme to oil ratio of 500 U/g, sodium phosphate buffer to oil ratio of 2:1 (w/w) and initial pH of 7.0. The reaction was conducted at 40 °C and stirred at 500 rpm under a nitrogen atmosphere. At specified time intervals, 0.50 g of samples was withdrawn from the reaction to determine degree

of hydrolysis. The reaction was terminated by adding 50 mL of solvent solution containing ethanol and acetone at the ratio at1:1 (v/v).

Optimisation of enzymatic hydrolysis

CCD was employed to evaluate the optimal conditions for enzymatic hydrolysis. The examined variables were initial pH, enzyme to oil ratio, buffer to oil ratio and reaction time. In general, the variables can be described according to the Equation (1):

$$x_i = \frac{(X_i - X_0)}{\Delta X_i} \quad (1)$$

where x_i is the coded value of the independent variable; X_i is the real value of the independent variable; X_0 is the real value of the independent variable at the centre point and $\triangle \mathbf{X_i}$ is the step change value.

Levels of the variables in CCD are shown in Table 1. The experimental design consisted of 30 treatments with a 2^4 full factorial design, 8 trials of axial points and 6 replicates of the central points. Degree of hydrolysis was selected as the response. The predictive model was expressed as a quadratic polynomial equation according to Equation (2):

$$Y = \beta_0 + \sum \beta_j X_j + \sum \beta_{jj} X_j^2 + \sum \beta_{jk} X_j X_k \quad (2)$$

where Y is the response; β_0 is the intercept; β_j, β_{jj} and β_{jk} are the linear, quadratic and interaction coefficients, respectively, and X_j and X_k are the coded values of the independent variables.

Enrichment of LA using urea complex fractionation

The fatty acids in hydrolysates were enriched through urea complex fractionation according to a previous study with modification.[18] Briefly, 15.0 g of the resulting fatty

acids was mixed with 100 mL of 25% (w/v) urea in ethanol at 60 °C with stirring until the mixture was turned into a clear homogeneous solution. The mixture was allowed to crystallise at 4°C for 10 h with stirring. The crystals were then removed by filtration. Finally, composition of total fatty acids in the crystals and filtrates were determined by gas chromatography.

Determination of the degree of hydrolysis

The degree of hydrolysis was determined by titrating of the released fatty acids against 25 mM NaOH solution. Briefly, the degree of hydrolysis is defined as the weight percent of the free fatty acids in the sample to the maximum theoretical amount according to Equation (3).[10]

$$Degree\ of\ hydrolysis\ (\%) = \frac{V_{Na} \times C_{Na} \times MW}{W_t \times f_0} \times 100 \qquad (3)$$

where V_{Na} is the required volume of NaOH solution during titration (L); C_{Na} is the molar concentration of NaOH (M); *MW* is the average molecular mass of fatty acids in sunflower oil; W_t is the weight of oil in the sample (g); and f_0 is the fraction of oil in mixture at the beginning of reaction.

Composition of fatty acids

During urea complex fractionation, composition of total fatty acids in the crystals and filtrates was analysed according to a previous report.[18] All the extracted fatty acids were methylated to fatty acid methyl esters (FAMEs) according to standard protocols.[26] Methylation of the fatty acids was performed with 14% (v/v) borontrifluoride in methanol solution at 55 °C for 5 min. FAMEs were extracted by addition of distilled water and *n*-hexane. The hexane layer was collected and analysed by a gas chromatography (Shimadzu GC 2014, Kyoto, Japan) equipped with a flame

ionisation detector. A capillary column (30 m x 0.25 µm x 0.25 µm; DB-5) was used. The injector and flame ionisation detector temperatures were 250 °C and 260 °C, respectively. The initial oven temperature was increased from 150 °C to 220 °C at 10 °C/min and held at 220 °C for 10 min. Helium was used as a carrier gas at 87.5 kPa. FAMEs were identified by comparison with standard and quantified as the weight percent.

Statistical analysis

Design Expert version 7.0.0 (Stat-Ease, Minneapolis, MN, USA) was used for statistical and graphical analysis of the experimental data. Statistical significance was evaluated using Fisher's *F*-test. Quality of the predictive model was statistically evaluated using the coefficient of determination. All experiments were carried out in triplicate and the averaged values are reported.

Results and discussion

Enzyme screening for oil hydrolysis

Several types of lipases were used to hydrolyse sunflower oil in the preparation of LA. In general, degree of hydrolysis was observed to initially increase and subsequently, remain at a constant level (Figure 1-2). When fungal lipases were used as a biocatalyst, degree of hydrolysis steadily increased within the first 6 h to a relatively stable rate at 27-43% (Figure 1). The higher conversion rate of 42.75% was obtained using MJL as a catalyst. When bacterial lipases PFL and BCL were used as a biocatalyst, the conversion rates were observed to gradually increase within the first 12 h to stable rates of 42.43% and 54.46%, respectively (Figure 2). When yeast lipase CRL was used as a biocatalyst, degree of hydrolysis rapidly increased within the first 6 h and steadily

improved to 67.12% (Figure 2). According to these results, the optimal lipase for hydrolysis of sunflower oil was found to be CRL. These results were comparable with several reports. CRL has been shown to be an effective biocatalyst with high conversion rates in the hydrolysis of oil from several sources.[20,27-29]

Typically, the catalytic activity of lipase toward triacylglycerols depends on its preference for fatty acids. This could be related to structure of the binding site. Crevice-, funnel- and tunnel-like conformations have been observed in most binding sites. Lipases containing tunnel-like binding sites prefer to hydrolyse plant oil with long-chain fatty acids more than enzymes with crevice- and funnel-like binding sites.[14] Moreover, a tunnel-like conformation is sterically suitable for OA.[12] This unique conformation was found only in CRL and *Geotrichum candidum* lipase. Lipases from *Pseudomonas* sp. and fungi, such as RML and RNL, exhibit a funnel- and crevice-like binding sites.[29] Utilisation of CRL as a biocatalyst for the preparation of polyunsaturated fatty acids (PUFAs) has been widely reported. For example, enzymatic preparation of α-linolenic acid from flax seed oil was performed using selective hydrolysis. Lipases from several sources, such as CRL, BCL, PFL and RML were examined. The results showed that CRL exhibited the highest degree of hydrolysis because it preferentially hydrolysed α-linolenic acid to a greater degree than the other lipases. The hydrolysis degree of 89.6% was obtained using enzyme loading of 300 U/g, buffer to oil ratio of 20:1 (v/w), initial pH of 7.5, temperature of 35 °C and reaction time of 2 h.[20] CRL was also found to be a suitable biocatalyst for the selective hydrolysis of soybean oil in the preparation of stearidonic acid. A reaction rate of 66.20% was obtained at 4 h of incubation time using enzyme loading of 3,218 U/g, oil to buffer ratio of 1:1.5 (w/v), initial pH of 7.5 and temperature of 40 °C.[29] Similarly, the selective

hydrolysis of salmon oil for enrichment of omega-3 PUFAs was examined. Among the tested lipases, CRL was found to be the most active biocatalyst in hydrolysis to produce the concentrated PUFAs of 27.81% (w/w). The highest hydrolysis degree of 91.89% was achieved after 24 h of incubation using oil to water ratio of 1:2 (w/v) and enzyme concentration of 40 U/g at 37 °C.[28]

Optimisation of the enzymatic hydrolysis

Once a suitable lipase for sunflower oil hydrolysis was identified, RSM was used as a tool to determine the optimal conditions for the enzymatic process. In this study, the variables supposed to affect degree of hydrolysis were initial pH, enzyme to oil ratio, buffer to oil ratio and reaction time. A CCD containing with 2^4 full factorial trials, 8 treatments of axial points and 6 replicates of centre points is displayed in Table 2. The results demonstrated that degree of hydrolysis fluctuated from 38.67 to 77.85%. The higher degree of hydrolysis (> 69%) was obtained when the independent variables were set to the centre point. Moreover, initial pH, and enzyme to oil and buffer to oil ratios appeared to be the variables that significantly affected degree of hydrolysis as evidenced by the significant differences in responses when these variables were altered (i.e., experiment number of 17-24). In addition, statistical analysis indicated that these variables significantly affected degree of hydrolysis ($p < 0.05$). The calculated *F*-values of 9.16 and the low *p*-values of the model implied that the model had a high level of confidence ($p < 0.01$). The goodness, degree of precision and reliability of the model were evaluated according to the coefficient of determination (R^2), the lack-of-fit and the coefficient of variation (CV). R^2 values of 0.8953 suggested a high correlation between the experimental and the predicted values. The non-sigificance of the lack-of-fit ($p > 0.05$) and the low values of CV (5.92%) indicated that the model was precise and

reliable (Table 3). According to Equation (4), the model consisted of three linear effects and two squared effects. The positive coefficients in each term of the equation suggested that the independent variables had a linear effects on the response.[21]

$$Y = 74.99 + 2.37\,X_1 - 1.80\,X_2 + 6.91\,X_3 - 2.81\,X_1^2 - 4.36\,X_4^2 \qquad (4)$$

where Y is the degree of hydrolysis; X_1, X_2, X_3, and X_4 are the coded values of enzyme to oil ratio, initial pH, buffer to oil ratio and reaction time, respectively.

A response surface plot was used to observe effects and interactions of the independent variables. Effects of enzyme to oil and buffer to oil ratios on degree of hydrolysis are shown in Figure 3. The influences of these two variables on the response were similar. Degree of hydrolysis improved at low levels to an approximately constant rate at higher levels. Likewise, Figure 4 shows effects of initial pH and buffer to oil ratio on degree of hydrolysis. At a low level of buffer to oil ratio, the increase in initial pH did not substantially affected degree of hydrolysis degree. By contrast, at a high level of buffer to oil ratio, the conversion rate decreased at elevated levels of initial pH. Apart from the type of biocatalyst, several reaction parameters could affect the reaction rate. In this study, the buffer to oil and enzyme to oil ratios as well as initial pH were found to be the parameters that significantly affected degree of hydrolysis ($p < 0.05$). Typically, lipase-catalysed hydrolysis would not occur because substrate could not access to the catalytic triad. To initiate activity of the enzyme, the lid has to leave the active site in order to gain substrate accessibility. Catalytic activity initially occurs after interfacial activation at the interface area of the water and oil phase.[13] In this case, contents of buffer and oil during hydrolysis could affect the interfacial dynamics between lipase and oil. At a high oil content, reduction of catalytic activity is observed due to the diminished access to the active centre and aggregation of oil droplets around the active

sites and substrate (Figure 3).[24,30] Therefore, effect of enzyme to oil ratio on the hydrolytic activity is associated with a limitation of the interfacial area between biocatalyst and oil. At a high content of enzyme loading, degree of hydrolysis does not significantly increase because the contact area is occupied by activated lipases and the accumulated intermediates (Figure 3). [22,23,25,27] In addition, at a high level of buffer to oil ratio, the conversion rate decreased with elevated levels of initial pH (Figure 4). This change could be attributed to the altered ionisation of protein structure, the ionic stage of substrate and the catalytic ability of enzyme.[23,25] These results were also reported in the previous studies. Statistical optimisation of erucic acid production from mustard oil was performed. CCD was used to optimise the enzymatic hydrolysis. Among several variables, initial pH and buffer concentration were found to significantly impact the yield of fatty acid ($p < 0.01$). Lipase denaturation and product degradation occurred at extreme pH values. However, the catalytic activity improved at a low buffer concentration. The optimal conditions improved the fatty acid yield to 43.08% (w/w).[25] The optimal conditions for lipase-catalysed hydrolysis of linseed oil were investigated. The effect of several parameters including temperature, initial pH, oil to aqueous phase ratio, enzyme loading and reaction time on degree of hydrolysis were evaluated using Box-Behken design. All variables were found to significantly affect the response ($p < 0.05$). A higher reaction temperature affected the response owing to the increased probability of substrate collision and the inactivation of enzyme. High values of oil to aqueous phase ratio and initial pH decreased the hydrolytic activity as the result of substrate inhibition and enzyme inactivation. High levels of enzyme loading did not improve the hydrolytic activity because the interfacial area was saturated with the enzyme molecules. In addition, long periods of reaction times diminished the enzyme

activity owing to the reduction of substrate concentration. The hydrolysis of linseed was enhanced to 93.93%.[30]

Model validation

Model validation was performed at the optimal conditions. The results revealed that difference between the predicted and the experimental responses was approximately 5%. To achieve hydrolysis degree of 76.07%, the optimal conditions for oil hydrolysis were at enzyme to oil ratio of 750.28 U/g, initial pH of 6.7, buffer to oil ratio of 4:1 (w/w) and reaction time of 19 h. The highest degree of hydrolysis was 96.72%, which was more than the minimum level of the response obtained from CCD.

Enrichment of LA using urea complex fractionation

Urea complex fractionation was used to concentrate LA in the hydrolysates. The fatty acid composition of the crystals and filtrates during fractionation is depicted in Table 4**.** The results demonstrated that most of the saturated fatty acids (i.e., palmitic and stearic acids) and some OA were removed from non-urea complex fraction. The LA content in the fraction was enhanced to 70.07% (w/w).

Conclusion

In the present study, enzymatic preparation of LA from sunflower oil was developed. The statistical approach was proof to be an effective tool for the optimisation of lipase-catalysed hydrolysis. The predicted model was reliable and displayed an adequate precision. Under the optimal conditions, degree of hydrolysis was enhanced to 76%. Urea complex fractionation was employed to achieve high purity of the LA product. In

this way, this bio-based LA can be directly used as a feed stock for nutraceutical and cosmetic industries.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Table 1. Levels and values of the independent variables for central composite design.

The examined variables	Symbols	Levels				
		-2	-1	0	+1	+2
Enzyme to oil ratio (U/g)	X_1	50	350	650	950	1250
Initial pH	X_2	6.0	6.5	7.0	7.5	8.0
Buffer to oil ratio (w/w)	X_3	1	2	3	4	5
Reaction time (h)	X_4	8	16	24	32	40

Table 2. Central composite design with the predicted and the experimental values of hydrolysis degree using CRL as a biocatalyst.

Experimental number	Levels of the coded variables				Degree of hydrolysis (%)	
	X_1	X_2	X_3	X_4	Predicted values	Actual values
1	-1	-1	-1	-1	56.83	59.19
2	+1	-1	-1	-1	61.89	59.90
3	-1	+1	-1	-1	56.12	60.75
4	+1	+1	-1	- 1	62.15	62.14
5	-1	-1	+1	-1	73.01	75.52
6	+1	-1	+1	-1	80.36	77.85
7	-1	+1	+1	-1	64.97	60.70
8	+1	+1	+1	-1	73.30	73.93
9	-1	-1	-1	+1	59.41	60.95
10	+1	-1	-1	+1	60.41	64.20
11	-1	+1	-1	+1	59.29	61.19
12	+1	+1	-1	+1	61.40	61.06
13	-1	-1	+1	+1	75.89	75.29
14	+1	-1	+1	+1	79.32	76.87
15	-1	+1	+1	+1	68.43	72.60
16	+1	+1	+1	+1	72.84	69.87
17	-2	0	0	0	59.01	53.68
18	+2	0	0	0	68.48	72.24
19	0	-2	0	0	75.57	75.09
20	0	+2	0	0	68.38	67.29

21	0	0	-2	0	43.76	38.67
22	0	0	+2	0	71.38	74.90
23	0	0	0	-2	72.09	72.20
24	0	0	0	+2	74.21	72.52
25	0	0	0	0	74.99	69.14
26	0	0	0	0	74.99	74.65
27	0	0	0	0	74.99	76.98
28	0	0	0	0	74.99	77.28
29	0	0	0	0	74.99	76.55
30	0	0	0	0	74.99	75.34

Table 3. Analysis of variance for the response of hydrolysis degree using CRL as a biocatalyst.

Source	Degrees of freedom	Mean Squares	*F*-values	*p*-values
Model	14	149.91	9.16	< 0.0001
X_1	1	134.26	8.20	0.0118
X_2	1	77.47	4.73	0.0460
X_3	1	1144.35	69.92	< 0.0001
X_4	1	6.72	0.41	0.5314
Lack of fit	10	19.94	2.16	0.2040
Residual	15	16.37		
Pure error	5	9.22		
Total	29			

$R^2 = 0.8953$, CV = 5.92%

Table 4. Composition of fatty acids in each fraction of urea complex fractionation.

Type of fatty acids	Oil hydrolysates (%)	Non-urea complex fraction (%)	Urea complex fraction (%)
Palmitic acid (16:0)	5.63 ± 0.57	1.10 ± 0.46	11.62 ± 1.25
Stearic acid (C18:0)	3.06 ± 0.16	0.48 ± 0.20	6.57 ± 1.62
Oleic acid (C18:1)	47.73 ± 0.25	27.91 ± 3.20	53.70 ± 2.60
Linoleic acid (C18:2)	43.48 ± 0.12	70.07 ± 3.25	27.73 ± 0.76
Others	0.61 ± 0.48	0.43 ± 0.01	0.49 ± 0.14

Figure captions

Figure 1. Time course of enzymatic hydrolysis using lipases from fungi as biocatalyst (■) ANL, (▲) MJL and (●) RNL; Hydrolysis was performed using enzyme loading of 500 U/g and buffer to oil ratio of 2:1 (w/w) at 40 °C.

Figure 2. Time course of enzymatic hydrolysis using lipases from bacteria and yeast as biocatalyst
(■) BCL and (●) PFL and (▲) CRL; Hydrolysis was performed using enzyme loading of 500 U/g and buffer to oil ratio of 2:1 (w/w) at 40 °C.

Figure 3. Three dimensional surface plot of the combined effects of enzyme to oil ratio and buffer to oil ratio on degree of hydrolysis using CRL as a biocatalyst with constant initial pH of 7.0 and reaction time of 24 h.

Figure 4. Three dimensional surface plot of the combined effects of initial pH and buffer to oil ratio on degree of hydrolysis using CRL as a biocatalyst with constant enzyme to oil ratio of 650 U/g and reaction time of 24 h.

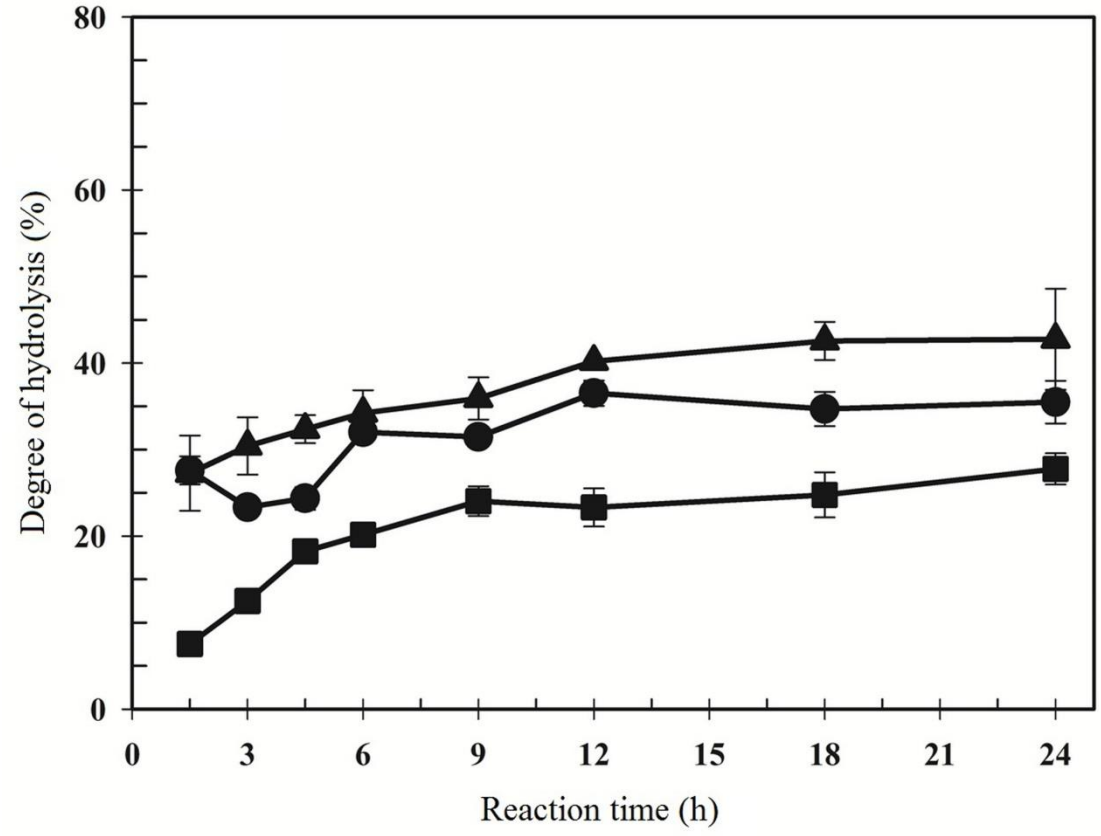
80
60
40
20
0
Degree of hydrolysis (%)
0
3
6
9
12
15
18
21
24
Reaction time (h)

Figure 1.

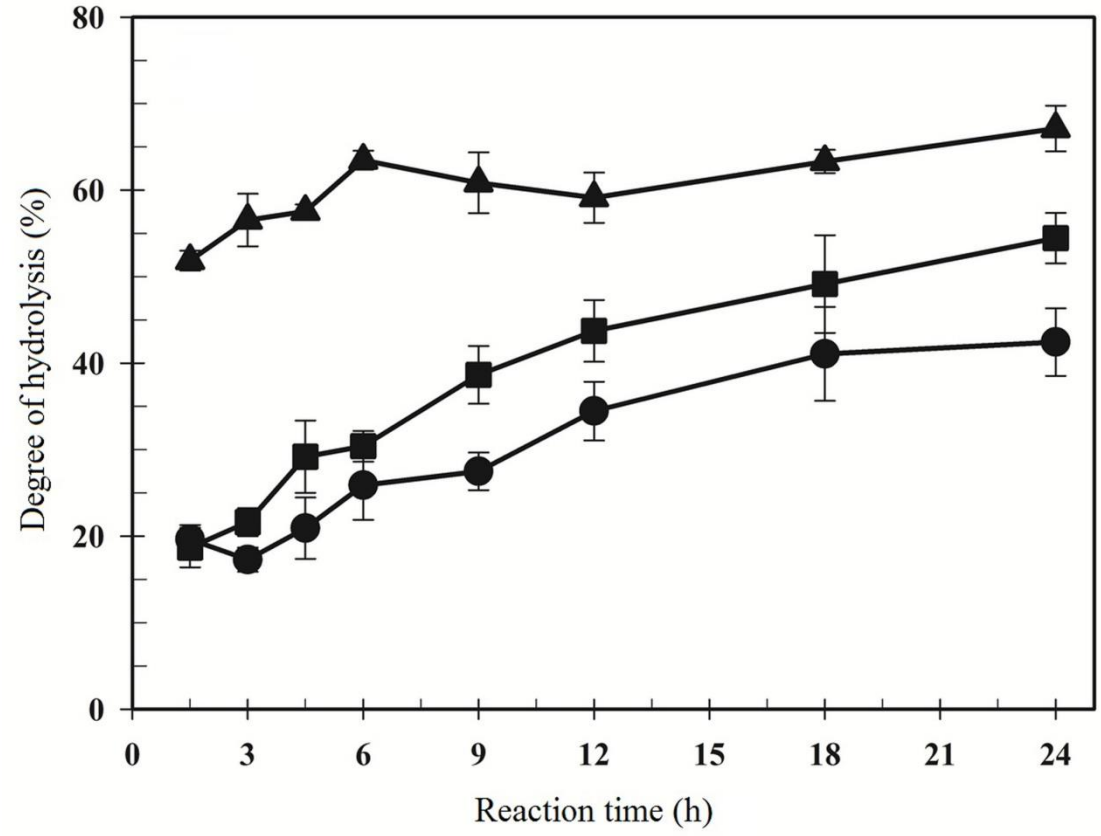
80
60
40
20
0
Degree of hydrolysis (%)
0
3
6
9
12
15
18
21
24
Reaction time (h)

Figure 2.

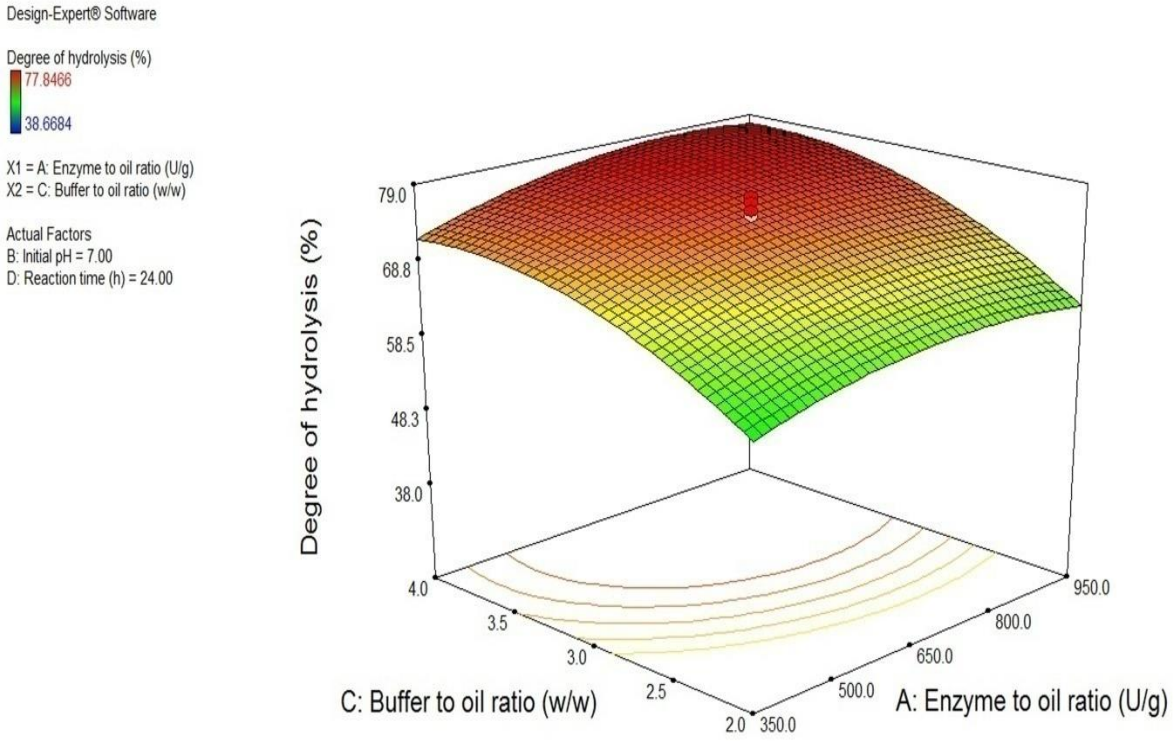
Design-Expert® Software
Degree of hydrolysis (%)
77.8466
38.6684
X1 = A: Enzyme to oil ratio (U/g)
X2 = C: Buffer to oil ratio (w/w)
Actual Factors
B: Initial pH = 7.00
D: Reaction time (h) = 24.00
Degree of hydrolysis (%)
79.0
68.8
58.5
48.3
38.0
C: Buffer to oil ratio (w/w)
4.0
3.5
3.0
2.5
2.0
A: Enzyme to oil ratio (U/g)
350.0
500.0
650.0
800.0
950.0

Figure 3.

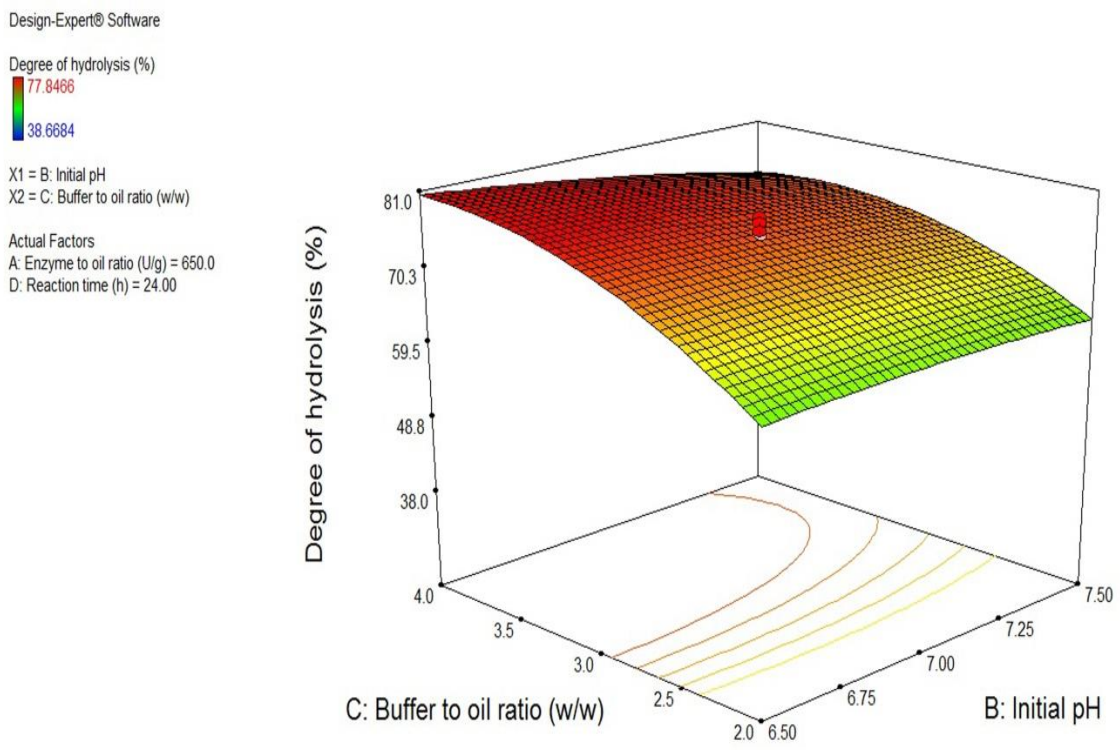
Design-Expert® Software
Degree of hydrolysis (%)
77.8466
38.6684
X1 = B: Initial pH
X2 = C: Buffer to oil ratio (w/w)
Actual Factors
A: Enzyme to oil ratio (U/g) = 650.0
D: Reaction time (h) = 24.00
Degree of hydrolysis (%)
81.0
70.3
59.5
48.8
38.0
4.0
3.5
3.0
2.5
2.0
C: Buffer to oil ratio (w/w)
6.50
6.75
7.00
7.25
7.50
B: Initial pH

Figure 4.