ENZYMATIC PRETREATMENT OF RICE BRAN ACID OIL BEFORE γ-ORYZANOL RECOVERY
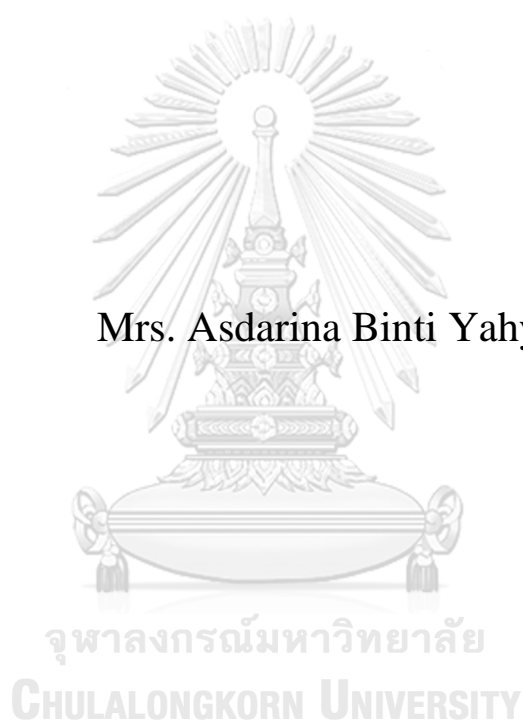
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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Engineering in Chemical Engineering
Department of Chemical Engineering
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การปรับสภาพด้วยเอนไซม์ของน้ำมันรำข้าวก่อนการกู้คืนสารแกมมาออริซานอล

นางอัสดารินา บินติ ยะยา

Thesis Title	ENZYMATIC PRETREATMENT OF RICE BRAN ACID OIL BEFORE γ-ORYZANOL RECOVERY
By	Mrs. Asdarina Binti Yahya
Field of Study	Chemical Engineering
Thesis Advisor	Professor Dr. ARTIWAN SHOTIPRUK, Ph.D.
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อัสดารินา บินติ ยะยา : การปรับสภาพด้วยเอนไซม์ของน้ำมันรำข้าวก่อนการกู้คืนสารแกมมาออริซา นอล. (ENZYMATIC PRETREATMENT OF RICE BRAN ACID OIL BEFORE γ-ORYZANOL RECOVERY) อ.ที่ปรึกษาหลัก : อาทิวรรณ โชติพฤกษ์, อ.ที่ปรึกษาร่วม : ชลเทพ อุสาคู

     งานวิจัยนี้มีวัตถุประสงค์ในการใช้ปฏิกิริยาของเอนไซม์ในการปรับสภาพน้ำมันกรดรำข้าวซึ่ง เป็นผลิตภัณฑ์ผลพลอยได้ที่สำคัญทางอุตสาหกรรมก่อนทำการกู้คืนสารแกมมาออริซานอล ซึ่งในน้ำมันกรดรำ ข้าวนี้ประกอบไปด้วยกรดไขมันอิสระ กลีเซอไรด์ และสารต้านอนุมูลอิสระออริซานอล โดยในการวิจัยจะแบ่ง ออกเป็นสองขั้นตอน ได้แก่ การย่อยด้วยเอนไซม์ และเอสเทอริฟิเคชัน/ทรานส์เอสเทอริฟิเคชัน ในขั้นตอนแรกจะ ใช้การย่อยด้วยเอนไซม์เพื่อกำจัดกลีเซอไรด์เนื่องจากเป็นสารที่มีขั้วใกล้เคียงกับแกมมาออริซานอล โดยจะ เริ่มต้นจากการประเมินสภาวะเบื้องต้นของการปฏิกิริยาโดยวิธีการทดลองทีละปัจจัย ซึ่งมีปัจจัยที่ศึกษาได้แก่ ระยะเวลาในการทำปฏิกิริยา อุณหภูมิ ปริมาณเอนไซม์ไลเปส อัตราส่วนของน้ำและน้ำมันกรดรำข้าว รวมไปถึง ความเร็วในการปั่นกวน ที่ส่งผลต่อความสามารถในการกำจัดกลีเซอไรด์และปริมาณแกมมาออริซานอลที่สูญเสีย ไป จากผลเบื้องต้นดังกล่าวนำไปสู่การศึกษาเพิ่มเติมถึงความเชื่อมโยงของปัจจัยด้านระยะเวลา อุณหภูมิและ อัตราส่วนของน้ำและน้ำมันกรดรำข้าวที่ส่งผลต่อตัวแปรตอบสนองคือความสามารถในการกำจัดกลีเซอไรด์ ปริมาณแกมมาออริซานอลที่สูญเสียไปและปริมาณของกรดไขมันอิสระที่สร้างขึ้น โดยใช้การออกแบบการ ทดลองแบบส วนประสมกลางและอาศัยแบบจำลองทางสถิติที่สามารถอธิบายความสัมพันธ์ของปัจจัยดังกล่าว ซึ่ง จะทำให้สามารถระบุตำแหน่งที่เหมาะสมที่สุดของการทดลอง ซึ่งสอดคล้องกับการกำจัดกลีเซอไรด์สูงสุดด้วย ขีดจำกัดบนที่กำหนดไว้ที่การสูญเสียออริซานอล 35% พบว่าสภาวะที่เหมาะสมจากแบบจำลองที่ระยะเวลา 22 ชั่วโมง อุณหภูมิ 48.5 °C และอัตราส่วนน้ำต่อน้ำมันกรดรำข้าว 1:1 โดยสภาวะนี้จะถูกนำมายืนยันความถูกต้อง ของแบบจำลองที่พบว่าสามารถกำจัดกลีเซอไรด์ได้เกือบสมบูรณ์ (99%) มีปริมาณการสูญเสียออริซานอลที่ต่ำ เพียง 32% และมีปริมาณกรดไขมันอิสระที่สร้างขึ้น 73-75% ในส่วนที่สองจะเริ่มต้นด้วยการประเมินสมรรถนะ ของปัจจัยต่าง ๆ ของสภาวะการทำงานด้วยวิธีการทดลองทีละปัจจัย ได้แก่ อัตราส่วนโมลของเอทานอลต่อน้ำมัน กรดรำข้าว กรดไขมันเอทิลเอสเทอร์ อุณหภูมิ ระยะเวลาของปฏิกิริยา ปริมาณเอนไซม์ และความเร็วในการปั่น กวน ที่ส่งผลปริมาณการสูญเสียออริซานอลและปริมาณกรดไขมันอิสระที่เหลืออยู่ จากการทดลองพบว่าสอง สภาวะการทดลองที่เหมาะสมที่อุณหภูมิ 40°C อัตราส่วนโมลของเอทานอลต่อน้ำมันกรดรำข้าว 3:1 และ 5:1 ปริมาณเอนไซม์ไลเปสที่ใช้ 10% ความเร็วรอบ 200 รอบต่อนาที และเวลาทำปฏิกิริยา 18 และ 24 ชั่วโมง ที่ สามารถการกำจัดกลีเซอไรด์ได้มากกว่า 98% จากนั้นปริมาณแกมมาออริซานอละถูกสกัดออกจากกรดไขมัน เอทิลเอสเทอร์โดยใช้ตัวทำละลายเอทานอลในน้ำที่มีโซเดียมไฮดรอกไซด์ที่ความเข้มข้น 2 โมลาร์ โดยพบว่า สามารถสกัดแกมมาออริซานอลได้ประมาณ 90% จากที่กล่าวไว้ในส่วนแรกว่าเป็นขั้นตอนของการกำจัดกลี เซอไรด์ออกจากน้ำมันกรดรำข้าวเพื่อสกัดออริซานอล โดยกลีเซอไรด์ที่ถูกกำจัดออกจะถูกเปลี่ยนเป็นสารที่แยก ได้ง่ายขึ้นเช่น กรดไขมันอิสระหรือกรดไขมันเอทิลเอสเทอร์ ซึ่งผลที่ศึกษานี้แสดงให้เห็นว่าการไฮโดรไลซิส ด้วยเอนไซม์และเอสเทอริฟิเคชัน/ทรานส์เอสเทอริฟิเคชันเป็นวิธีที่มีแนวโน้มในการกำจัดกลีเซอไรด์ก่อนที่จะ นำกลับแกมมาออริซานอจากน้ำมันกรดรำข้าว และแบบจำลองทางสถิติให้การคาดการณ์การตอบสนองที่แม่นยำ และจะเป็นประโยชน์สำหรับการ เป็นตัวเร่งปฏิกิริยาชีวภาพสำหรับการออกแบบกระบวนการในเชิงอุตสาหกรรมที่ เป็นมิตรต่อสิ่งแวดล้อม นอกจากนี้อาจจำเป็นต้องปรับเปลี่ยนกระบวนการเพิ่มเติมเพื่อลดปริมาณกลีเซอไรด์ ให้ มากขึ้น การสูญเสีย แกมมาออริซานอลจากทั้งสองวิธียังดีกว่าวิธีเดิมมากและน่าสนใจที่จะศึกษาต่อไป

สาขาวิชา	วิศวกรรมเคมี	ลายมือชื่อนิสิต ....
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KEYWORD: Rice bran acid oil, γ-oryzanol, Enzymatic hydrolysis, Enzymatic esterification/transesterification, Glycerides, Free fatty acids, Fatty acid ethyl ester

Asdarina Binti Yahya : ENZYMATIC PRETREATMENT OF RICE BRAN ACID OIL BEFORE γ-ORYZANOL RECOVERY. Advisor: Prof. Dr. ARTIWAN SHOTIPRUK, Ph.D. Co-advisor: Chonlatep Usaku, Ph.D.

This study aims to apply enzymatic reactions for pretreating the major byproduct of rice bran oil (RBO) industry, rice bran acid oil (RBAO) before γ-oryzanol recovery. RBAO contains free fatty acids (FFAs), glycerides, and is rich in the super-antioxidant, γ-oryzanol. As a primary step to recover γ-oryzanol from RBAO, glycerides must be removed because of having similar polarity with γ-oryzanol. This study is conducted in two parts, hydrolysis and esterification/transesterification. In the first part, enzymatic hydrolysis. The study evaluates the performance of operating conditions using one factor at a time (OFAT) on the reaction time, temperature, lipase loading, water:RBAO ratio, and speed where glycerides removal and γ-oryzanol loss are measured as responses. From these, Face-centered central composite rotatable design (FCCD) was used to investigate the effects of three independent variables: time, temperature, and water:RBAO ratio, and their interactions on the responses: glyceride removal, γ-oryzanol loss, and FFAs production, and to determine the statistical models describing their relationships. In addition, by applying the desirability function approach, the optimal location was identified, corresponding to maximal glyceride removal with an imposed upper limit of 35% γ-oryzanol loss. Lastly, the last section is the confirmation of the validity of the model and at an established optimal condition of 22 h, 48.5 °C and 1:1 water:RBAO ratio, confirmed the validity of the models: glyceride removal approached completion (99%), γ-oryzanol loss was as low as 32%, and the FFAs production was 73–75%. Then the second part is enzymatic esterification/transesterification. First is to evaluate the performance of operating conditions using OFAT of ethanol to RBAO molar ratio, temperature, time of reaction, lipase loading, and speed on the glycerides removal, Fatty acid ethyl ester (FAEE/biodiesel), γ-oryzanol loss, and FFAs remaining. Two optimal conditions at 40°C, 3:1 and 5:1 mol ratio of ethanol to RBAO,10% of lipase loading,200 rpm, and 18 and 24 h reaction time, respectively with >98% glyceride removal. Then, the extraction of γ-oryzanol from FAEE using aqueous ethanolic NaOH is conducted and resultant that around 90% of γ-oryzanol can be extracted at 2M. As a primary step to recover γ-oryzanol from RBAO, glycerides must be removed by conversion into more easily separable components such as FFAs or FAEE. The results of this study demonstrated that enzymatic hydrolysis and esterification/transesterification are promising methods for glyceride removal prior to recovery of γ-oryzanol from RBAO and that the statistical models gave accurate predictions of responses and would be useful for further industrial design of the process also is environmentally friendly biocatalysts. Further modifications in the process may be needed to reduce the content of glycerides. Moreover, the losses of γ-oryzanol from these two methods are much better than the conventional method and are interesting to explore further.

Field of Study:	Chemical Engineering	Student's Signature ....
Academic Year:	2022	Advisor's Signature ....
		Co-advisor's Signature ....

ACKNOWLEDGEMENTS

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จุฬาลงกรณ์มหาวิทยาลัย

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LIST OF ABBREVIATIONS

RBO	Rice bran oil
RBAO	Rice bran acid oil
HCL	Hydrochloric acid
H_2SO_4	Sulfuric acid
FFAs	Free fatty acid
MPa	Megapascal
°C	Degree Celsius
CCRD	Central Composite Rotatable Design
RSM	Response Surface Methodology
DOE	Design of experiments
FCCD	Face Centered Composite Design
OFAT	One factor at time
LDL	Low-density lipoproteins
HDL	High-density lipoproteins
NaOH	Sodium hydroxide
KOH	Potassium hydroxide
$CaCO_3$	Calcium Carbonate
>	More than
<	Less than
CRL	Candida Rugosa Lipase
PPL	Porcine Pancreas lipase
H_2O	Water
HPLC- ELSD	High Performance Liquid Chromatography – Evaporative Light

Scattering Detector

GC-FID	Gas Chromatography - Flame Ionization Detector
U	Unit per activity
g	gram
M	Molarity
L	Liter
μl	Microliter
I.D	Internal diameter
ANOVA	Analysis of Variance
t-test	Turkey Test
%	Percentage
hr	Hour
min	Minute
rpm	Rotation per minutes
mL	Milliliter

จุฬาลงกรณ์มหาวิทยาลัย
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CHAPTER 1

INTRODUCTION

1.1 Motivation

Thailand is one of the top rice producers in the world, accounting for ca. 19% of the worldwide rice export market value in 2019 (ca. 4.2 billion dollars) (USDA, 2019). White rice, which is the final product distributed domestically and globally, is produced from rice milling (Sowcharoensuk, 2019). During the milling process, rice bran is obtained as a byproduct. Normally, rice bran is used as animal feed, however, since rice bran contains high oil content of 15-21%wt (Zhuang, Yin, Han, & Zhang, 2019), it is used as raw material for rice bran oil (RBO) production (Sohail, Rakha, But, Iqbal, & Rashid, 2017). Rice bran is found to naturally contain γ-oryzanol (Ferreira, Fernández, Bilbao, & Fernández, 2019), an important antioxidative (Yu, Nehus, Badger, & Fang, 2007) and anti-inflammatory (Peanparkdeea & Iwamotoa, 2019) agent which has wide application in medical, pharmaceutical, food industries. It is also used for treatments of chronic diseases including diabetes (Francisqueti et al., 2017), cancer (Islam et al., 2014), hyperlipidemia (R. D. Sharma & Rukmini, 1986), liver injury (Chotimarkorn & Ushio, 2008), and heart disease (Shao & Bao, 2019). Therefore, the demand for γ-oryzanol is continuously growing, which is predicted to expand from 115,000 tons in 2014 to 320,000 tons in 2020 (GrandViewResearch, 2016).

Thailand is one of the biggest RBO producers with its RBO produciton capacity of 61,000 metric tonnes in 2018(Boonlai, 2019), which is behind only China and India. Since γ-oryzanol exists in the oil fraction of rice bran, during RBO extraction, γ-oryzanol mostly comes out with the extracted crude RBO. However, during the RBO refining, the majortiy of the γ-oryzanol is lost; up to ca. 90% of original γ-oryzanol is lost into rice bran acid oil (RBAO), a major byproduct from the neutralization step which is the acidulated rice bran oil soapstock with H_2SO_4 (A. G. G. Krishna et al., 2001; Pestana et al., 2008; Wongwaiwech, Weerawatanakorn, Tharatha, & Ho, 2019). Since RBAO contains high levels of γ-oryzanol, many studies have attempted to utilize RBAO as feedstock for γ-oryzanol production. However, the yield of γ-oryzanol recovery from RBAO is limited due to the existing impurities in RBAO. Glycerides are considered to be the major impurity in RBAO since they have similar physicochemical propeties (i.e. polarity) to the γ-oryzanol (Narayan, Barhate, & Raghavarao, 2006). Thus, they are required to be removed or at least reduced before the γ-oryzanol recovery (Joshi et al., 2016; Kaewboonnum, Vechpanich, Santiwattana, & Shotipruk, 2010; Narayan et al., 2006; Sahu, Ghosh, & Bhattacharyya, 2019; Xu & Godber, 2001)

Hydrolysis is the common method which has been employed to remove glycerides in RBAO, in which glycerides are converted into more separable FFAs and glycerol (Meedam, Usaku, Daisuk, & Shotipruk, 2020). This reaction can be conducted with/without a catalyst. Base-catalyzed hydrolysis, e.g. with either NaOH or KOH, (Carrillo, Lis, Colom, M.Lo´pez-Mesas, & Valldeperas, 2005; Meedam et al., 2020) is commonly used, which involves short reaction times and can be

performed at relatively fair conditions, i.e. atmospheric pressure and 60-100°C. Nevertheless, soap formation can be seen during the reaction. Acid-catalyzed hydrolysis, e.g. with either HCl or H_2SO_4 (Aguilar, Ramırez, Garrote, & M.Vazquez, 2002) can also be used although it provides relatively low glycerides conversion . These methods with chemical catalyst require a subsequent neutralization step (Meedam et al., 2020). Another alternative is hydrolysis at subcritical water conditions, which is considered to be a green method (Russell L. Holliday et al., 1997) since no chemical catalyst, and thus subsequent treatments for use of a chemical catalyst, is required. Despite high glycerides conversion; more than 95% glycerides conversion in RBAO (Meedam et al., 2020), this method is rather energy intensive (Holliday, King, & List, 1997). For the application of glycerides removal prior to recovery of γ-oryzanol, certain degrees of γ-oryzanol loss due to these hydrolysis methods is seen. Around 40-50% of original γ-oryzanol in RBAO was hydrolyzed by alkali hydrolysis and subcritical water hydrolysis though >90% glycerides conversion was achieved (Meedam et al., 2020). The obtained high γ-oryzanol loss in turn lowers recovery yields of the bioactive compound in the later separation and purification steps.

Enzymatic hydrolysis, in which glycerides are hydrolyzed with the aid of lipase enzymes, is a promising alternative to the physicochemical approaches for glycerides removal. It is considered as a less energy intensive, safe, and environmentally friendly method (Najafpour, 2015; Reetz, 2002), while it provides high specificity to the reaction substrates (Reetz, 2002; Ribeiro, Castro, Coelho, & Freire, 2011) and does not require a neutralization step after reaction (Gupta, 2016 ; Pourali, Asghari, & Yoshida, 2009   ). Enzymatic hydrolysis has been extensively

explored for the conversion of glycerides, mostly from vegetable oils, toward free fatty acid (FFAs) production, nevertheless studies on by enzymatic hydrolysis of glycerides in RBAO are still limited. Ghosh and Bhattacharyya (1995) demonstrated utilization of enzymatic hydrolysis of RBAO for FFAs production. However, effects on remaining contents of the γ-oryzanol were not clearly reported.

Simultaneous esterification/transesterification is another method for glycerides removal in RBAO, in which FFAs and glycerides are simultaneously converted into fatty acid alkyl esters of a short-chain alcohol, or biodiesel. With addition of an alcohol (e.g. methanol, ethanol, and butanol) (Rodrigues, Volpato, Wada, & Ayub, 2008), the reactions take place simultaneously with a typical catalyst; either an acid or a supercritical/subcritical condition. Ju and Zullaikah (2013) employed acid-catalyzed esterification/ transesterification to produce biodiesel from a byproduct from crystallization of RBO and showed that all FFAs and glycerides could be converted into biodiesel, though 36% of γ-oryzanol was lost. The study by Sombutsuwan et al. (2018) also employed acid-catalyzed esterification/transesterification to convert glycerides/FFAs in RBAO to biodiesel, and also demonstrated γ-oryzanol recovery from the obtained via extraction with an aqueous ethanolic NaOH solvent. By converting RBAO into biodiesel, it helps reduce viscosity and thus improve the extraction of γ-oryzanol, which resulted in 78% γ-oryzanol recovery. Because this extraction method can operate at room temperature with short times, it provides considerable cost savings in terms of energy consumption, operational efficiency, and solvent usage.

Enzymatic esterification/transesterification, in which conversion of glycerides/FFAs into biodiesel is catalyzed by lipase enzyme, is a promising

alternative to the physiochemical methods due to its above-discussed advantageous traits. While the majority of studies have focused on its applications for biodiesel production from glycerides/FFAs in vegetable oils, which generally report high glycerides/FFAs conversion and biodiesel yields (Atadashi, Aroua, Aziz, & Sulaiman, 2012; Encinar, Gonza´lez, Rodrı´guez, & Tejedor, 2002; Ondul, Dizge, Keskinler, & Albayrak, 2015), its application for glycerides removal in RBAO before γ-oryzanol recovery is still scare. An early study by Ghosh and Bhattacharyya (1995) showed that microbial lipase-catalyzed hydrolysis, esterification, and alcoholysis reactions were carried out on acid oils from coconut, soybean, mustard, sunflower, and rice bran for the free fatty acids production. The various monohydric alcohol esters of fatty acids of the acid oils recent study by N. Choi, Lee, Kwak, Lee, and H (2016) also applied enzymatic esterification/transesterification with immobilized lipases and ethanol to produce biodiesel from RBAO. This study showed ca. 92% yield of biodiesel were nevertheless, the reaction effects on γ-oryzanol in RBAO were not clearly reported. Therefore, further studies are still in need when this method is used as a pretreatment step toward initial glycerides removal followed by γ-oryzanol recovery.

Provided the above-described potential applications of enzymatic hydrolysis and enzymatic simultaneous esterification/transesterification for glycerides removal in RBAO before γ-oryzanol recovery, this thesis aims to study effects of reaction conditions in order to suggest optimal/suitable conditions which give highest glycerides removal while keeping γ-oryzanol loss to a minimum. The study in this thesis is divided into two parts: enzymatic hydrolysis and enzymatic esterification/transesterification. First, effects of conditions for enzymatic hydrolysis

of RBAO: reaction time, water: RBAO ratio, temperature, lipase loading and speed are investigated on degrees of glycerides conversion and γ-oryzanol loss. Three significant conditions are then selected for optimization using response surface methodology (RSM) with central composite rotatable design (CCRD) toward maximizing glycerides removal with minimum loss of γ-oryzanol. In the second part, effects of reaction conditions for enzymatic esterification/transesterification of RBAO: ethanol to RBAO molar ratio, temperature, reaction time, lipase loading and speed on glycerides removal, γ-oryzanol loss, FAEE produced and FFAs remaining are studied. The results from this research on enzymatic hydrolysis and enzymatic esterification/transesterification can be a basis for further determination of the suitable method for the recovery of γ-oryzanol from RBAO.

1.2   Research Objective:

The objective of this research are:

1.2.1   To investigate effects of reaction conditions of interest (reaction time, water:RBAO ratio. temperature, lipase loading and speed) for enzymatic hydrolysis of RBAO on glycerides removal and the losses of γ-oryzanol.

1.2.2   To investigate effects of reaction conditions of interest (ethanol to RBAO molar ratio, temperature, reaction time, lipase loading and speed) for

enzymatic esterification/transesterification of RBAO on glycerides removal, γ-oryzanol loss, FAEE produced as well as FFAs remaining.

1.3   Working Scope of Research

This research will be divided into two parts.

Part 1

1.3.1   Evaluate the effects of reaction conditions for the enzymatic hydrolysis using *Aspergillus Niger* on the degree of glycerides removal using the one-factor-at-a-time (OFAT) method: reaction time (6 to 30 hr), speed (200 – 500 rpm), RBAO to water ratio (1:1 to 1:5), temperature (30 to 60°C) and the amount of enzyme loading (5 to 15%).

1.3.2   Study the effects of reaction conditions of interest simultaneously in order to determine the optimum condition using Design of Experiment (DoE) with the face central composite rotatable design (FCCRD) method. The 3 selected conditions are used for the optimization, with 5 levels, and 3 responses, including glycerides removal, FFAs produced, and γ-oryzanol loss in hydrolyzed RBAO, are selected.

Part 2

1.3.3 Evaluate the effects of reaction conditions for the enzymatic esterification/transesterification using *Aspergillus Niger* on glycerides removal, γ-oryzanol loss, FAEE produced, and FFAs remaining using the one-

factor-at-a-time (OFAT) method: reaction time (6-24 hr), ethanol to RBAO ratio (1:1- 1:9 molar ratio), temperature (30-60°C) and the amount of lipase (5-15%).

1.3.4   Determine the amount of γ-oryzanol extraction at different molarity of acid-base solution using the method modified from Sombutsuwan et al., (2018); 1.0 to 4.0 M aqueous ethanolic NaOH

1.4   Significant of Research/Output of the Research

This research will provide information on the application of enzymatic hydrolysis and enzymatic simultaneous esterification/transesterification for glycerides removal in RBAO before recovery of γ-oryzanol. The results from this study will provide effects of reaction conditions on glycerides removal, FFAs production/ remaining, γ-oryzanol loss, with biodiesel production in the case of esterification/transesterification, which are important for determination of optimal conditions where glycerides conversion is maximized and high γ-oryzanol recovery as well as biodiesel contents could be obtained. The determined suitable reaction condition for both enzymatic hydrolysis and simultaneous esterification/ transesterification can be the basis for development of industrial process of γ-oryzanol recovery from RBAO. Compared to the other methods, the enzymatic method is greener and environmentally friendly, and when used in industries, it will help reduce the operation cost and financial planning of the company.

CHAPTER 2

BACKGROUND & LITERATURE REVIEW

2.1   Introduction

The rice bran oil (RBO) industry is a large-scale industry in a country that produces rice. In many instances, it is economically feasible to extract oil from rice bran and purify it by physical and chemical refining either for food or industrial usage. Therefore, rice bran is a desirable byproduct of the rice processing industry, which is obtained from the outer layer of the brown rice kernel during milling to produce polished rice. Figure 2.1 shows the whole structure of the rice kernel that contains 70-72% of starch endosperm, 20% of hull, 7-8% of rice bran and 2-3% of embryo (Ferreira et al., 2019)
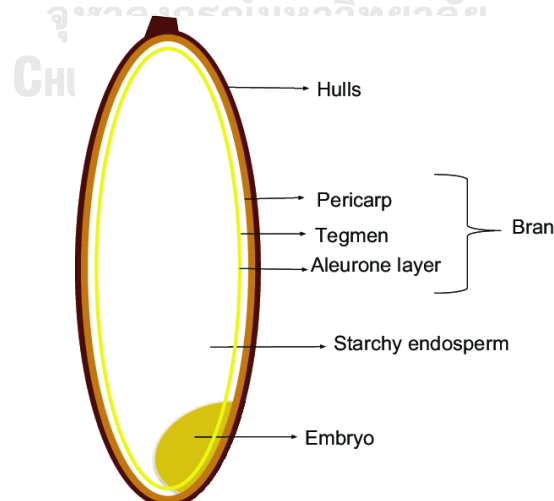
Figure 1: Structure of rice kernel

Traditionally, in many Asian countries, rice bran is largely being fed to animals and used as detergent (Wangdee & Onsaard, 2018). Overall, rice bran contains more than 15% – 20% of fat, 12% – 16% of proteins, 23% – 28% of dietary fiber, and 7% – 10% of ashes. Its composition depends on many factors like botanical diversity, agronomic environmental conditions, and processing (Yılmaz, 2016). There are some antioxidants and micronutrients in the rice bran such as γ-oryzanol, phytic acid, tocopherols, tocotrienols, thiamine, riboflavin, and niacin. Rice bran contains oil accounting for ca. 15-23 %wt., rich in lipids, and therefore, the oil extraction would be commercially feasible (Aladedunye, Przybylski, Rudzinska, & Klensporf-Pawlik, 2013). The production of RBO requires, , as shown in Figure 2, multiple processes such as degumming, neutralization, winterization, and bleaching, and generates a number of byproducts (Singanusong & Garba, 2019).

There are remaining components in RBO byproducts like γ-oryzanol, tocopherols, FFAs, and triglycerides (Narayan et al., 2006). During the caustic refining process, ca. 20-60% γ-oryzanol loss to byproduct (A. G. G. Krishna et al., 2001). Therefore, researchers are still interested in recovering these compounds. Typically, rice bran acid oil (RBAO) which has pH 5, moisture content of approximately 1.25-2.00 %, contains γ-oryzanol (8.55 ± 3.10), glycerides (4.75 ± 1.19) and FFAs (47.83 ± 12.92) on dry basis. Rice bran oil soapstock (RBOS) and RBAO which produced after the acidulation of RBOS, increase agricultural yields and reduce pollution from the industrial waste of RBO. Table 1 shows the amount of bioactive compounds remaining in some of the RBO's commercial products and the content of γ-oryzanol in the RBAO is the highest compared to other products (A. G.

G. Krishna et al., 2001; Pestana-Bauer, Zambiazi, Mendonça, Beneito-Cambra, & Ramis-Ramos, 2012; Singanusong & Garba, 2019; Wongwaiwech et al., 2019).
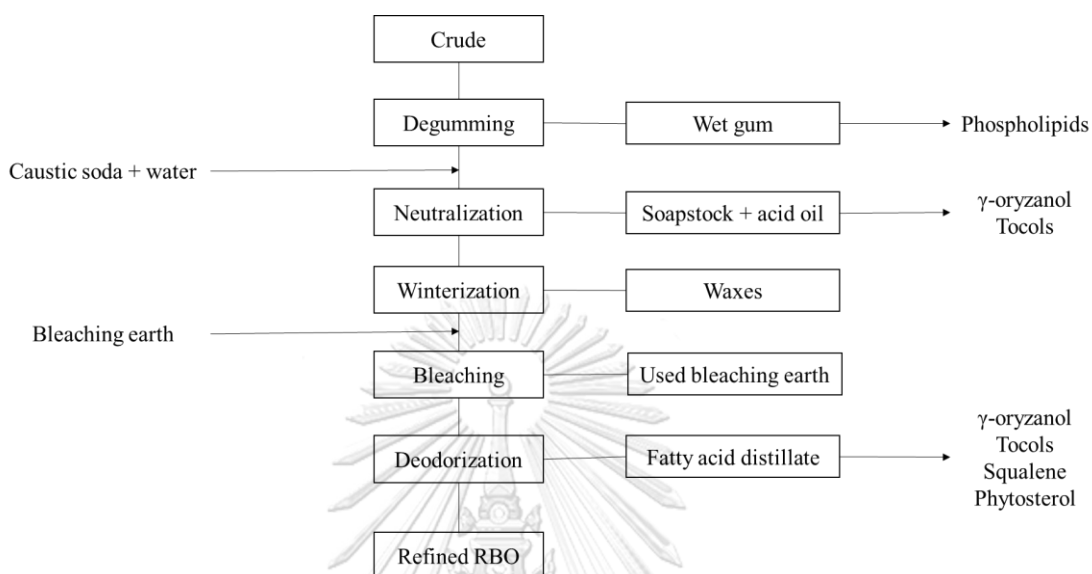
Figure 2: Production of rice bran oil

Table 1: Micronutrients contents in some commercial products of the RBO industry

Micronutrients (%)	Crude RBO	Degummed RBO	Refined RBO	Deodorizer distillate	Rice Bran Soapstock (RBOS)	Rice Bran acid oil (RBAO)
γ-oryzanol	1.7-2.1	1.71	0.19-0.20	0.79	2.21-6.9	3.28-7.36
Tocopherol	0.0264	0.0045	0.02-0.08	0.579	0.0039	0.01365
Tocotrienols	0.007		0.025-0.17	0.05		0.10693
Phytosterols	1.362 – 1.37		0.858 – 0.1037	8.5		0.5994
Squalene	0.756		0.3-0.4	3.5		
Phospholipids	4.2		1.3			

RBAO thus offers such a good opportunity for the commercial production of γ-oryzanol. The influence on the recovery of γ-oryzanol is related to the impurities in

RBAO, which is essential to design a large-scale recovery process. The major impurities which potentially complicate subsequent isolation and purification of γ-oryzanol include glycerides (Narayan et al., 2006).Glycerides have the same polarity as γ-oryzanol, rendering these bioactive compounds less pure and difficult to be recovered (Narayan et al., 2006). In order to recover a high amount of bioactive compounds, glycerides can thereafter be converted into free fatty acid (FFAs) and glycerol using the hydrolysis method (Narayan et al., 2006). FFAs can be transformed into biofuel after hydrolysis via esterification (Mannion, Furey, & Kilcawley, 2016) or as a precursor for cleaning industries.

γ-oryzanol ($C_{40}H_{58}O_4$) is the mixture of major components found in the rice grains including cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, campesteryl ferulate and sistosteryl ferulate as in Figure 3 (Ghatak & Panchal, 2011; Hakala et al., 2002). γ-oryzanol is greatly demanded worldwide thanks to its numerous health benefits and is now one of the important antioxidants (Figure 4). It can be used as an antistatic agent in reducing low-density lipoproteins (LDL), boost high-density lipoproteins (HDL) cholesterol, antioxidants (Srikaeo, 2014), and antidepression (Mizuta & Itaya, 1978). It also helps to reduce plasma cholesterols, platelet aggregations, high cholesterol diet adsorption, and aortic fatty streaks as in Figure 4 (Oryza Oil & Fat Chemical Co., 2012). Besides that, γ-oryzanol can also be an ingredient for an sunscreen agent (Sapino et al., 2013)
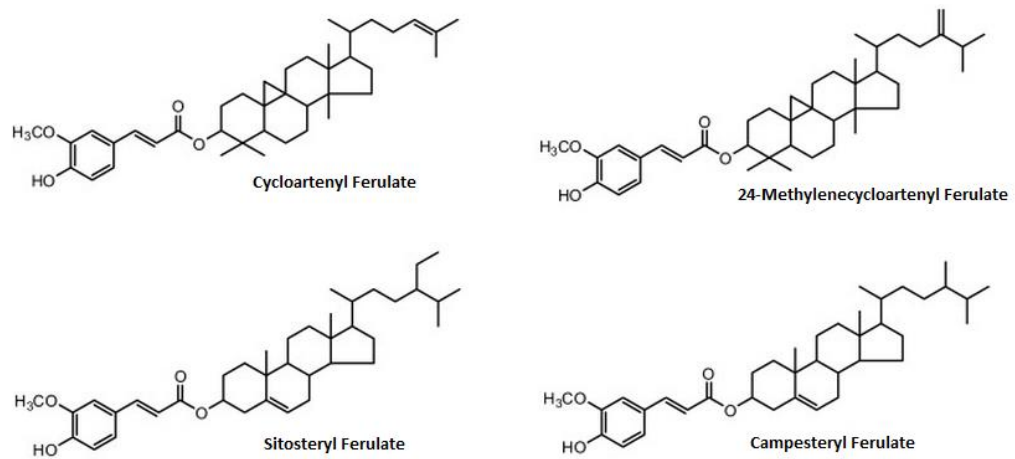
Figure 3: Chemical structure of γ-oryzanol
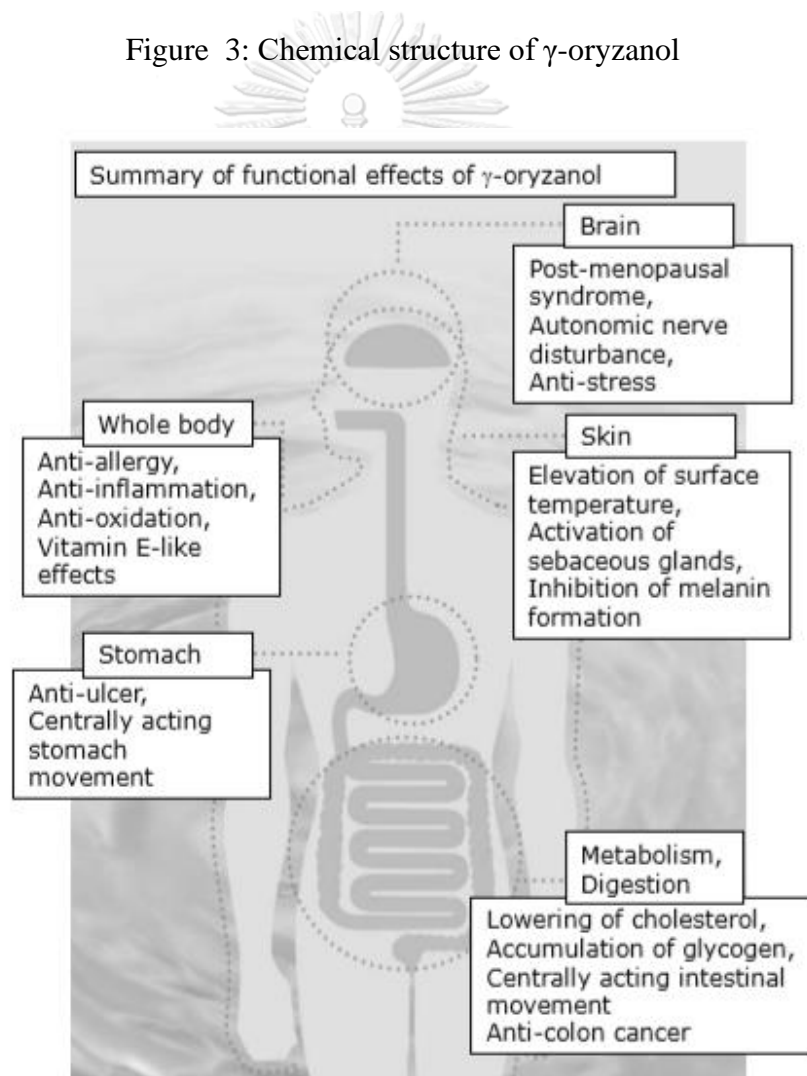
Figure 4: Benefits of γ-oryzanol

Research has been conducted for the recovery of γ-oryzanol recovery through a number of techniques. Soxhlet extraction using ethyl acetate recovers 87% of γ-oryzanol with 24% purity (Wangdee & Onsaard, 2018) and 98% of γ-oryzanol with 38% purity (Kaewboonnum et al., 2010) from RBOS. Almost 98% recovery with 84% purity of γ-oryzanol is obtained using solvent extraction of biodiesel residue from RBO using a combination of hexane and ethyl acetate (Novy S. Kasim, Chen, & Ju, 2007). Sombutsuwan et al. (2018) recovered γ-oryzanol (ca 57%) from RBAO after converting it into biodiesel using acid esterification/transesterification with ethanol. Pestana-Bauer et al. (2012) reported around 6.7% of tocopherol in deodorization distillate and almost 97% of γ-oryzanol in RBOS but no research on these bioactive compounds recovered.

2.2   Hydrolysis

More specifically, a glyceride molecule is converted during oils and fats hydrolysis either with or without a catalyst (acid/base/enzymatic) and with water into FFAs and glycerol as in Figure 5. There are four major methods commonly used for this use, including high or low pressure steam fracturing (no catalyst used), alkaline hydrolysis, acid hydrolysis, and enzymatic hydrolysis. Table 2 summarizes comparison between these type of ctalaysts (Gog, Roman, Tos, Paizs, & Irimie, 2012; Jahnavi, Prashanthi, Sravanthi, & Rao, 2017; Lerma-García, Herrero-Martínez, Simó-Alfonso, Mendonça, & Ramis-Ramos, 2009; Meedam et al., 2020; Murty, Bhat, & Muniswaran, 2002a; Tronia, Silva, Meirelles, & Ceriani, 2013)

$$\begin{array}{l}\text{CH}_2\text{OOR'} \\ | \\ \text{CHOOR''} \\ | \\ \text{CH}_2\text{OOR'''}\end{array} + \quad 3\text{H}_2\text{O} \quad \longrightarrow \quad \begin{array}{l}\text{HOR'} \\ \\ \text{HOR''} \\ \\ \text{HOR'''}\end{array} \quad + \quad \begin{array}{l}\text{CH}_2\text{OH} \\ | \\ \text{CHOH} \\ | \\ \text{CH}_2\text{OH}\end{array}$$

Triglycerides   Water   Fatty acids   Glycerol
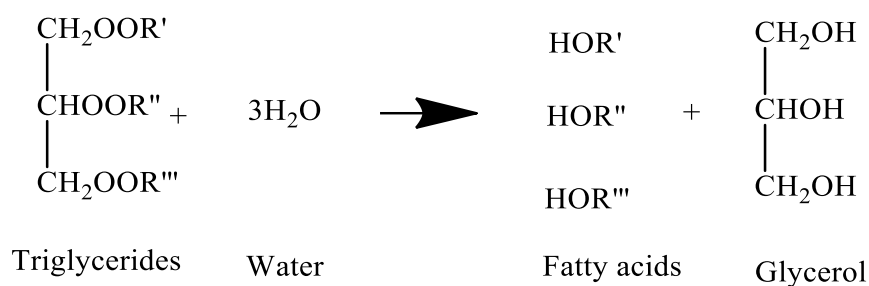
Figure 5: Hydrolysis reaction

Table 2: Comparison of types of catalyst for hydrolysis

	Free catalyst	Alkaline catalyzed	Acid-catalyzed	Enzymatic catalyzed
Type of catalyze	None	NaOH, KOH, CaCO$_3$,	H$_2$SO$_4$, HCl,	Lipase
Temperature (°C)	Subcritical (< 100) Supercritical (> 200)	60 – 70	80 - 200	30 – 60
Conversion	High conversion and could destroy other bioactive compounds	High conversion and could destroy other bioactive compounds	High conversion and could destroy other bioactive compounds	High conversion and can recover remaining high bioactive compounds
Cost of catalyst	Low	Low	Low	High
Time of hydrolysis	Short	Shorter	Medium	Longer
Waste product	Low. Wastewater treatment not needed	High. Need wastewater treatment	High. Need wastewater treatment	Low. Wastewater treatment not needed
Catalyzed recovery	Not required	Difficult, need to neutralize by acid	Difficult, need to neutralize, can destroy bioactive compounds	Easy and reusable
Energy cost	High	Medium	High	Low
Product	Fatty acids and glycerol	Salt of fatty acid or soap and glycerol	Glycerol and fatty acids	Fatty acids and glycerol

To enhance the γ-oryzanol recovery from RBAO, this hydrolysis stage is where significant quantities of γ-oryzanol are lost. Apart from glycerides, γ-oryzanol can also be hydrolyzed and can degrade due to heating environments during the hydrolysis reaction itself. Brenes, García, Dobarganes, Velasco, and Romero (2002); Nissiotis and Tasioula-Margari (2002) found that the depletion of antioxidants, including derivatives of γ-oryzanol, α-tocopherol, hydroxytyrosol, and tyrosol, is caused by thermal oxidation at 60-100°C. V. Van Hoed et al. (2006) had, while studying these bioactive compounds in the RBO during alkaline hydrolysis, found a significant loss of γ-oryzanol during the reaction. Furthermore, as soap is produced by the alkaline hydrolysis process, a further step is required to remove soap after application, followed by soap acidification. These chemical catalyzed hydrolysis methods also require a lot of excess water and thus produce wastewater, which will also increase the process cost. Meedam et al. (2020) showed that base-catalyzed hydrolysis provided complete glycerides removal in RBAO. Despite a decrease in γ-oryzanol hydrolysis when using higher amounts of NaOH for hydrolysis, only 57–66% remained in the product. Using subcritical water hydrolysis at 200°C also provided similar γ-oryzanol recovery of 51–75%.

Enzymatic hydrolysis of glycerides occurs under relatively mild conditions (low temperature and atmospheric pressure). This method requires a longer reaction time due to its slow reaction rate. This method also has issues involving lipase stability, such as how much time lipase can be recovered and used for subsequent hydrolysis before it loses its function

2.3   Lipase Enzyme

Lipases are a special category of esterase belonging to the hydrolytic enzyme (E.C.3.1.1.3) due to its affinity to carboxylic acid ester bonds, which are useful for chiral-alcohol-bearing ester resolution or symmetry (Patil & Mohapatra, 2016). The popular lipases are *Candida sp., Aspergillus sp., Rhizomucor sp., Rhizopus sp., Humicola sp., Yarrowia lipolytica and Pseudomonas sp*. which are produced by fungal, yeast, and bacterial species (Guerrand, 2017). Lipase also can act as a catalyst for the esterification/transesterification reaction in alcohol. Lipases have become a popular biocatalyst because of their innovative and multifold uses in oleochemistry, organic synthesis, detergent formulation, and nutrition. Lipases are also used for renewable energy production due to its ability to catalyze reactions including hydrolysis and esterification/transesterification. Lipases are specialized in catalyzing fat hydrolysis into FFAs and glycerol, reversing the non - aqueous media reaction (A. Sharma, Chaurasia, & Dalaic, 2013) and converting FFAs/glycerides into fatty acid alkyl ester (biodiesel) with help of alcohol (Yomi Watanabe et al., 2005), which all take place at the water lipid interface. As shown in Figure 6, lipases act as biocatalysts for alcoholysis, acidolysis, esterification, and aminolysis (Gunasekaran & Das, 2005; Ribeiro et al., 2011).

Lipases can be made available from various sources, which vary greatly in their reaction specificities. Lipase possesses the unique features of interface between an aqueous and non-aqueous phase. In theory, some of the reactions, the chemical and lipase-catalyzed reactions, however, differ widely. Like all other catalysts, enzymes are characterized by two fundamental properties. To begin with, they speed up

chemical processes without being ingested or changed in any way. To begin with, they speed up chemical processes without being ingested or changed in any way. However, it may be a downside as lipases can only function under moderate conditions, i.e. lipase is much slower than chemical catalysts. Lipase, secondly, is selective for acyl groups in substrates at certain positions/stereoisomers, which means it may speed up processes without upsetting the chemical equilibrium between reactants and products. This specificity allows the modification of oils and fats more sophisticatedly (R. Sharma, Chisti, & Banerjee, 2001)
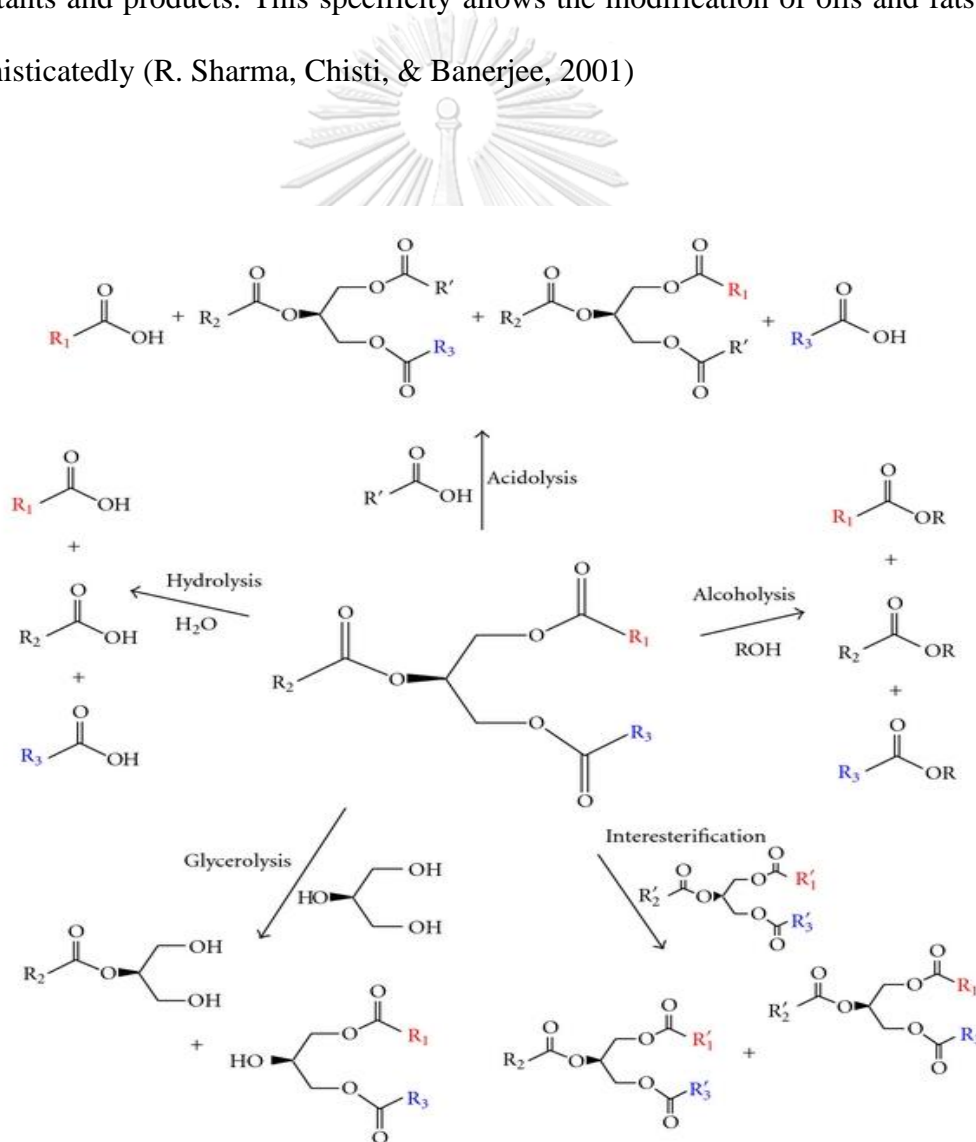
Figure 6: Reaction mechanism of lipase

2.4   Enzymatic Hydrolysis

Enzymatic hydrolysis can be performed at ambient conditions (low temperature and atmospheric pressure), making the hydrolysis process more efficient when compared to other catalyzed methods. Lipase enzymes that are specifically catalyzed would normally catalyze the reaction of the glycerides into FFAs and glycerol at the interface between oil and aqueous phases. The specificity of different lipases towards reaction substrates depends on the type of amino acid active site present in their structure. Figure 7 a)describes the general mechanism of hydrolysis reaction to convert glycerides to FFAs using an enzymatic method (A. Sharma et al., 2013) and Figure 7 b) 2-D four-step reaction scheme of action accepted for lipases catalyzing the hydrolysis of a substrate at the aqueous/lipid interface explained by Stergiou et al. (2013). A number of studies showed that lipases have a different mode of action for glycerides hydrolysis present in oil/fat depending on whether it is originated from the plant, animal or microorganism source (R. Sharma, Thakur, Sharma, & Birkeland, 2013). Cavalcanti-Oliveira, Silva, Ramos, Aranda, and Freire (2011) demonstrated hydrolysis of soybean oil at curtain levels of temperature, enzyme loading, and substrate to buffer ratio with *Lipozyme TL 100L*, and found that 85% of FFAs were obtained. Raspe, Filho, and Silva (2013) used *Lipozyme RM IM* for hydrolysis of Macauba kernel oil and investigated effect of enzyme loading and substrate to buffer ratio and showed that 60% of FFAs were produced. Table 3 summarizes results and reaction conditions from a number of studies in which enzymatic hydrolysis was performed.

a)
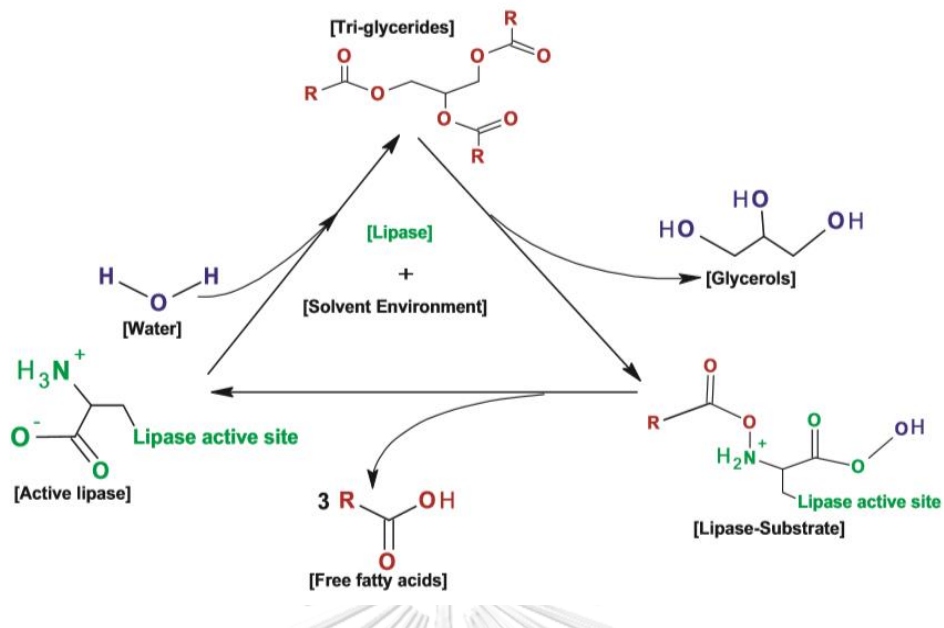
b)
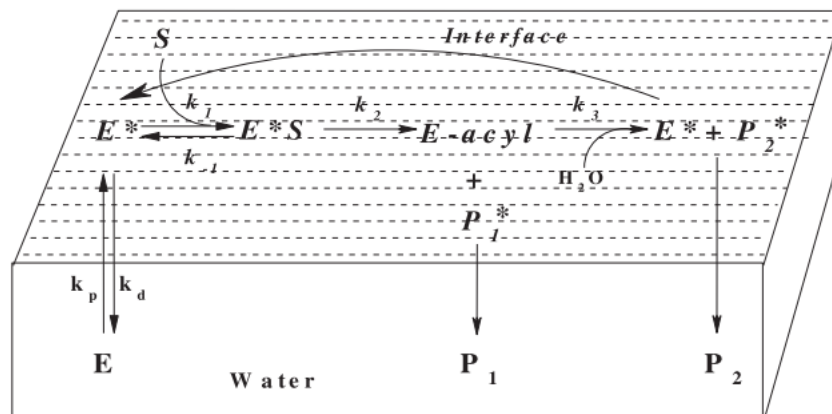
Figure 7: a) Reaction mechanism of triglycerides molecule via hydrolysis by lipase and b) 2-D four-step reaction scheme of action accepted for lipases catalyzing the hydrolysis of a substrate at the aqueous/lipid interface.

Table 3: Summarize on the reaction conditions of various types of enzymatic catalysts used in the hydrolysis reaction

Enzyme	Substrate	Buffer	Reaction condition						Result	Remarks	Reference
			Oil to buffer ratio	Temp (°C)	Time	pH	Agitation Speed	Enzyme loading (%wt substrate)	Yield (FFAs or TG conversion) (%)		
Candida Rugosa	Soybean oil Palm oil	Water	2:1 (w/w)	30	24 hr	7	200 rpm	4.5%	89%	This method is converting triglycerides to produce high amount of FFAs	(Adachi et al., 2012)
Ricinus communis Lipase	Acid oil	0.1 mol L^{-1} sodium acetate buffer	50% (v/v)	30	6 hr	4	-	2.5% (w/v)	99.6%	High efficiency with high oil concentration and without organic solvent and emulsifier	(Erika C.G. Aguieiras, Cavalcanti-Oliveira, Castro, Langonec, & Freire, 2014)
Ricinus Communis Lipase	Canola oil	Buffer acetate 100 mmol L^{-1}	22- 50 wt%	37.5	2 hr	4.5	1000 rpm	2 g	100%	Lipase from dormant castor bean seeds. Optimization of FFAs conversions	(Avelar et al., 2013)
Thermomyces lanuginosus	Soybean oil	0.1 mol L^{-1} sodium acetate buffer	50% v/v	47 – 63	19 hr	4	-	0.5-9.5%	99%	Hydrolyzed FFAs very promising biocatalyst for biodiesel synthesis	(Cavalcanti-Oliveira et al., 2011)
Jatropha curcas L.	Refined palm oil, Crude palm oil,	0.1 M Tris–HCl buffer	10% (w/v)	40	2 hr	8	200 rpm	10%	87%	Different carbon chain substrate	(Sousa, Cavalcanti-Oliveira, Aranda, &

Enzyme	Substrate	Buffer	Reaction condition						Result	Remarks	Reference
			Oil to buffer ratio	Temp (°C)	Time	pH	Agitation Speed	Enzyme loading (%wt substrate)	Yield (FFAs or TG conversion) (%)		
	Olive oil, physic nut oil, Castor oil, biodiesel waste, animal tallow										Freire, 2010)
Candida cylindracea	Sunflower oil, coconut oil, mustard oil, soybean oil, rice bran oil	Water	(60% by weight of the neutral triglycerides present)	35	48 hr	-	-	0.4%	Sunflower oil 38%, mustard oil -76.5%, Soybean oil –79%, Coconut oil -69%, Rice bran oil – 56%	Catalytic route to produce high-quality acids and esters in high yield.	(Ghosh & Bhattacharyya, 1995)
Immobilized candida cylindracea	Jatropha curcas oil	Water	1:0.5	30 - 50	24 hr	7.5	100 – 400 rpm	1 – 10%	78	Effect of agitation is studied to optimize the hydrolysis efficiency.	(Kabbashi, Mohammed, Alama, & Mirghani, 2015)
Mucor Circinelloides	Waste sardine oil	Sodium nitrate, Potassium Dihydrogen	10:2	25 - 40	Up to 72 hr	4-7	170 rpm	-	12.56% increase of FFAs at pH 5 and 35°C	Increase of FFAs content in oil when compare with crude oil	(Purwanto, Maretha, Wahyudi, &

Enzyme	Substrate	Buffer	Reaction condition						Result	Remarks	Reference
			Oil to buffer ratio	Temp (°C)	Time	pH	Agitation Speed	Enzyme loading (%wt substrate)	Yield (FFAs or TG conversion) (%)		
		Phosphate									Goeltom, 2015)
Candida sp 99-125	Waste cooking oil	Water	200 g oil: 240 g water	40	15 hr	-	-	0.3%	99.2%	Can be used to FFAs removal	(M. Wang et al., 2014)
Lipozyme TL IM, lipozyme RM IM, lecitase ultra, Aspergillus niger, Candida rugose	Palm olein	Water	40 - 50% (w/w)	30 - 80	2 – 10 hr	-	150 rpm	2 – 10 % of oil	*Lecitase ultra* (96%), *lipozyme TL IM* (77%), *lipozyme RM IM* (73%), *Aspergillus niger* (70%), *Candida rugose* (57%)	Optimization using RSM (Central Composite Design method)(Talukder, #109)	(Mardani, Farmani, & Kenari, 2015)
Y. Lipolytica	Soybean oil	Water	200 g oil: 120 ml water	40	48 hr	-	180 rpm	20,000 Uw lipase	90%	Small scale and large-scale production	(Meng et al., 2011)
Immobilized candida cylindracea	Rice bran oil	0.1M phosphate buffer	1:1	42	5 hr	7.2	-	0.25–6.0 mg/mL	69.5%	Effect of immobilization of enzyme concentration and recycle of enzyme	(Murty, Bhat, & Muniswaran, 2002b)
Candida rugose	Soy deodorized		4:6 (w/w)	45	3 hr	-	-	200 U/g	71%	SC CO_2 is used for this research. Optimization using	(Nagesha, Manohar, & Sankar,

24

Enzyme	Substrate	Buffer	Reaction condition						Result Yield (FFAs or TG conversion) (%)	Remarks	Reference
			Oil to buffer ratio	Temp (°C)	Time	pH	Agitation Speed	Enzyme loading (%wt substrate)			
	distillate									CCRD	2004)
Lipase SP398	Palm oil	0.1 M acetate buffer	25 g oil: 1 liter water	40	90 min	6	200 – 2000 rpm	25 mg /l	100%	The interface of water and oil controls the hydrolysis. Hydrolysis in bioreactor	(I.M. Noor, Hasan, & Ramachandran, 2003)
Candida rugose (CRL),Porcine pancreas lipase (PPL)	Virgin coconut oil	CRL - phosphate buffer, PPL - borate buffer	1:1 to 1:6	30 - 50	Up to 28 hr	CRL (6-8), PPL (7-9)	10000 rpm	0.5 – 2.5%	CRL -47% and PPL – 44%	Can be used to hydrolyzed virgin coconut oil	(T. A. V. Nguyen, Le, Phan, & Tran, 2018)
Rhizimuc or miehei	Palm oil	Water	1.5 -20% w/w	45 – 85	8hr		100 - 500 rpm	5- 20%	OFAT on diacylglycerol (DAG)	Investigate the influence factors on glycerides synthesis.	(Phuah, Laia, Choong, Tan, & Lo, 2012)
Pancreatic lipase	Olive oil	0.05 M sodium phosphate buffer	50% (v/v/)	40	8 hr	8-9.5	-	5g in 100 ml buffer	38% increase	Biphase enzyme membrane reactor (EMR) was used for this research	(Pugazhenthi & Kumar, 2004)
A. Oryzae	Castor oil	phosphate buffer	0.25:1 and 1:1	35	15 min to 72 hr.	7	2500 - 4000 rpm	0.34 mg/ml	70%	Hydrolysis process occurs in oil in water emulsions by optimizing the usage of enzyme	(Puthli, Rathod, & Pandit, 2006)
Lipozyme	Macauba	sodium	1:20, - 1:1	35 to	6 hr	5.7	400 to	5 to 20%	82 wt% at	To investigate the	(Raspe et

Enzyme	Substrate	Buffer	Oil to buffer ratio	Temp (°C)	Time	pH	Agitation Speed	Enzyme loading (%wt substrate)	Result Yield (FFAs or TG conversion) (%)	Remarks	Reference
RM IM, Lipozyme TL IM, and Lipozyme 435	kernel oil (Cocal Brasil)	phosphate buffer		55		to 8.0	2000 rpm		buffer to oil mass ratio of 1:2, pH 8.0, 15% of the enzyme, 400 rpm, and 6 hours	enzymatic hydrolysis of macauba oil conditions for obtaining a hydrolysate rich in FFAs.	al., 2013)
Lipolase	Castor oil	0.2 M sodium phosphate buffer	3:1	35-40	24 hr	7	-	0.2 - 1.2%	36%	Additives such as salts and isooctane harm hydrolysis	(Rathod & Pandit, 2009)
Candida Rugosa type VII	Palm oil	phosphate buffer	1:10 (w/v)	45	90 min	7.5	200 rpm	10%	92%	Kinetic model using *Michaelis-Menten* equation	(Serri, Kamarudin, & Rahaman, 2008)
Candida rugose	Waste cooking oil	Water	1: 0. to 1:3 (v/v)	30	10 hr	-	250 rpm	0.01 – 0.1 (%)	100%	An enzyme can be recycled up to 5 times. The performance of the enzyme decreases every recycles time	(Talukder, Wu, & Chua, 2010)
Candida Rugosa	Crude palm oil	0.1 M sodium phosphate buffer	1:0.25 to 1:2 (v/v)	30 – 40	4 hr	7	250 rpm	0.01–0.1%	100%	Enzyme can be recycled up to 10 cycles	(Talukder, Wu, Fen, & Melissa, 2010)
Porcine pancreas, Immobiliz	Coconut oil	0.15 M borate solution.	15-90%	30 - 60	5 hr	7.5 - 9	6000 rpm	1%	Porcine pancreas-68%	Analysis factor that influences the hydrolysis rate	(V. T. A. Nguyen, Hoa, Lam,

Enzyme	Substrate	Buffer	Reaction condition						Result Yield (FFAs or TG conversion) (%)	Remarks	Reference
			Oil to buffer ratio	Temp (°C)	Time	pH	Agitation Speed	Enzyme loading (%wt substrate)			
ed porcine pancreas									immobilized porcine pancreas - 72%		& Ai, 2016)
C. rugose type VII	Soybean oil	Water	10:1, 10:3, 10:5, 10:7, 10:9 (w/w)	40	5 hr	-	-	0.3% (v/v)	10:1 - 33.10% 10:3 - 37.71% 10:5 - 41.40% 10:7 - 45.08% 10:9 - 36.04%	Water ratio is to maintain its enzymatic activity varies depending on the water partitioning among all the components of the system.	(Ting, Huang, Giridhar, & Wu, 2008)
R. Oryzae	Triolein	0.1M phosphate buffer	10 g triolein, 12 g tert-butanol, 1.46 g methanol, and 0.3 g phosphate buffer	35	Up to 120 hr	6.8	130 rpm	-	>80 %	Two-step reaction (hydrolysis and esterification) using immobilized enzyme	(W. Li, Li, Li, Du, & Liu, 2010)
Candida rugose	Acid oil	Water	6:4 (w/w)	30	24 hr	-	500 rpm	0.2 g/mL	66%	Powder enzyme is used.	(Yomi Watanabe, Nagao, Nishida, Takagi, & Shimada,

Enzyme	Substrate	Buffer	Reaction condition							Result	Remarks	Reference
			Oil to buffer ratio	Temp (°C)	Time	pH	Agitation Speed	Enzyme loading (%wt substrate)		Yield (FFAs or TG conversion) (%)		
												2007)

2.5   Enzymatic Esterification/Transesterification/

Another promising method to remove glycerides is simultaneous esterification/transesterification in which FFAs/glycerides are converted into fatty acid alkyl ester (biodiesel). Biodiesel is a promising alternative source to petrodiesel. Industrial biodiesel production typically employs esterification/transesterification of FFAs/glycerides with a short-chain alcohol and either with or without a catalysts (acid, base, enzyme or sub/super critical conditions) (Awad, Paraschiv, Geo, & Tazerout, 2013; Rodrigues et al., 2008). Esterification/transesterification is a three-step consecutive reaction producing diglycerides and monoglycerides as intermediate compounds occurring at one time of reaction as in Figure 8. It is a reversible reaction and stoichiometry (Luković, Knežević-Jugović, & Bezbradica, 2011). Besides fatty acid alkyl ester use as a fuel, it also used as solvent for oil recovery surfactant, pesticides, asphalt rejuvenator, detergent, and cosmetics industry (Cardinali, Pizzeghello, & Zanin, 2015; Karis et al., 2022; Keng et al., 2009).
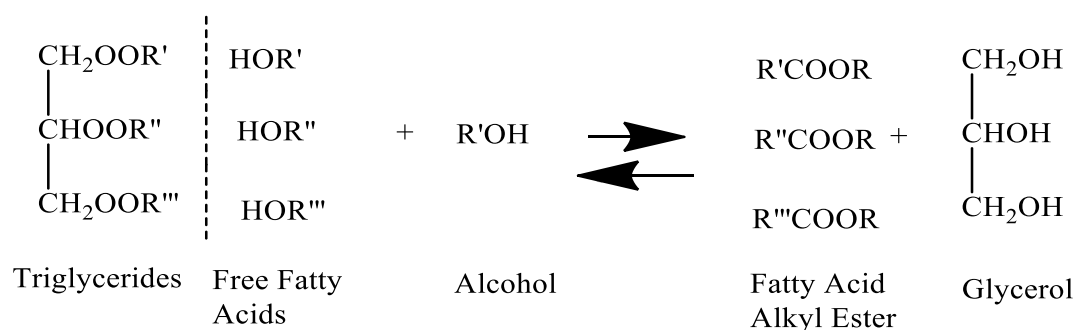
Figure 8: Esterification/transesterification reaction of triglycerides

The key benefits of using lipases as a biocatalyst include mild reaction conditions, fast recovery of glycerol without subsequent purifying and producing chemical waste, and high-purity product (Sandoval et al., 2017). Furthermore the amount of FFAs/glycerides in oils can be completely converted to biodiesel without soap formation, thereby increasing biodiesel yield and reducing fuel purification costs (Silva et al., 2019). There are also drawbacks associated with chemical-catalyzed approaches such as high energy consumption, high cost of catalyst recovery and purification as well as the need for wastewater treatment (Norjannah, Hwai Chyuan Ong, H. H Masjuki, Juan, & W.T Chong, 2016). This enzyme characteristic enables the use of high FFAs or water content products such as non-edible oils, waste cooking oils, and agricultural waste oil (Rodrigues et al., 2008).

Esterification/transesterification of FFAs/glycerides using lipases is considered one of the most efficient waste oil production methods. Clearly, the base catalyst processing process is more complicated than the enzymatic catalyst Despite multiple advantages, enzyme processes have disadvantages such as low reaction rate, industrial enzyme cost relative to alkali catalyst, low enzyme stability in the presence of excess alcohol (Bajaj, Lohan, Jha, & Mehrotra, 2010; Fjerbaek, Christensen, & Norddahl, 2009). A number of different factors influence the enzymatic biodiesel synthesis, such as the source of oil, reaction temperature, choice of acyl acceptors, acyl acceptors to oil molar ratio, amount of water in the system or water activity, amount of enzyme loading, and the presence of organic solvent in the mixture (Xiao, Li, & Pan, 2017). Nagesha et al. (2004) also esterified FFAs from hydrolyzed soy deodorizer distillate with butanol using *Mucor miehei* lipase as a biocatalyst, producing 100% biodiesel at 120bar,36°C, 3hr of reaction. Then, enzymatic biodiesel

production from plant oil hydrolysates, an *Aspergillus oryzae* whole-cell biocatalyst that expresses *Candida antarctica* lipase B (r-CALB) with high esterification activity was developed for soybean and palm oil (Adachi et al., 2012). Table 4 shows the summary of previous research regarding the reaction condition on enzymatic esterification/transesterification of biodiesel from different type of oil, enzyme, and solvent.

Table 4: Summarize of biodiesel production via enzymatic esterification/transesterification.

Enzyme	Substrate	Solvent	Reaction Condition					Result	Remarks	Reference
			Ratio (substrate : solvent)	Temp (°C)	Time	Enzyme loading (%)	Speed (rpm)	Yield		
Candida antarctica lipase B (r-CALB)	Soybean and palm oil	Methanol	1:0.5- 1:2	30 – soybean 50 – palm oil	24 hr	4.5% of oil	150	90% biodiesel produce after 6 h with the addition of 1.5 M of methanol	This research is further research after enzymatic hydrolysis to produce biodiesel	(Adachi et al., 2012)
Pseudomonas cepacia, lipase immobilized polyethyleneimine coated magnetic silica nanocomposite particles (L-PEI-MS) and lipase immobilized polyacrylic acid(PAA) coated magnetic silica (L-PAA-MS)	canola oil, sunflower, soybean and palm oil	Methanol and ethanol	1:2 – 1:5	50	24 hr	2%	200	L-PEI-MS > L-PAA-MS> free lipase FAEE> FAME	Ethanol give better performance for enzymatic transesterification of acid oils.	(Ahranjani, Kazemiini, & Arpanaei, 2020)
Novozym 435, Lipozyme RM IM, and Lipozyme TL IM	RBAO	Ethanol	1:2 - 1:6	10-60	24	2.5-15% of RBAO	300	93% conversion using Novozym 435 (1:5, 40°C, &10% enzyme), 80% conversion using Lipozyme RM IM (1:5, 30°C, &5% enzyme), &	Two step of conversion to biodiesel also evaluate and combination using Novizyme 435 with	(N. Choi et al., 2016)

Enzyme	Substrate	Solvent	Reaction Condition					Result	Remarks	Reference
								88% of removal using Lipozyme TL IM (1:5, 30°C, & 15% enzyme)	Lipizyme RM IM and Novozyme 435 with Lipizyme TL IM produce 92% of biodiesel produce.	(Chowdhury & Mitra, 2015)
Novozyme 435	Waste cooking oil	Octanol	1:1 – 1:4	40 - 60	1.5 – 4.5	1.25 - 5% of FFAs	400	95.1 % oc conversion at 60°C, 5 wt% of FFAs, 2.5:1 octanol: WCO, 3 h.	To optimize the various process variables for the production of octyl esters by applying Taguchi L9 orthogonal design	
Combi-lipase composition of 75 % Novozym 435 + 10 % Lipozyme TL-IM and 15 % Lipozyme RM-IM	Soybean oil	Ethanol	1:3 – 1:9	40	1	5, 15 to 25 %	30 - 70 duty cycle ultrasonic	Optimum condition obtained at 15 % enzyme, ethanol:oil molar ratio of 3:1; ultrasonic amplitude of 30 %, duty cycle of 50 % and time pulse of 15 sec. The combi-lipase produced 75 % biodiesel than the individual lipases (55 %) in 5 h under	This research evaluates the performance on combining lipase for biodiesel production. The reason for this is to reduce the cost of Novozym 435.	(Freitas, Matte, Rodrigues, & Ayub, 2019)
Immobilized R. oryzae NRRL 3562	Methyl Butyrate	methanol	(0.2–10M) & (0.1–10%) of water.	28-40	2–20 hr	20–120 U	100-200 rpm	70.42 % was obtained in a 200 rpm, 0.2% water, 6h, 0.6M methanol in vinyl butyrate, 80U immobilized lipase at 36°C for methyl butyrate.	To synthesize the flavor esters through immobilized lipase mediated transesterification under solvent-free conditions	(Garlapati & Banerjee, 2013)
M. miehei	coconut, soybean, mustard,	Various typeof alcohol	1:1	60	4 hr	10% of RBAO		91 % - Coconut (C_{16} alcohol), 88% - soybean (C_8 alcohol),	This is combination of enzymatic hydrolysis and	(Ghosh & Bhattacharyya, 1995)

Enzyme	Substrate	Solvent	Reaction Condition					Result	Remarks	Reference
	sunflower, and RBAO	(C$_4$ -C$_{18}$)						90% -mustard (C$_8$-C$_{18}$), 76% -sunflower(C$_{10}$-C$_{12}$), 78% -RBAO (C$_8$)	esterification in producing biodiesel from various type of acid oils	
B. cepacia	Soybean, soybean soapstock acid oil, beef tallow & waste cooking oil	Ethanol	1:3	40	48 hr	12%	200	95% conversion of Fatty acid Soybean Soapstock acid oil	The selection of Soybean oil soapstock acid oil for the packed bed column biodiesel production.	(Soares, Pinto, Gonçalves, Mitchell, & Krieger, 2013)
	Soybean soapstock acid oil		1:1	40	31hr		200	93% biodiesel produce		
Cross-linked protein coated microcrystalline (CL-PCMC), Novozyme 435, Lipolase 100T, PCMC	Palm fatty acid distillate	Ethanol	1:2- 1:16	50	Up to 25hr	20%		81% at 1:4, 6hr using CL-PCMC CL-PCMC > PCMC > Novozyme 435 > Lipolase 100T	Provides a promising approach for production of biodiesel from inexpensive feedstocks with high FFAs contents. CL-PCMC can be recycled 8 times	(Raita, Laothanachareon, Champreda, & Laosiripojan, 2011)
NS81006	Oleic acid	Ethanol	1:3.5 – 1:5.5	45	6 hr	1650 - 8260 U	500 – 1300	>85% biodiesel produce at 1,000 rpm, 4,950 U, 1:4.5,	Optimization using CCD	(Ren, Li, Du, & Liu, 2013)
Lipozyme RM-IM,	Soybean oil deodorizer distillate	Ethanol	1:1 – 1:4	45 – 78	2.5 hr	1.5-9 wt.%		60% production at 1:1, 50°C, 3% enzyme	Novozym 435 > Lipozyme RM-IM > Lipozyme TL-IM. Immobilized lipase	(Souza, Aguieiras, Silva, & Langone, 2009)

Enzyme	Substrate	Solvent	Reaction Condition					Result	Remarks	Reference
Lipozyme RM-IM, Lipozyme TL-IM, and Novozym 435			1:1	50	2.5 hr	3%		80% using Novozym 435	offered better conversions that solid acid catalyst for the same reaction time.	
Candida Sp 99-125	Safflower, soybean, linseed, corn, palm oil	Methanol	1:3	40	30 hr	15%	170	Safflower oil (91.8%), soybean oil (91%), linseed oil (90.8%), corn oil (88.5%), palm oil (88.5%)	The immobilized lipase on the membrane could be reused six times with conversion up to 90%, and the half-life of the immobilized lipase was more than 200 h.	(Tan, Nie, & Wang, 2006)
Candida Sp 99-125	Oleic acid	Ethanol	1:1	15 - 50	Up to 25 hr	Up to 18%	190	>80% 1:1, 14%, 30°C, 5 hr	Under optimum condition, it can be recycled up to 19 times produce more than 80% biodiesel	(W. N. Li, Chen, & Tan, 2011)
Immobilized Candida antarctica	Acid oil	Methanol	1:1 – 1:10	30	2 hr	20U/g	130	96% conversion at 1:5 molar ratio	Two-step enzymatic process: hydrolysis of acylglycerols by C. rugosa lipase, followed by methyl esterification of FFAs in the resulting oil layer by C. antarctica lipase.	(Yomi Watanabe, Pinsirodom, et al., 2007)

2.6   Conclusion

Based on previous research, enzymatic as a catalyst a promising method to convert glycerides to FFAs and biodiesel via both methods either hydrolysis or esterification/transesterification reaction. Different types of enzymes with different type of acid oil used, produce different results. In addition, effects of several factors of reaction conditions have been studied on the performance of lipase during hydrolysis and esterification/transesterification reaction namely pH, type of buffer/solvent used, ratio of substrate to buffer/solvent, amount of enzyme loading, and temperature as summarized in Table 3 and 4. From this, temperature is one of the most significant factors as reported by most studies since enzymes are very sensitive to heat. Also, solvent to oil ratio and enzyme loading play a role in the performance of glycerides removal. Last but not least, the suitable reaction time and speed makes the reaction more efficient. Besides that, the most important part is identifying the suitable operating condition for glycerides removal. Therefore, this research is conducted mainly to evaluate the lipase performance on glycerides removal using hydrolysis and esterification/transesterification method on the various reaction condition in order to convert the highest glycerides in the RBAO.

CHAPTER 3

ENZYMATIC HYDROLYSIS AS A GREEN ALTERNATIVE FOR GLYCERIDES REMOVAL FROM RICE BRAN ACID OIL BEFORE γ-ORYZANOL RECOVERY: STATISTICAL PROCESS OPTIMIZATION

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Abstracts

Rice bran acid oil (RBAO) is a byproduct of rice bran oil refinery, comprising of free fatty acids (FFAs), glycerides, and considerable amount of the natural antioxidant, γ-oryzanol. As a primary step to recover γ-oryzanol from RBAO, glycerides must be removed by conversion into more easily separable components such as FFAs. In this study, enzymatic hydrolysis was proposed as a green alternative to conventional base-catalyzed hydrolysis for glycerides removal from RBAO. The face-centered central composite rotatable design was used to investigate the effects of three independent variables: time, temperature, and water:RBAO ratio, and their interactions on the responses: glycerides removal, γ-oryzanol loss, and FFAs production, and to determine the statistical models describing their relationships. In addition, by applying the desirability function approach, the optimal location was identified, corresponding to maximal glycerides removal with an imposed upper limit of 35% γ-oryzanol loss. At an established optimal condition of 22 h, 48.5 °C and 1:1 water:RBAO ratio, the experiments in which the enzymatic hydrolysis was carried out at 1x and 10x reaction scales confirmed the validity of the models: glycerides removal approached completion (99%), γ-oryzanol loss was as low as 32%, and the FFAs production was 73–75%, falling within the 95% prediction interval. The results of this study demonstrated that the enzymatic hydrolysis is a promising method for glycerides removal prior to recovery of γ-oryzanol from RBAO, and that the statistical models gave accurate predictions of responses and would be useful further industrial design of the process.

3.1 Introduction

Rice bran oil (RBO) is regarded as "healthy oil" as it contains a balanced ratio of saturated (17–21.5% palmitic acid), monounsaturated (38.4–42.3% oleic acid), and polyunsaturated (33.1–37.0% linoleic acid) fatty acids at the ratio of about 0.6:1.1:1 (O.-M. Lai, Jacoby, Leong, & Lai, 2019). Another important characteristic pertaining specifically to RBO is its high level (ca. 1.8%–2.2%, (O.-M. Lai et al., 2019) of γ-oryzanol, a mixture of ferulic acid esters of triterpene alcohols and plant sterols (Ito et al., 2019) which is known to exhibit super-antioxidant, cholesterol-lowering, and anticarcinogenic properties (Hirsch et al., 2015; Minatel, Francisqueti, Corrêa, & Lima, 2016; Szcześniak, Ostaszewski, & A. Ciecierska, 2015). To obtain stabilized RBO industrially, crude RBO must first be refined by removing unwanted compounds, commonly via either a chemical or a physical refining process (Gharby, 2022). The two approaches differ significantly in the deacidification step, in which free fatty acids (FFAs) are removed. Nevertheless, the chemical refining remains the most currently adopted practice industrially, largely due to the desirable RBO qualities it affords (Vera Van Hoed, Ayala, Czarnowska, Greyt, & Verhé, 2010). During the refining, FFAs in the crude RBO are neutralized with alkali, resulting in the formation of soaps, which then phase-separate readily from glycerides or neutral oil. In this process, large amounts of glycerides and γ-oryzanol are lost as they become trapped in the rice bran oil soapstock (RBOS) byproduct. Specifically, the glycerides loss of 2.5–3 folds the content of FFAs in the crude RBO (Prasad, 2006) and the γ-oryzanol loss of up to 96% of its original content (A. G. G. Krishna et al., 2001; Pestana et al., 2008), were reported. Derived from re-acidulation of RBOS,

followed by water removal, rice bran acid oil (RBAO) is a more stable byproduct of RBO refinery, which is composed mainly of 40–80% FFAs and 20–50% glycerides (Ghosh & Bhattacharyya, 1995) and is a rich source (3.8–9%) of γ-oryzanol (Meedam et al., 2020; Wongwaiwech et al., 2019).

RBAO is normally sold as low-cost animal feed (Michael J. Haas, Michalski, Runyon, Nunez, & Scott, 2003) or utilized as a feedstock for biodiesel production (N. Choi et al., 2016) Nevertheless, owing to containing relatively high γ-oryzanol content, RBAO has drawn attention for its potential use as raw material for isolation/production of γ-oryzanol. To do so, γ-oryzanol must be separated from the main components in RBAO, namely FFAs and glycerides. Since FFAs are relatively volatile and polar compared to γ-oryzanol and glycerides, separation of FFAs from γ-oryzanol can easily be achieved through molecular distillation (Das, Chaudhuri, Kaimal, & Bhalerao, 1998; Yasuo Watanabe, Arawaka, Kitatamagun, & Iwasaki, 1969) and chromatography (Anjinta, Usaku, Boonnoun, Daisuk, & Shotipruk, 2023). The FFAs can thereafter be used for the production of valuable chemicals such as fuels (Atadashi et al., 2012) and pharmaceuticals (Hackett, Zaro, Shen, Guley, & Cho, 2013; Katdare, Thakkar, Dhepale, Khunt, & Misra, 2018). By contrast, separation of glycerides from γ-oryzanol is more challenging due to the limitations imposed by their phase equilibria. As a consequence, prior glycerides removal is a necessary step, which generally involves an initial conversion of glycerides into components that are more easily separable.

Hydrolysis reaction converts glycerides into FFAs and glycerol can be applied to break down glycerides from RBOS and RBAO, prior to γ-oryzanol recovery from these byproducts (Kaewboonnum et al., 2010; Meedam et al., 2020). The reaction can be catalyzed by strong acids (e.g. HCl and H_2SO_4), bases (e.g. NaOH and KOH), or non-catalytically, under supercritical/subcritical water conditions. Base-catalyzed hydrolysis was found to be the most suitable among the above physicochemical processes for glycerides removal from RBAO, potentially driving glycerides conversion to completion (Das et al., 1998; Jesus, Grimaldi, & Hense, 2010; Meedam et al., 2020). Nevertheless, owing to the harshness of such alkaline conditions, up to 60% of original γ-oryzanol loss is found in this process (Das et al., 1998; Kaewboonnum et al., 2010; Meedam et al., 2020). An appealing alternative for hydrolysis of glycerides involves using a lipase enzyme known to selectively bind with and cleave the ester bonds in fats and oils and has been examined for the production of FFAs from various sources of glycerides, such as macaw kernel and pulp oils, microalgal oil (Machado, Rós, Castro, & Giordani, 2021), and fish oil (A. Sharma et al., 2013). Enzymatic processes have also recently been actively investigated for deacidification of crude RBO through various reactions, such as amidation (X. Wang, Wang, & Wang, 2017) and esterification (Sun et al., 2021) which offer several advantages over the traditional chemical and physical processes including high catalytic specificity and minimal generation of toxic waste. While the enzymatic processes have generally provided high substrate conversion, findings on their effects on other valuable compounds in the feedstock, e.g. γ-oryzanol in RBO derivatives, are still limited. The enzymatic hydrolysis was previously used for the production of FFAs and fatty acid esters from RBAO (Ghosh & Bhattacharyya, 1995)

, nonetheless, its effects on γ-oryzanol remaining in the product have not been reported. Recently, (D. Li et al., 2021) showed that mild reaction conditions of enzymatic deacidification and degumming of crude RBO led to the high retention levels of γ-oryzanol (ca. 70%), while the enzymatic deacidification described in the study by (Sun et al., 2021) did not noticeably affect γ-oryzanol content. The results from these previous studies suggest high potential for the application of enzymatic hydrolysis for glycerides removal from RBAO prior to γ-oryzanol isolation which, to the best of our knowledge, has not been thoroughly and systematically studied before.

In this investigation, we sought to maximize the glycerides conversion in the original RBAO, while at the same time maintaining an acceptable level of γ-oryzanol. Response surface methodology (RSM), a powerful tool used widely to provide solutions to optimization problems (Myers, Montgomery, & Anderson-Cook, 2016; Ravn, Damstrup, & Meyer, 2012) requiring a minimum number of designed experimental runs, was employed. Specifically, a face-centered central composite rotational design (CCRD) was applied to evaluate the effects of the reaction variables and their interactions on multiple responses: glycerides removal, γ-oryzanol loss, as well as FFAs production, and to statistically model the factor-response relationships. From the models, the desirability function approach was then used to optimize the reaction conditions. The practicability of the proposed green enzymatic hydrolysis was further evaluated at a selected set of conditions by carrying out independent validation experiments. Results are then discussed and compared with those typical of conventional base-catalyzed hydrolysis.

3.2   Materials and Methods

3.2.1   Materials

RBAO (pH = 5) and RBO used in this study was kindly supplied by Thai Edible

Oil Co., Ltd. (Samutprakarn, Thailand). The composition of RBAO as summarized in

Table 5 was determined by HPLC (Section 3.2.3). The lipase used herein was of

industrial grade (SinoBios Imp. & Exp. Co., Ltd., Shanghai, China) and originated

from Aspergillus Niger. Its bioactivity is stated as 10,000 U/g as per the

manufacturers' instructions (1 U is defined as 1 μmol FFAs per min being liberated

from triglycerides using 1 g of lipase at pH 7.5 and 40 °C)

Table 5: Composition of RBAO

Components	Amount (%wt)
FFAs	40
Glycerides	23
γ-oryzanol	9
Others	28

3.2.2   Enzymatic Hydrolysis of RBAO

Enzymatic hydrolysis was performed by modifying the method described in

Kabbashi et al. (2015). For each experimental run, RBAO (2 g) was mixed with water

at a specified water:RBAO weight ratio (g/g) in a 50 mL Erlenmeyer flask. Into this

mixture, lipase was added at a specified percentage by weight (%wt) of RBAO. The

mixture was then heated to a specified temperature (°C) in a water bath with magnetic bar stirring at a specified speed (rpm). After a specified reaction time, the hydrolyzed RBAO was separated from the aqueous/lipase phase by centrifugation (4500 rpm and 10 min). The hydrolyzed sample was stored at 4 °C until analyzed for composition.

3.3.3   Quantification of Glycerides, γ-oryzanol, and FFAs

The amounts of glycerides, γ-oryzanol, and FFAs in the starting RBAO and the hydrolyzed RBAO samples were quantified using HPLC. The HPLC apparatus consisted of a separation module (Waters, USA) with a ELSD detector (Sedere, France). Prior to analysis, each hydrolyzed sample (20 μL) was dissolved in ethyl acetate (3 mL) and filtered with a 0.45 μm syringe filter. The filtered sample (5 μL) was then injected onto a 4.6 mm × 100 mm Sunfire® C18 column (Waters, USA) maintained at room temperature, and eluted with a mobile phase (60:40 v/v methanol:isopropanol) at 0.5 mL/min. Levels of γ-oryzanol, glycerides, and FFAs were determined based on the calibration curves constructed using a γ-oryzanol standard, and RBO and oleic acid standards, respectively. The extent of glycerides removal, γ-oryzanol loss, and FFAs production were defined as follows (Eqs. (1)-(3)):

Glycerides removal (%) =

$$\frac{\text{Initial amount of glycerides - Amount of remaining glycerides after hydrolysis}}{\text{Initial amount of glycerides}} \times 100 \qquad (1)$$

γ-oryzanol loss (%) =

$$\frac{\text{Initial amount of γ-oryzanol - Amount of remaining γ-oryzanol after hydrolysis}}{\text{Initial amount of γ-oryzanol}} \times 100 \qquad (2)$$

FFAs production (%) =

$$\frac{\text{Amount of FFAs present after hydrolysis - Initial amount of FFAs}}{\text{Initial amount of FFAs}} \times 100 \qquad (3)$$

3.2.4 Experimental Design and Statistical Model

A face-centered CCRD was applied to optimize the enzymatic hydrolysis conditions on three responses: glycerides removal (Y_1), γ-oryzanol loss (Y_2), and FFAs production (Y_3). As a preliminary study, one-factor-at-a-time (OFAT) experiments were carried out, and the significance of the effects of five variables (hydrolysis time, temperature, water:RBAO ratio, percentage of lipase loading, and speed) was evaluated on glycerides removal and γ-oryzanol loss. Of these variables, the three displaying the most significant effects (the lowest p-values) were selected for the CCRD experiments. The total number of the CCRD experiments was 19, consisting of 8 factorial points, 6 axial points, and 5 replicates at the center point. All experiments were carried out in duplicate and in a randomized order to minimize the effects of any unexplained sequence variabilities or those caused by extrinsic factors. The relationship between the variables and each response was then modeled by the following quadratic regression equation (Eq. (4)):

$$Y = \beta 0 + \sum_{i=1}^{3} \beta i X i + \sum_{i=1}^{3} \beta i i X i 2 + \sum_{i-1}^{1} \sum_{j=i\_1}^{3} \beta i j X i X j \qquad (4)$$

In which β0 is a constant, βi are the linear coefficients, βii are the quadratic coefficients, and βij are the interaction regression coefficients. Xi and Xj are the dimensionless coded values of the ith and jth independent variables which were estimated according to Eq. (5):

$$X_i = \frac{x_i - x_{i,0}}{\Delta x_i} \tag{5}$$

where x_i is the actual value, $x_{i,0}$ is the actual value at the center of the domain, and Δx_i is the step change value. Based on the coded quadratic model equation, analysis of variance (ANOVA) was employed to calculate the statistical parameters including the model lack of fit, the coefficient of determination (R^2), the adjusted R^2, the predicted R^2, and the adequate precision, from which the goodness-of-fit and the predictability of the statistical models were evaluated. For further statistical analysis of the experimental data, the response surface plots were constructed, and the statistical significances of the variable effects were determined by a two-tailed Student's t-test. In addition, to improve the goodness-of-fit and the predictability, model reduction was performed through a backward stepwise selection to identify only relevant predictors of the responses. Both the experimental design and the statistical model analysis were conducted through the Design-Expert software (version 11.1.0.1, Stat-Ease, Inc., Minneapolis, MN, USA).

3.2.5   Optimization of Hydrolysis Conditions

To obtain the locations for optimal conditions for the enzymatic hydrolysis of RBAO, the desirability analysis was carried out using the Design-Expert software, in which the individual desirability function ($d_i(Y_i)$), whose values vary between 0 and 1.00 (the lowest to the highest desirability), was assigned to each individual response i (Y_i). Since the main purpose of glycerides hydrolysis was to maximize the glycerides removal (Y_1), while maintaining the γ-oryzanol amount in the hydrolyzed RBAO at an acceptable level, we decided to set the upper limit of γ-oryzanol loss (Y_2) as 35% based on the best obtainable value from base-catalyzed hydrolysis previously determined at the most suitable condition (Meedam et al., 2020). . The individual desirability functions for d_1 (Y_1) and d_2 (Y_2) were therefore defined according to Eqs. (6)-(7): Individual desirability of Y_1 :

$$d_1(Y_1) = \begin{cases} 0 & \text{if } Y_1(X_i) < 0 \\ \frac{Y_1(X_i)}{100} & \text{if } 0 \leq Y_1(X_i) \leq 100 \\ 1 & \text{if } Y_1(X_i) > 100 \end{cases} \tag{6}$$

Individual desirability of Y2 :

$$d_2(Y_2) = \begin{cases} 0 & \text{if } Y_2(X_i) > 35 \\ 1 & \text{if } Y_2(X_i) \leq 35 \end{cases} \tag{7}$$

Since FFAs produced by the reaction can be easily separated, their production is taken to have no detrimental effect on the subsequent γ-oryzanol recovery step. As a result,

d_3 (Y_3) was set equal to 1.00 since there was no constraint for the percentage of FFAs production (Y_3).

The optimal location was therefore identified as the region of enzymatic hydrolysis conditions giving the highest overall desirability, D, defined as the geometric mean of the individual desirability functions according to Eq. (8).

Overall desirability (D):

$$D = \left(d_1(Y_1) \cdot d_2(Y_2) \cdot d_3(Y_3) \right)^{\frac{1}{3}} \hspace{3cm} (8)$$

3.2.6   Model Validation

To validate the model, two sets of independent hydrolysis experiments were carried out at different scales, both under the conditions from the model's predicted optimal range, and the results were compared with the predicted responses at the same condition. Both the small scale (2 g of RBAO, 1x) and the large scale (20 g of RBAO, 10x) experiments were carried in pentaplicate and triplicate, respectively, in the same manner according to the enzymatic hydrolysis procedure previously described (Section 3.2.2), and the results were expressed as means ± standard deviation. The statistical difference and pairwise comparison between mean values were evaluated by one-way ANOVA using Tukey test at the 95% confidence level ($\alpha = 0.05$) in SPSS 21.0 (IBM SPSS Statistics, New York, NY, USA).

3.3   Results and Discussion

3.3.1   Experimental Design and Statistical Model

The initial concentrations of primary components in RBAO, namely glycerides, FFAs, and γ-oryzanol, were determined prior to the enzymatic hydrolysis of RBAO. As summarized in Table 6, FFAs and glycerides were the most abundant with the content of 398.1 and 228.7 mg per g RBAO, or around 40 and 23 wt%, which were within their previously reported content; 40–80% and 20–50%, respectively (Ghosh & Bhattacharyya, 1995). A relatively high amount of γ-oryzanol, 89.8 mg per g RBAO (ca. 9 wt%) was observed in the herein used RBAO, compared to that in RBAO reported in the study by (Wongwaiwech et al., 2019) with 38.3 mg per g RBAO. The content of these components can be variable depending on the process conditions used in the deacidification step during chemical refining (A. G. G. Krishna et al., 2001). . Prior to the optimization study, OFAT experiments were carried out to investigate the effects of various process variables on glycerides removal and γ-oryzanol loss. Here, time, temperature, and water:RBAO ratio were found to be the most significant (Figure 9 - 13).
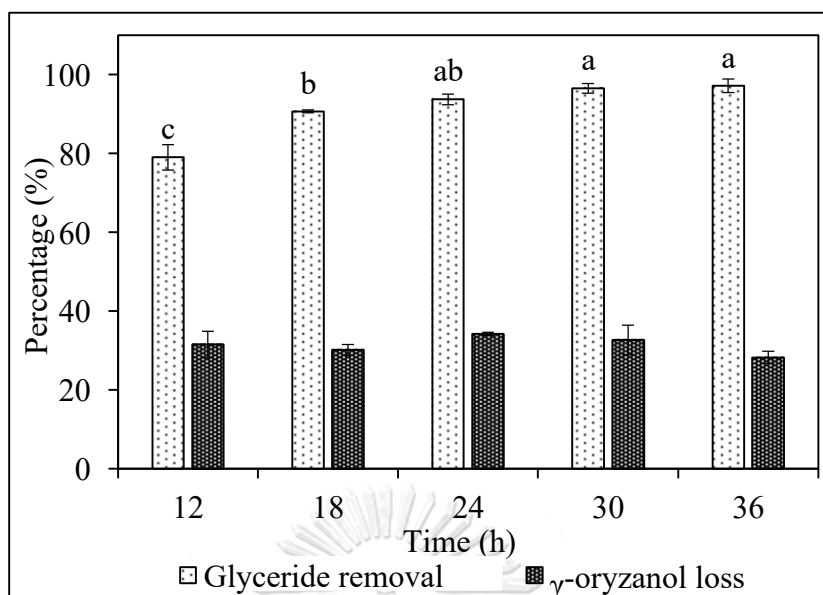
Figure 9: Glycerides removal and γ-oryzanol loss using different reaction times at 40°C, 300 rpm, 10% lipase loading, and 3:1 water:RBAO ratio (n=3). Glycerides removal was significantly different (p = 1.48×10^{-6}) but γ-oryzanol loss was not ($p = 0.10$)
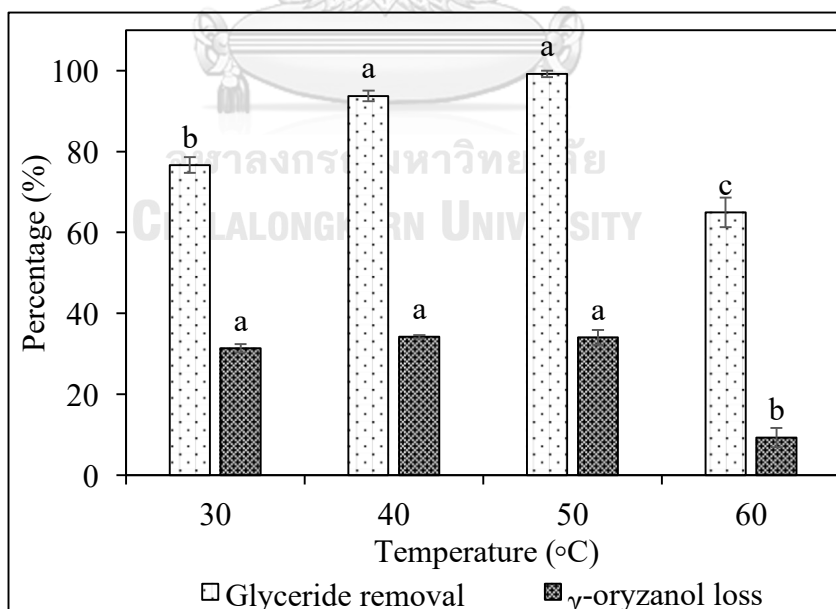
Figure 10: Glycerides removal and γ- oryzanol loss using different temperatures at 300 rpm, 3:1 water:RBAO ratio, 10% lipase loading, and 24 h reaction time (n=3).

There was significant difference in Glycerides removal ($p = 2.07 \times 10^{-7}$) and γ-oryzanol loss ($p = 1.42 \times 10^{-7}$)
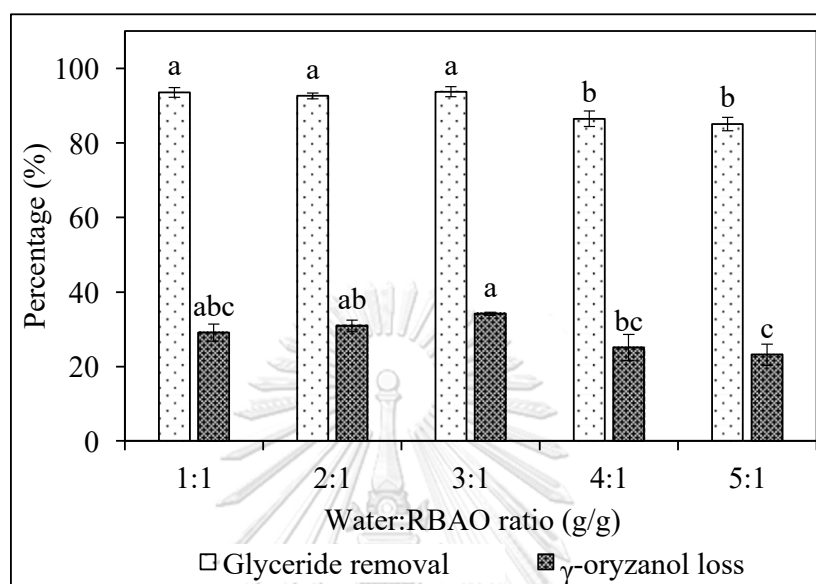
Figure 11: Glycerides removal and γ-oryzanol loss using different water:RBAO ratios at 40°C, 300 rpm, 10% lipase loading, and 24 h (n=3). There was significant difference in Glycerides removal (p = 5.37 × 10-5) and γ-oryzanol loss ($p = 0.001$)
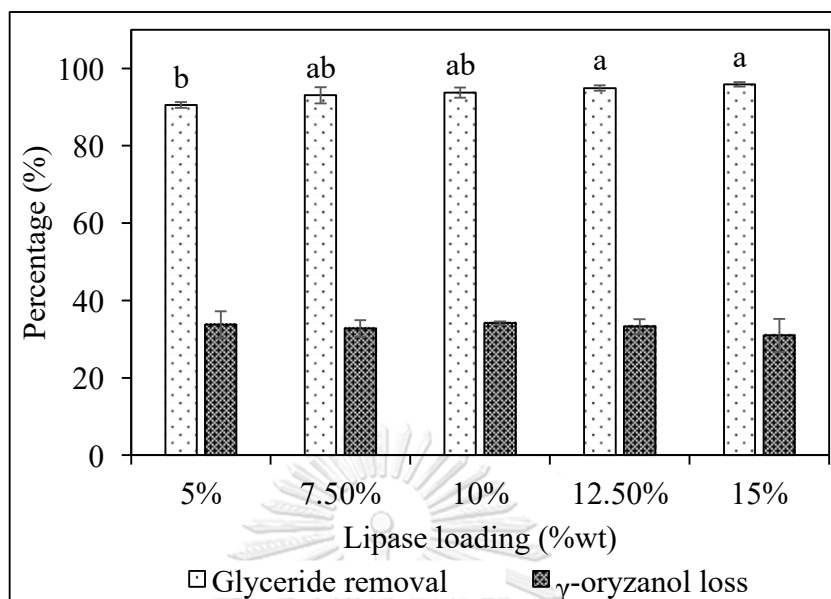
Figure 12: Glycerides removal and γ- oryzanol loss using different values of lipase

loading at 40°C, 300 rpm, 3:1 water:RBAO ratio, and 24 h (n=3). Glycerides

removal (p = 0.003) was significantly different while γ-oryzanol loss was not (p =

0.666)
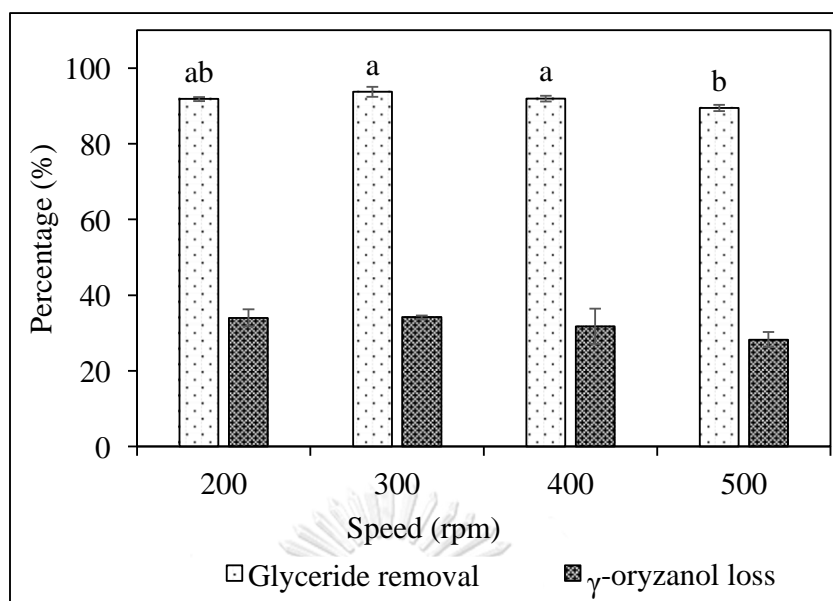
Figure 13: Glycerides removal and γ- oryzanol loss at different speeds at 40°C, 3:1

water:RBAO ratio, 10% lipase loading, and 24 h (n=3). Glycerides removal was

significantly different ($p = 0.003$) while γ-oryzanol loss was not ($p = 0.097$)

These parameters were therefore chosen as the design variables for the subsequent CCRD. The total of 19 CCRD experiments in Tabl 6 were carried out to evaluate the effects of the three variables: time, temperature, and water:RBAO ratio on multiple responses, namely glycerides removal, γ-oryzanol loss, and FFAs production. The results in Table 6 reveal that, at certain hydrolysis conditions (i.e. using 50 °C and 24 h for any water:RBAO ratios), nearly complete glycerides removal (> 99%) was achieved. The results were in good agreement with those presented in (Ghosh & Bhattacharyya, 1995), which primarily aimed to convert glycerides into either FFAs or alcohol esters through enzymatic reactions. More specifically, >95% of glycerides could be hydrolyzed using *Candida cylindracea*

lipase at 35 °C for 48 h. Nonetheless, the effects of the reaction on γ-oryzanol loss and FFAs production were not revealed in their study (Ghosh & Bhattacharyya, 1995). Our results demonstrated that at the conditions employed, the γ-oryzanol loss ranged from 15 to 41%, which could be a result of hydrolysis, as suggested by Andreas Miller, Lina Majauskaite, and Engel (2004) who reported that γ-oryzanol could undergo hydrolysis in presence of certain mammalian lipases, e.g. porcine pancreas (Andreas Miller et al., 2004). Although the *A. Niger* lipase had shown no γ-oryzanol loss in Miller et al.'s study, our results showed that the enzyme could in deed hydrolyze γ-oryzanol under our albeit rather different applied conditions. As their studied systems needed to serve as a model reaction proceeding in vivo, the hydrolysis conditions resembled the condition of living cells (i.e. at 37 °C). Our study, by contrast, was not limited to physiological conditions and so the higher temperatures (up to 60 °C) and consequent higher lipase activity could lead to the observed higher γ-oryzanol loss.

Comparing the results in Table 6 with those obtained with other conventional hydrolysis methods, the glycerides removal was much higher than those of acid-catalyzed hydrolysis (Meedam et al., 2020), in which up to ca. 30% glyceride conversion was resulted for the conditions studied (1–3 N H_2SO_4 , 70–100 °C, and 10–180 min). Base-catalyzed hydrolysis, however, yielded more favorable glycerides conversion with shorter reaction times. For this reason, this method is commonly used to remove glycerides from RBOS and RBAO prior to γ-oryzanol recovery in various works (Das et al., 1998; Kaewboonnum et al., 2010; Meedam et al., 2020). By optimizing the reaction conditions, this loss could be minimized, as was suggested by Meedam et al. (2020) that, at the best base hydrolyzed condition (2.5 N NaOH, 90 °C,

and 5–10 min), complete glycerides conversion could be achieved with only 35% γ-oryzanol loss.

Table 6: Actual and coded values of independent variables for face centered CCRD and corresponding experimental results of responses.

Run*	Time (hr), X_1	Temperature (°C), X_2	Water to RBAO ratio (g/g), X_3	Glycerides removal (%), Y_1	γ-oryzanol loss (%), Y_2	FFAs produced (%), Y_3
1	12(-1)	60(+1)	5(+1)	41.80	17.88	38.21
2	12(-1)	40(-1)	1(-1)	78.47	25.65	19.43
3	36(+1)	40(-1)	5(+1)	92.83	35.04	80.43
4	24(0)	50(0)	3(0)	99.22	35.43	89.5
5	36(+1)	60(+1)	1(-1)	87.93	24.85	45.43
6	12(-1)	60(+1)	1(-1)	63.32	15.08	31.28
7	24(0)	50(0)	3(0)	98.22	36.17	87.02
8	36(+1)	60(+1)	5(+1)	64.54	29.41	58.13
9	12(-1)	40(-1)	5(+1)	76.03	28.45	53.92
10	36(+1)	40(-1)	1(-1)	93.37	32.134	94.74
11	36(+1)	50(0)	3(0)	100	35.15	86.98
12	24(0)	60(+1)	3(0)	62.58	17.75	43.5
13	24(0)	40(-1)	3(0)	88.30	39.92	65.45
14	12(-1)	50(0)	3(0)	90.81	27.88	78.34
15	24(0)	50(0)	1(-1)	97.31	30.74	78.95
16	24(0)	50(0)	3(0)	99.89	41.02	81.41
17	24(0)	50(0)	3(0)	97.66	38.2	88.25
18	24(0)	50(0)	3(0)	99.41	41.11	82.42
19	24(0)	50(0)	5(+1)	98.75	39.70	73.12

*All runs were performed using 10% lipase loading and speed of 300 rpm

Presented also in Table 7 is the FFAs production obtained at various enzymatic hydrolysis conditions. Since in the enzymatic hydrolysis of glycerides, water attacks glycerides-lipase intermediates to produce FFAs and glycerol (Vaysse, Ly, Moulin, & Dubreucq, 2002), there expected to be positive correlations between the levels of glycerides removal and FFAs production. Overall, at most of the

experimental conditions favorable for high glycerides removal, high amounts of FFAs were produced (between 73 and 89%). Nonetheless, at certain runs, especially at low water:RBAO ratio (1:1), the FFAs production was observed to be relatively low despite high glycerides removal. This could be due to the low amount of water that glycerides in the intermediates would not readily be converted to FFAs. With base-catalyzed hydrolysis, on the other hand, FFAs originally present in the RBAO were depleted as they reacted with the base catalyst to form soap. Although soap can be readily separated, FFAs are more valuable, as they are important precursors of a significant number of high-value products, including coatings, adhesives, surfactants, pharmaceuticals, and biofuels. This is an additional reason, other than the environmental concern, that the enzymatic hydrolysis would be more advantageous method for glycerides removal from RBAO. The produced FFAs can further be separated from γ-oryzanol in the reaction product relatively easily via liquid-liquid extraction using deep eutectic solvents (Norjannah et al., 2016; Zullaikah, Wibowo, Wahyudi, & Rachimoellah, 2019) or semi-preparative chromatography (Anjinta et al., 2023).

To quantitatively evaluate the effects of enzymatic hydrolysis variables on the responses, the experimental data (Table 6) were fitted with the coded quadratic polynomial equation (Eq. (4)) through multiple regression. The intercept, linear (direct), interaction, quadratic coefficients of the resulting equations, and their associated *p*-values are summarized in Table 7. The effects of the different variables in RBAO enzymatic hydrolysis on the responses could also be analyzed considering the surface plots of the obtained quadratic equations (Figure 14). Increasing hydrolysis time was found to increase glycerides removal, FFAs production, and γ-

oryzanol loss (Figure. 14a, c, and d), largely in agreement with the statistical analysis (Table 7) showing the significant positive effect on all the responses (X_1, $p < 0.05$) of prolonged reaction time.

The responses to temperature, on the other hand, exhibited a convex response surface (Figure 14a-d) in which the highest levels of glycerides removal, γ-oryzanol loss, and FFAs production were observed within the temperature range studied (40–60°C), and the temperature between 45 and 50°C was where complete glycerides removal could be achieved. This is indicated by the significant negative direct effect of temperature (X_2) and its significant negative quadratic effect (X_2^2), on all the responses ($p < 0.05$). In a typical catalytic hydrolysis, the rate of reaction generally increases as the temperature increases. Nevertheless, this was the case only between ca. 40–50°C, beyond which all responses decreased. This peaking is likely due to enzyme denaturation at higher temperatures (Laidler & Peterman, 1979), similar lipase behavior has been described by Linfield, Barauskas, Sivieri, Serota, and Sr (1984) and is also supported by the A. niger lipase manufacturer's specification, which claims 55 °C to afford the maximum hydrolytic activity.

For the effect of water:RBAO ratio, it would be expected that the rate of RBAO hydrolysis would increase with water content, as lipase would in general be activated at the water-oil interface (R. Sharma et al., 2013). The results in Table 7, on the ot.her hand, reveal a significant negative direct effect of water:RBAO ratio (X_3) and a significant negative interacting effect between temperature and water:RBAO ratio (X_2X_3) on glycerides removal ($p < 0.05$). In other words, higher water:RBAO ratios are less effective for glycerides removal, particularly at higher temperatures.

This drop-off could be attributed to the competitive binding between water and lipase with the glycerides substrate in presence of excess water (Phuah et al., 2012; A. Sharma et al., 2013), which limited accessibility of the active sites by the glycerides substrate (Chew et al., 2008). The range of conditions that causes this negative effect on glycerides conversion has been reported to vary depending on the type of lipase employed (T. A. V. Nguyen et al., 2018). In addition, with the negative effect of temperature, the decrease in the hydrolysis rate is further substantiated.

Table 7: Regression coefficients of fitted coded second-order polynomials and their associated p-values from the two-tailed t-test[a]

Variables	Effects[b] (p-value)		
	Y_1	Y_2	Y_3
Intercept	98.81 (>0.0001)	37.24 (>0.0001)	86.85 (>0.0001)
X_1	8.82 (0.0004)	4.16 (0.021)	14.45 (0.001)
X_2	-10.88 (<0.0001)	-5.62 (0.005)	-9.74 (0.008)
X_3	-4.65 (0.012)	2.20 (0.160)	3.40 (0.241)
$X_1 X_2$	1.96 (0.244)	1.03 (0.533)	-8.47 (0.024)
$X_1 X_3$	0.004 (0.998)	0.23 (0.886)	-5.38 (0.113)
$X_2 X_3$	-5.24 (0.0113)	0.21 (0.899)	-0.067 (0.982)
X_1^2	-2.37 (0.415)	-4.20 (0.176)	-0.39 (0.943)
X_2^2	-22.33 (<0.0001)	-6.88 (0.043)	-28.57 (0.001)
X_3^2	0.26 (0.927)	-0.49 (0.864)	-7.01 (0.225)

[a]Corresponding t-values from the two-tailed Student's t-test
[b]Bold numbers indicate significant effects ($p<0.05$)
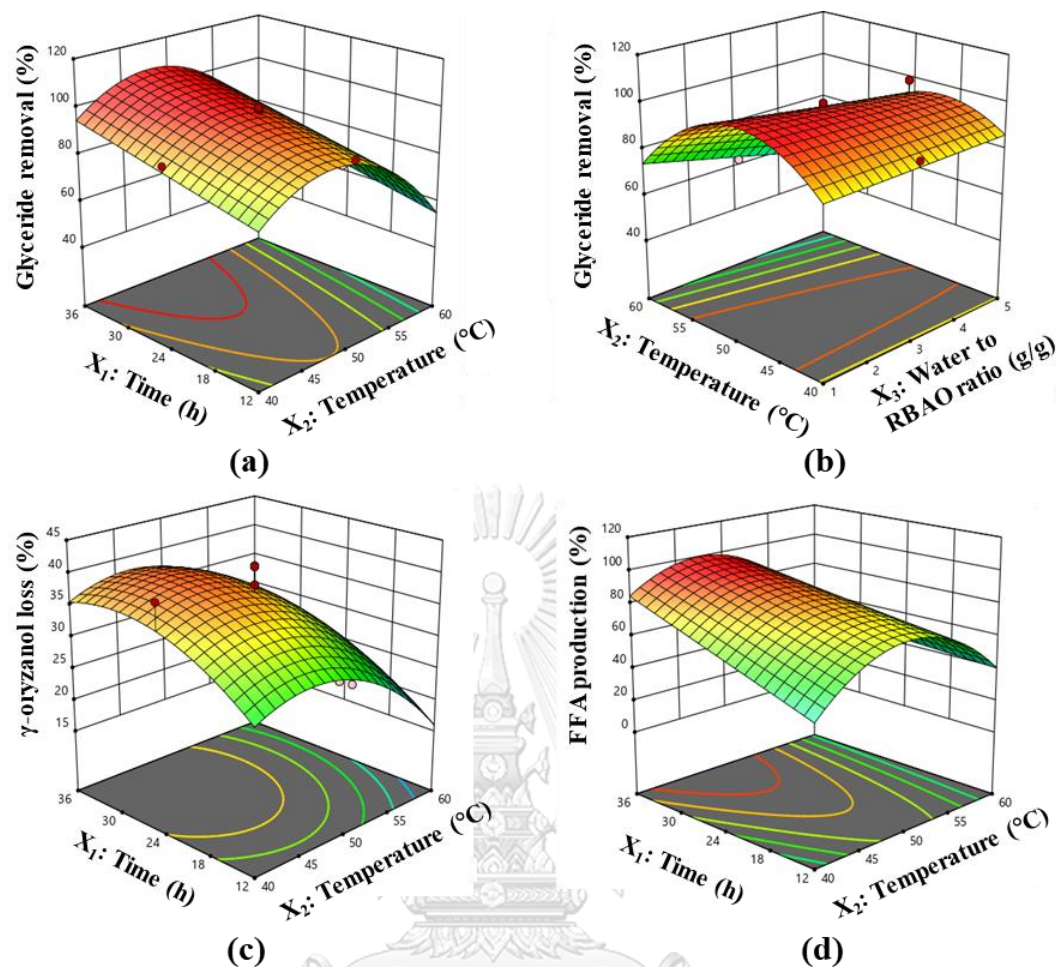
Figure 14: Response surfaces for: (a and b) effects on glycerides removal at

water:RBAO = 3:1 and time = 24 h, respectively; (c) effect on γ-oryzanol loss at

water:RBAO = 3:1; (d) effects on FFAs production at water:RBAO = 3:1.

For the ANOVA of the statistical models, the adequacy of the developed

coded models was first checked considering the plots of residuals versus normal

probability, model predictions, and run numbers for each response (Figure 15 - 17). It

is evident from these results that the residuals from all the equations were found

randomly distributed with constant variance, independent from one another, and were

normally distributed. These observations collectively suggest that the assumption required for the statistical analysis was met. Based on the f-test ANOVA with 95% confidence level (Table 8), the coded quadratic equations were suggested (not aliased) to fit the experimental data. The models were found to be significant (regression, $p < 0.05$), and the "lack of fit" insignificant ($p > 0.05$), while the R^2 and the adjusted R^2 were close to unity, suggesting that the equations describe the experimental data adequately. The "adequate precision", representing the ratio of the predicted value at the design points to the average prediction error (signal-to-noise ratios) for all models was greater than 4, indicating that the equations can be used to guide the design space. However, for each model, the predicted R^2 was considerably less than their corresponding adjusted R^2 (the difference being greater than $\pm 20\%$), suggesting that the equations contain too many insignificant terms, thus requiring further model reduction.
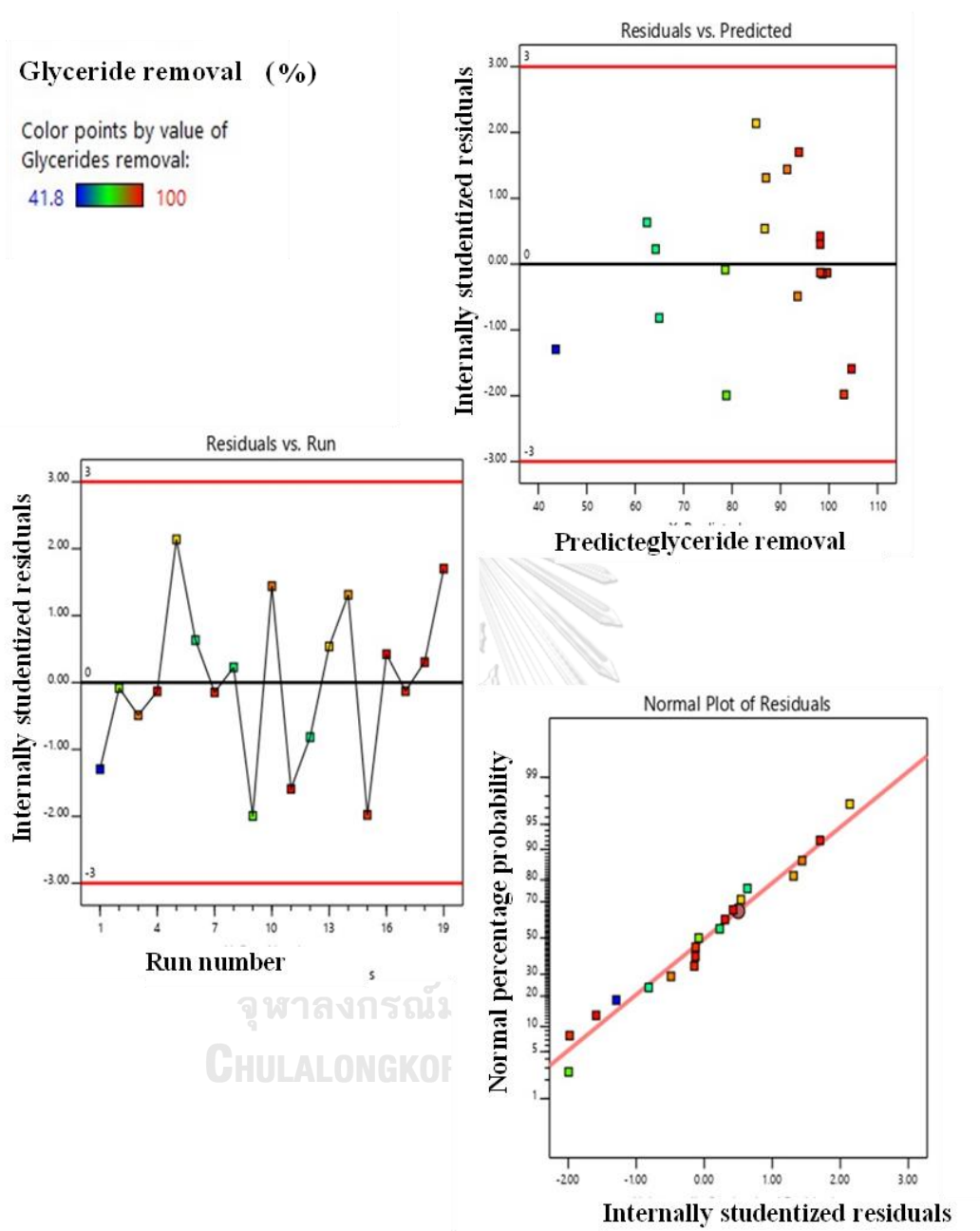
Figure 15: Plots of residuals versus normal probability, model predictions, and run

numbers for statistical analysis of glycerides removal (%)
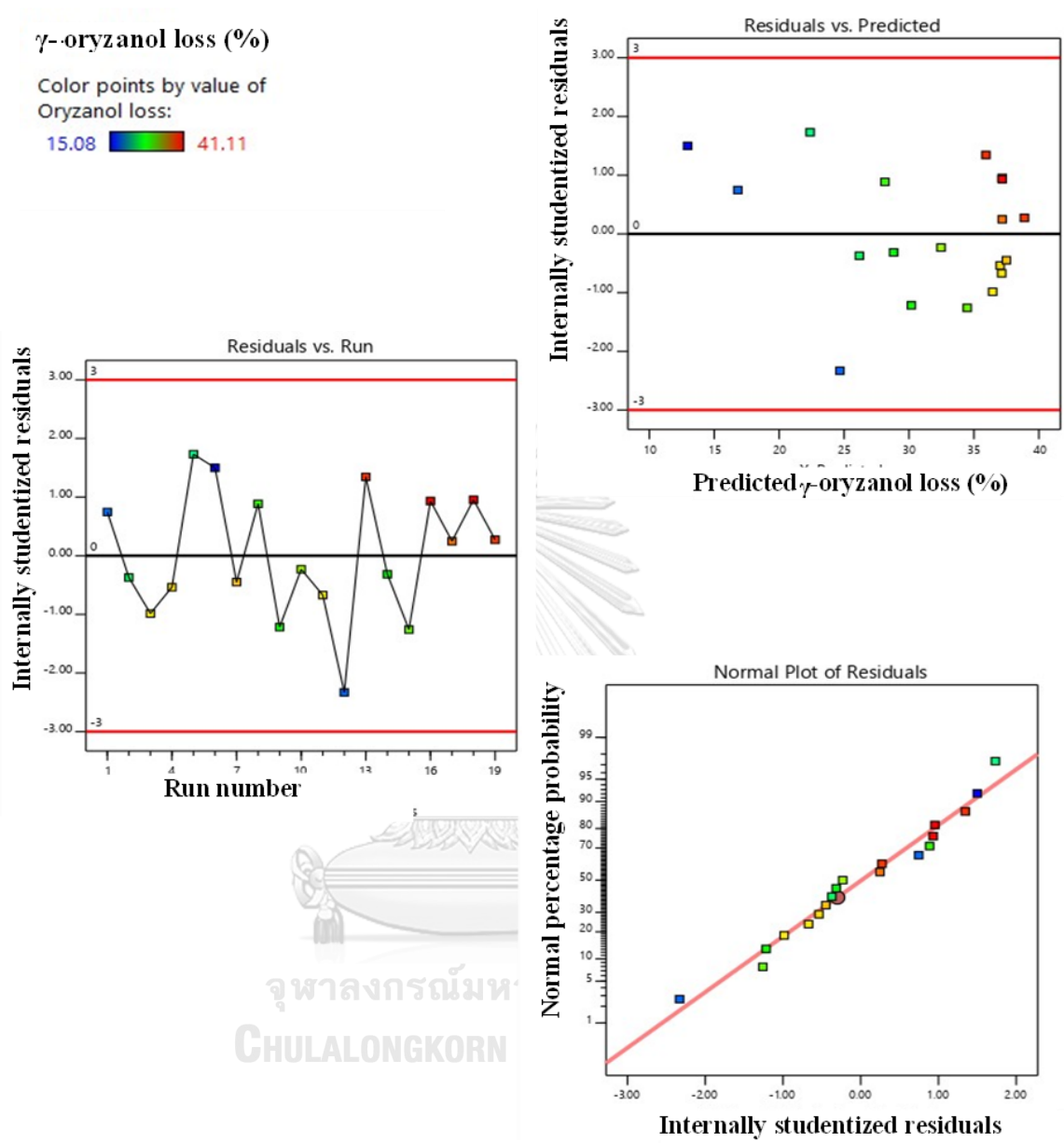
Figure 16: Plots of residuals versus normal probability, model predictions, and run numbers for statistical analysis of γ-oryzanol loss (%)
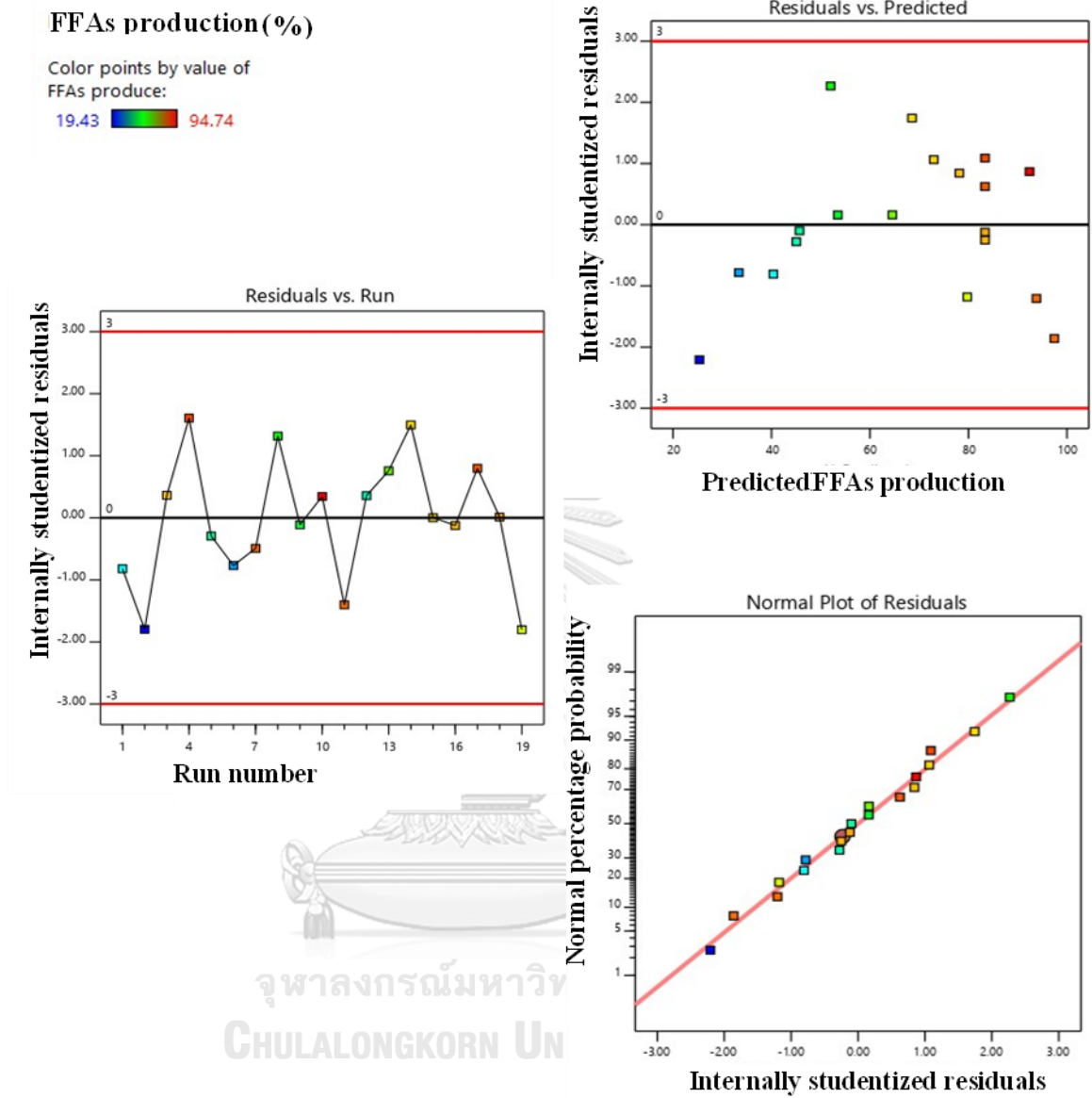
Figure 17: Plots of residuals versus normal probability, model predictions, and run numbers for statistical analysis of FFAs production (%)

Table 8: Analysis of variance (ANOVA) of quadratic coded equations before model reduction: Glycerides removal (Y_1), γ-oryzanol loss (Y_2), and FFAs production (Y_3).

Factors	Sum of squared (Degree of freedom)			Mean square			p-values (f-values)		
	Y_1	Y_2	Y_3	Y_1	Y_2	Y_3	Y_1	Y_2	Y_3
Regression	4128.1 (9)	832.3 (9)	7185.3 (9)	458.7	92.5	798.4	0.0002 (24.22)	0.0268 (4.7)	0.0021 (11.32)
Residual	132.6 (7)	137.8 (7)	493.6 (7)	18.9	19.7	70.5			
▪ Lack of fit	129.8 (5)	132.3 (5)	466.3 (5)	26.0	26.5	93.3	0.0512 (18.84)	0.0964 (9.67)	0.1324 (6.84)
▪ Pure error	2.76 (2)	5.48 (2)	27.3 (2)	1.4	2.7	13.6			
Total	5084.9	1177.0	9098.7						
R^2	0.969	0.858	0.936						
Adjusted R^2	0.929	0.675	0.853						
Predicted R^2	0.318	-1.312	-0.385						
Adeq Precision	17.65	7.36	10.8						

3.3.2 Model Reduction

Prior to the optimization, model reduction was applied to the equations using a backward stepwise selection in the Design-Expert software, in which the least significant variables (or terms) were removed sequentially from the original coded quadratic equations based on their corresponding p-values. By varying a p-value used as the criteria to exclude the terms, it turned out that the $p > 0.1$ criterion reasonably improved the goodness-of-fit and the predictive capability of the equations. The final reduced equations describing the relationships between the coded variables (X_1, X_2, and X_3) and the three responses (Y_1, Y_2, and Y_3) are summarized in Eqs. (9)–(11):

Glycerides removal (%),

$$Y_1 = 98.00+8.82X_1-10.88X_2-4.65X_3-5.24X_2X_3-23.10X_2^2 \tag{9}$$

γ-oryzanol loss (%),

$$Y_2 = 37.10+4.16X_1-5.62X_2+2.20X_3-4.33X_1^2-7.01X_2^2 \tag{10}$$

FFAs production (%),

$$Y_3 = 84.00+14.45X_1-9.74X_2+3.40X_3-8.47X_1X_2-5.38X_1X_3-31.28X_2^2 \tag{11}$$

Following model reduction, the assumption required for the statistical analysis was still met and all the equations remained significant (regression, $p < 0.05$) with their insignificant "lack of fit" ($p > 0.05$) (Table 9). The R^2 and the adjusted R^2 values were all still relatively close to unity, while the adequate precision values were all still larger than 4; but the values for the predicted R^2 were all improved. These suggested that the goodness of fit and predictability of the reduced models were improved. The equation for glycerides removal showed the highest predicted R^2 (0.850) and was the closest to the adjusted R^2 values (0.939). The reduced equation for glycerides removal (Eq. (9)) therefore would be expected to give more accurate predictions than those for γ-oryzanol loss (Eq. (10)) and FFAs production (Eq. (11)), where the predicted R^2 were quite small (0.516 and 0.638, respectively) and considerably lower than their corresponding adjusted R^2 (0.779 and 0.868, respectively). This was supported by the coefficient of variance of the glycerides removal model (4.68%), which was smaller than the permissible upper standard limit of 10%, ensuring the reproducibility of the model (Lim et al., 2016)

Table 9: Analysis of variance (ANOVA) of final reduced coded quadratic polynomial equations

Factors	Sum of squares (Df[b])			Mean square			p-values (f-values)		
	Y_1	Y_2	Y_3	Y_1	Y_2	Y_3	Y_1	Y_2	Y_3
Regression	4082.9 (5)	822.5 (5)	7046.5 (6)	816.6	164.5	1174.4	<0.0001 (50.53)	0.0003 (12.25)	<0.0001 (18.57)
Residual	177.8 (11)	147.7 (11)	632.3 (10)	16.2	13.4	63.2			
▪ Lack of fit	175.0 (9)	142.2 (9)	605.1 (8)	19.5	15.8	75.6	0.0679 (14.12)	0.1564 (5.77)	0.1616 (5.55)
▪ Pure error	2.76 (2)	5.48 (2)	27.3 (2)	1.4	2.7	13.6			
Total	5084.9	1177.0	9098.7						
R^2	0.958	0.848	0.918						
Adjusted R^2	0.939	0.779	0.868						
Predicted R^2	0.850	0.516	0.638						
Adeq Precision	23.33	10.658	12.86						

3.3.3 Optimization of Enzymatic Hydrolysis Conditions and Experimental Validation

3.3.3.1 Optimization by Desirability Analysis

To find the optimal range of conditions, a desirability analysis was performed on the reduced equations with the constraints of maximizing glycerides removal and maintaining the γ-oryzanol loss below 35%. The results suggest that the highest overall desirability of 1.00 could be obtained at various conditions. For example, as shown by the 3D desirability plot in Fig. 18a, for the water:RBAO ratio of 1:1, the hydrolysis times in the range of 20–36 h and temperatures between 47 and 53 °C were identified to give the optimal results, satisfying the desired constraints on glycerides

removal (Y_1) and γ-oryzanol loss (Y_2). It could be seen from the optimal results that, for the reaction to satisfy the constraints, when using low temperatures between 47 and 49 °C, relatively short reactions of 22–24 h were required, while longer reaction times up to 36 h would be needed with higher temperatures. This followed the observed effects of time and temperature on the enzymatic reaction as above discussed in Section 3.3. The patterned areas (area 1 and area 2) in the corresponding overlay plot of the glycerides removal and the γ-oryzanol loss at the water:RBAO ratio of 1:1 in Fig. 18b represents the location of reaction conditions that simultaneously satisfies the desired constraints of complete glycerides removal (G:100) and <35% γ-oryzanol loss (O:35). In other words, the regions of optimal conditions are bounded by the contour lines G:100 and O:35. On account of our combined results, the test hydrolysis conditions selected for our validation experiments were: 1:1 water:RBAO ratio, a relatively short reaction time (22 h) and low temperature (48.5 °C).
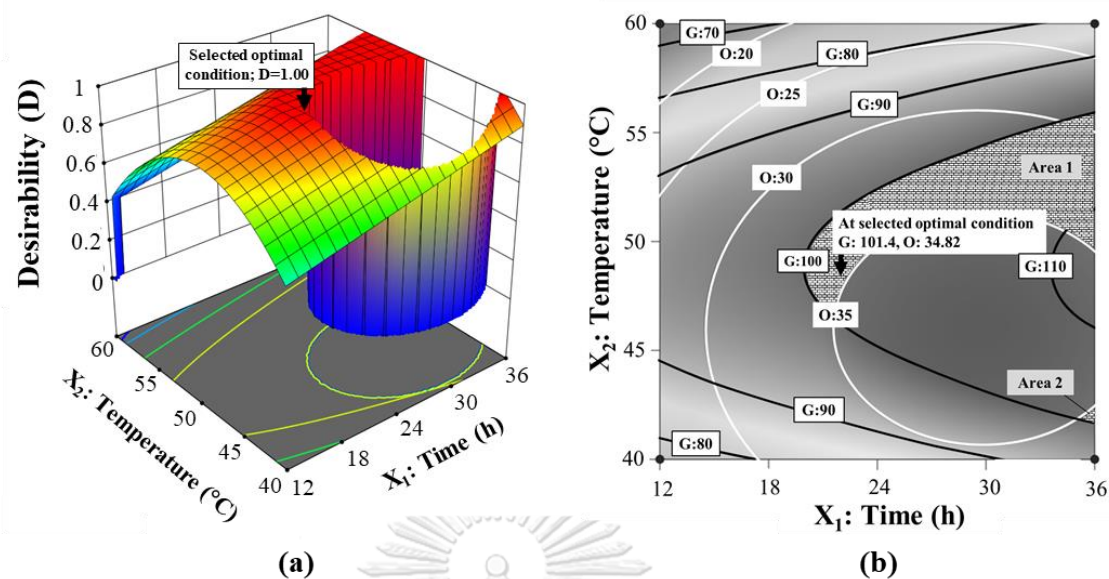
Figure 18: . Location of optimal hydrolysis time and temperature at 1:1 water:RBAO ratio from desirability analysis; (a) contour plot of desirability, D, and (b) overlay plot of glycerides removal, G, and γ-oryzanol loss, O; Patterned areas are locations of optimal conditions.

3.3.3.2   Validation Experiments

At the selected optimal condition of 22 h, 48.5 °C, and 1:1 water:RBAO ratio, the predicted responses were compared with the experimental data obtained from enzymatic hydrolysis carried out at small (2 g of RBAO, 1x) and large (20 g of RBAO, 10x) scales. The model-predicted responses of glycerides removal, γ-oryzanol loss, and FFAs production based on Eqs. (9)–(11) were 101.40%, 34.82%, and 77.96%, respectively (Figure 19). The corresponding responses from the 1x-scale experiment were 98.82%, 31.13%, and 73.23%, and those from the 10x-scale

experiment 98.52%, 40.29%, and 75.01%, respectively, which gave the final concentrations of glycerides, γ-oryzanol, and FFAs in the hydrolyzed product of around 2.7–3.4, 53.6–61.8, and 689.7–696.8 mg per g RBAO, respectively. For each response, there appeared to be no significant difference ($p < 0.05$) between the results experimentally obtained at both scales by oneway ANOVA with post-hoc Tukey test, suggesting the reaction was reproducible even at larger scales. Furthermore, it suggested that all the experimental data fell within the 95% prediction interval, confirming the validity of the model prediction (as summarized in Table 10). Despite the relatively low values of predicted R^2 of Eqs. (10) and (11) for γ-oryzanol loss and FFAs production, compared with that of Eq. (9), the results here confirmed that all the equations gave reasonably accurate predictions of experimental responses for the enzymatic hydrolysis of RBAO.

Table 10: 95% prediction interval (PI) of the selected condition

Response	Predicted Mean	Standard error	95% PI low	95% PI high	Experimental data of 2g RBAO: n =5	Experimental data of 20g RBAO: n =3
Glycerides removal (%)	101.40	2.74	95.28	107.33	98.82	98.52
γ-oryzanol loss (%)	34.82	2.65	31.12	41.12	31.13	40.29
FFAs production (%)	77.96	5.42	73.23	90.12	73.23	75.01
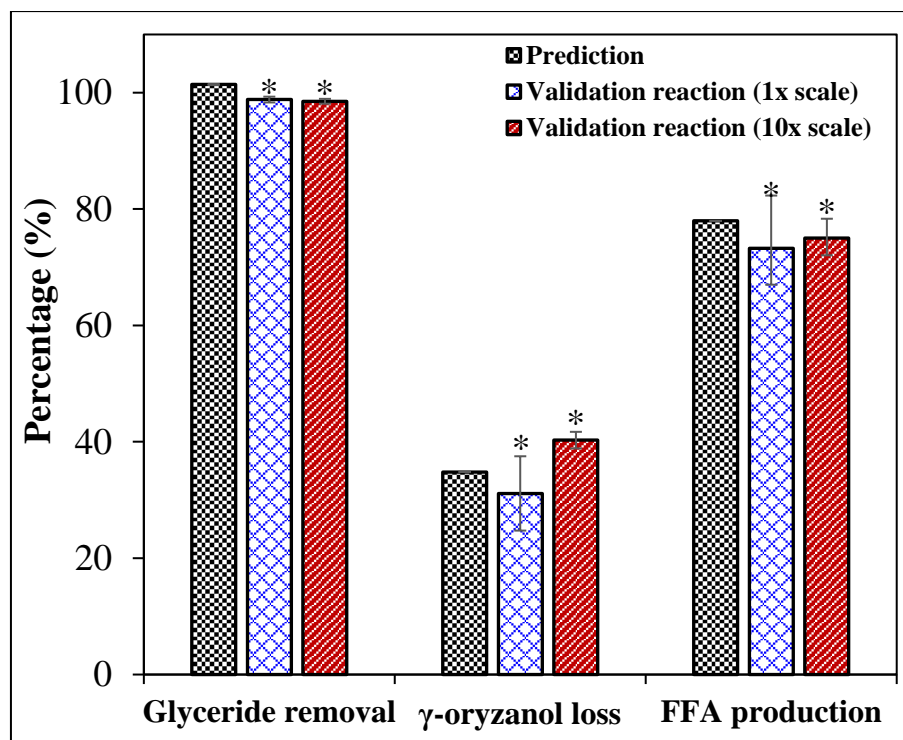
Figure 19: Glycerides removal (%), γ-oryzanol loss (%), and FFAs production (%) from model predictions (▨), experimental results of enzymatic hydrolysis using the same method as in CCRD (▨, n=5), and enzymatic hydrolysis using 10-fold increase in RBAO (▨, n=3) at selected optimal condition (22 h, 48.5°C, and 1:1 water:RBAO ratio). * indicates no statistical difference in each response. * indicates no statistical difference.

3.4   Conclusions

Enzymatic hydrolysis was demonstrated to be an effective and greener alternative to the conventional base-catalyzed hydrolysis for glycerides removal in RBAO, prior to subsequent recovery of γ-oryzanol. Here, the effects of three main variables, namely time, temperature, and water:RBAO ratio, on the degrees of

glycerides removal, γ-oryzanol loss, and FFAs production were revealed through RSM. Based on the statistical models for these responses, optimal variable levels: 20–36 h and 47–53 °C at 1:1 water:RBAO ratio, were found to give complete glycerides removal with γ-oryzanol loss below 35%. At the selected optimal condition of 22 h, 48.5 °C, and 1:1 water:RBAO ratio, the predicted and experimental enzymatic hydrolysis results agreed, confirming the predictability of the models, and yielded nearly complete glycerides removal (99%), with relatively small γ-oryzanol loss (ca. 30–35%). To further improve the γ-oryzanol retention in the hydrolyzed product, further research is underway to search for the most suitable enzymes with higher reaction selectivity.
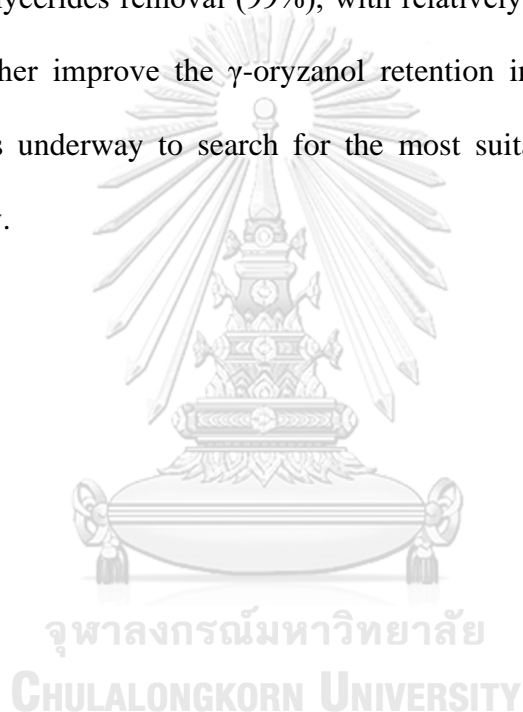
CHAPTER 4

ENZYMATIC ESTERIFICATION/TRANSESTERIFICATION AS A PRETREATMENT STEP FOR GLYCERIDES REMOVAL BEFORE γ-ORYZANOL RECOVERY FROM RICE BRAN ACID OIL

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Abstracts

This paper investigates glycerol removal via the esterification/ transesterification process from rice bran acid oil (RBAO) and γ-oryzanol recovery from biodiesel (FAEE) produced from RBAO. FAEE was synthesized in a biocatalyst using free lipase during the esterification/transesterification reaction. In this study, lipase from Aspergillus Niger was employed for biodiesel production RBAO. The optimal conditions for processing 2g RBOA: 40°C, 3:1 and 5:1 mol ratio of ethanol to RBAO,10% of lipase loading,200 rpm, and 18 and 24 h reaction time., respectively. At optimal conditions, 100% glycerides removal could be achieved. Five process variables, namely ethanol to RBAO ratio, temperature, time of reaction, lipase loading, and speed were investigated. Throughout the whole range of process variables, the extraction efficiency was relatively high. After the extraction, glycerides removal content was higher than the limit for all samples, but the γ-oryzanol remaining content was within the range comply with the hydrolysis method. The selected extraction solvent has proved to be efficient for γ-oryzanol extraction in the range of conditions, but further process modifications and FAEE production will be needed to reduce the content of glycerides. This shows that the Aspergillus Niger lipase in esterification/transesterification is a potential environmentally friendly biocatalyst for the biodiesel industry before the γ-oryzanol extraction.

4.1   Introduction

Simultaneous esterification/transesterification is a well-known method for fuel production from oils with high free fatty acid (FFAs) content, in which FFAs/glycerides in the oils are reacted with a short-chain alcohol to form fatty acid alkyl esters, or biodiesel (Demirbas, 2006). The most commonly utilized alcohols for this purpose include methanol and ethanol, which produce fatty acid methyl esters (FAME) and fatty acid ethyl esters (FAEE), respectively (Musa, 2016). These reactions can be catalyzed by an acid (H_2SO_4 and HCl) (Thein, Jindal, Jindal, & Yoswathana, 2019; Yasmin, Zullaikah, Permatasari, Marita, & Rachimoellah, 2019), a base (NaOH, KOH, CH_3ONa, and CH_3OK) (Sales, 2011; Yusup & Khan, 2010), heterogeneous (La_2O_3, SrO, BaO KNO_3, KF, CaO, $CaCO_3$) (Borges & Díaz, 2012; Bressani, Garcia, Hirata, & Mendes, 2015), or even without a catalyst (subcritical /supercritical conditions (Chen, Chen, Chang, Lai, & Tu, 2010; Novy Srihartati Kasim, Tsai, Gunawan, & Ju, 2009). Alternatively, esterification/transesterification can be catalysed by the lipase enzyme, which has several advantages over the physicochemical methods including high specificity and selectivity toward substates, as well as the ability to operate under mild conditions (Haar, Stäbler, Wichmann, & Schweiggert-Weisz, 2015; S. H. Krishna & Karanth, 2002; C.-C. Lai, Zullaikah, Vali, & Ju, 2005; Sendzikiene, Santaraite, & Makareviciene, 2020).

Biodiesel offers advantages over traditional fossil fuels, including renewability biodegradability, non-toxic, low emissions of smoke and hydrocarbon, and marginal effects on engine durability/carbon deposits (Arya, Rout, & Samanta, 2022). The raw

material that is utilized in the production of biodiesel may vary, however FFAs-rich oils which are agricultural/industrial byproducts/wastes are the most cost-effective options. Rice bran acid oil (RBAO) is the promising biodiesel raw material since it is the low-cost byproduct from the rice bran oil (RBO) refinery and is rich with FFAs (46wt%) and glycerides (37wt%). During the neutralization process, rice bran oil soapstock (RBOS) being produced and then is acidulated with H_2SO_4 to form RBAO (Wongwaiwech et al., 2019). Several studies have been carried out on the production of biodiesel from RBO (J.-S. Choi et al., 2015; Gunawan, Maulana, Anwar, & Widjaja, 2011; Ju & Vali, 2005; Lin, Ying, Chaitep, & Vittayapadung, 2009; Yasmin et al., 2019) and RBAO (N. Choi et al., 2016; López et al., 2015; S., Aparna, & Baskaran, 2019; Sombutsuwan et al., 2018) by employing acid-catalyzed and enzymatic esterification/transesterification processes. Andrade, Martín, Errico, and Christensen (2019)focused their attention on contrasting the effectiveness of two manufacturing methods: acid-catalyzed processes and enzymatic reactions of transesterification of castor oil and resulting that both approaches were successful in producing high conversion rates; however, the enzymatic process resulted in a biodiesel of superior quality that included far less acid and water.

Besides FFAs and glycerides, RBAO contains a large amount of γ-oryzanol (9 wt%). γ-oryzanol is a mixture of esters of sterols and ferulic acid and it is gaining popularity (Kaewboonnum et al., 2010; Wongwaiwech et al., 2019) since it has a number of health benefits, including lowering cholesterol and high antioxidant activity. It is thus used as a sunscreen agent, an antioxidant, and a preservative in cosmetics and food preparations (Berger et al., 2005; Heidtmann-Bemvenuti, Nora, & Badiale-Furlong, 2012; Lemus, Angelis, Halabalaki, & Skaltsounis, 2014; Oryza Oil

& Fat Chemical Co., 2012). Separating γ-oryzanol from RBAO typically involves initial removal of glycerides in the raw material, which are considered as the main impurities complicating the subsequent separation steps due to their similar physical properties (Narayan et al., 2006).

Several studies investigated glycerides removal via esterification/transesterification as a pretreatment step in which FFAs/glycerides are converted into biodiesel. Sombutsuwan et al. (2018) employed acid-catalyzed reactions to convert FFAs/glycerides to FAEE and employed acid-base extraction to recover γ-oryzanol. They however did not report the amount of γ-oryzanol remaining after the reactions. With the advantages of the enzymatic method, the application of enzymatic esterification/transesterification with glycerides removal in RBAO would help improve subsequent γ-oryzanol recovery, and to the best of our knowledge, has not been reported before.

In this study, enzymatic simutanous esterification/transesterification followed by acid-base extraction was applied with RBAO for initial glycerides removal, and subsequent γ-oryzanol recovery. Here, the reactions were catalyzed by *Aspergillus niger* lipase, and suitable reaction conditions for the reactions and the extraction were determined. To achieved this, effects of reaction conditions: ethanol:RBAO molar ratio, temperature, reaction time, lipase loading, and agitation speed, and effects of ethanolic NaOH concentration, were evaluated on degrees of glycerides removal, γ-oryzanol loss, biodiesel content, and FFAs production, and degrees of γ-oryzanol recovery, respectively. The contribution of this work offers new source of raw

materials for biodiesel production with greener approaches and a value added to the rice industry.

4.2 Materials and Methods

4.2.1 Materials and Chemicals

Rice bran acid oil (RBAO), with its composition as shown in Table 11, were obtained from Thai Edible Oil Co., Ltd Samutprakarn, Thailand. RBO (Thai Edible Oil Co., Ltd, Thailand), γ-oryzanol (CAS No. 11042-64-1, Wako Pure Chemical, Japan), oleic acid (CAS No. 112–80-1, Sigma-Aldrich, Singapore), and fatty acid ethyl esters (FAEEs, Cat. No. 49454-U, Sigma-Aldrich, Singapore) were used as standards for glycerides, γ-oryzanol, FFAs, and biodiesel, respectively. All solvents used in this study, including ethyl acetate (CAS No. 057-03371, Sigma-Aldrich, Singapore) methanol (CAS No. 67–56-1, Fisher Scientific, Belgium), isopropanol (CAS No. 67–63-0, Loba Chemie Pvt Ltd, India), ethanol (CAS No. 64-17-5, Qrec, New Zealand), and hexane (CAS No. 110-54-3, Qrec, New Zealand) were of analytical grade. *Aspergillus Niger* lipase (E.C. 3.1.1.3) was purchased from Sinobios Imp. & Exp. Co. Ltd, Shanghai, China was of industrial grade with the activity of 10,000 U/g, in which 1 U is defined as 1 µmol FFAs per min being liberated from triglycerides using 1 g of lipase at pH 7.0 and 40°C.

Table 11: Composition of RBAO

Components	Amount (%wt)
FFAs	46
Glycerides	36.15
γ-oryzanol	9.53
Tocopherols	1.32
Moisture content	1.37

4.2.2   Enzymatic esterification/transesterification of RBAO

Enzymatic esterification/transesterification of RBAO was herein used to primarily remove glycerides before subsequent γ-oryzanol extraction. Effects of reaction conditions on glycerides, FFAs, biodiesel, and γ-oryzanol were investigated according to the studied ranges as summarized in Table 12, in which each condition effect was studied in the order and suitable levels of each condition were accordingly suggested.

Table 12: Reaction conditions and their studied ranges

Order	Reaction variable	Studied range
1	Ethanol: RBAO molar ratio	1:1 – 9:1
2	Temperature	30 – 60°C
3	Reaction time	1.5 – 30 hr
4	Lipase loading	5 – 15 wt% of RBAO
5	Speed	200 – 400 rpm

The method for enzymatic esterification/transesterification of RBAO was modified from those described in (Erika C. G. Aguieiras, Cavalcanti-Oliveira, Castro, Langone, & Freire, 2017; N. Choi et al., 2016). Briefly, RBAO was mixed with ethanol at a specified molar ratio (wt/wt) and lipase loading (wt% of RBAO), the mixture was heated in a water bath under a specified speed with a 4cm magnetic bar

to a reaction temperature. After the reaction was allowed at the specified time, the mixture was separated from lipase from the crude biodiesel using a centrifuge at 4500 rpm for 10 minutes. Then, the top layer is collected and washed with water to remove the excess ethanol and glycerol since both of it dissolved in water. The biodiesel is then analyzed to determine the contents of glycerides, γ-oryzanol, FFAs, and FAEE. The average molecular weight of RBAO was calculated as follows (Equation 12) where Mn: Average molecular weight, Mi: Molecular weight of fatty acids, and Ni: Number of moles of fatty acids, and the molar ratio of RBAO to ethanol was calculated based on 2 g total weight.

$$Mn = \frac{\sum(Mi \; x \; Ni)}{Ni} \qquad\qquad (12)$$

4.2.3   Extraction of γ-oryzanol

The γ-oryzanol was extracted from crude FAEE through the acid-base extraction method, which was carried out by modified method from Sombutsuwan et al. (2018). Briefly, one gram of crude FAEE was placed in a 50-mL Erlenmeyer flask. Then, hexane was added and followed by a solution of aqueous ethanolic NaOH (4ml) at different molarity (1.0M – 4.0M) with 75.91％（v/v) Ethanol in water and 20.59 % hexane in the total volume of hexane and aqueous ethanol (hexane + ethanol = 100 mL). Then, the mixture was stirred for 1 min and after that, it was transferred to a centrifuge tube and centrifuged at 4500rpm for 10 min and produce 2 layers, the top layer is FAEE in hexane solution and the bottom is aqueous ethanolic NaOH solution

(pH 14). The top layer is removed, and the lower phase was washed once with hexane to remove the remaining crude FAEE and then adjusted the solution to pH 7 with glacial acetic acid. 20 µl of the neutralized of aqueous ethanolic NaOH is diluted with 3ml of ethyl acetate and filtered using 0.45 µm before analysis using HPLC ELSD on the amount of γ-oryzanol.

4.2.4 Quantification of glycerides, γ-oryzanol, and FFAs

Contents of glycerides, γ-oryzanol, and FFAs in the sample were quantified using HPLC ELSD using the method modified from Kaewboonnum et al. (2010). 20 µl of the collected biodiesel phase was dissolved in 3 ml ethyl acetate, and then filtered using a 0.45 µm syringe filter. The HPLC apparatus consisted of a pump (Waters ELSD e2695, USA), and was equipped with an evaporative light scattering detector (ELSD, SEDEX 80, USA). The detector condition was set at the tube temperature of 50°C, and nitrogen gas flow rate of 0.3 L/min, and the impactor was turned off. The analysis was carried out at room temperature in which 5 µl of the sample solution was injected into a Sunfire C18, 100 mm x 4.60 mm x 3.5 μm I.D. column. The mobile phase consisted of methanol and isopropanol at the ratio of 60:40 v/v, and the flow rate was controlled at 0.5 ml/min. Here, the standard calibration curves were constructed using oleic acid (FFAs), RBO (glycerides), and standard γ-oryzanol. The analyses were performed in triplicate and the standard deviation was less than 5%. The degrees of glyceride removal, γ-oryzanol loss, and FFAs remaining (wt%) were calculated as in the following equation 13- 15.

Glycerides removal (%) =

$$\frac{\text{Initial amount of glycerides - Amount glycerides remaining after hydrolysis}}{\text{Initial amount of glycerides}} \times 100 \qquad (13)$$

γ-oryzanol loss (%) =

$$\frac{\text{initial amount of γ-oryzanol - amount of γ-oryzanol remaining after hydrolysis}}{\text{initial amount of γ-oryzanol}} \times 100 \qquad (14)$$

FFAs remaining (%)=

$$\frac{\text{Initial amount of FFAs - Amount of FFAs after hydrolysis}}{\text{Initial amount of FFAs}} \times 100\% \qquad (15)$$

4.2.5   Quantification of biodiesel

20 μl of samples of biodiesel corresponding to the different reaction conditions are dissolved in 3 mL of hexane, filtered using 0.45 µm, and analyzed using gas chromatograph Model Agilent 122-7032 (Agilent Tech, USA) equipped with a DB-Wax column (30 m×0.25 mm x 2.5 μm i.d) modified method from (D.Surendhiran & M.Vijay, 2014). Samples were injected in a split mode (50:1 split ratio) at an oven temperature of 60°C with an injection volume of 1μl. The oven temperature program consisted of an initial hold at 60°C for 0 min, ramping to 70°C and then to 230°C with a final hold for 8min. The injector was kept at 230°C, the flow rate of carrier gas was 1 mL/ min, and the ionization energy was 70 eV. MS conditions were: ion source temperature 200 0 C, interface temperature 240 0 C, scan range-40-1000m/z, solvent cut time-5 min, MS start time-4min, end time-27min and ionization-EI (70eV). Biodiesel was quantified by using an Fatty Acid Ethyl Ester (FAEE) mixture standard

(Sigma–Aldrich, Singapore) and analysis is conducted at Mahidol University, Salaya.

The FAEE produced (wt%) was calculated as in the following equation 16.

FAEE produced (%)=

$$\frac{\text{Weight of FAEEs in crude FAEE}}{\text{Weight of FFAs+Glycerides+\gamma-oryzanol+FAEEs in biodiesel phase}} \times 100 \qquad (16)$$

4.2.6   Statistical Analysis

All experiments were performed in triplication and the data presented were mean values. The variations were presented as standard deviation and Microsoft Excel software was used for the analyses. Analysis of variance (ANOVA) using SPSS Version 21 and Tukey's test was used to compare between mean values at a significant level $p<0.05$.

4.3   Results and Discussions

In the following subsections, to find suitable levels of reaction variables in the enzymatic esterification/transesterification of RBAO to obtain optimum yield of the desired output; complete glycerides removal while keeping γ-oryzanol loss to a minimum, their effects on the contents of glycerides, γ-oryzanol, biodiesel and FFAs are discussed. The variables included ethanol to RBAO molar ratio, temperature, reaction time, lipase loading, and speed of the biocatalyst during the esterification transesterification reaction. Normally, methanol, butanol, ethanol were used as alkyl

acceptor in the reactions, however ethanol was suggested more suitable when dealing with the enzymatic reactions (N. Choi et al., 2016). . In addition, ethanol is renewable since it can be produced from agriculture waste.

4.3.1   Effect of Ethanol to RBAO Molar Ratio

This study evaluated the effect of the molar ratio of ethanol to RBAO molar ratio on the of glycerides removal, γ-oryzanol loss, FAEE production and FFAs remaining (Figure 20) and indicates that glycerides were maximally removed by 3:1 and 5:1, with almost 100% removal. The reason for this is due to the stoichiometric reaction between ethanol and glycerides, in which three moles of ethanol are needed to produce one mole of biodiesel. Therefore, the higher the molar ratio of alcohol to oil, the faster the reaction.

Musa (2016) mentioned that the stoichiometric molar ratio of alcohol to oil for the transesterification is 3:1 and the reaction is reversible. To improve the miscibility and promote better interaction between alcohol molecules and glycerides, higher molar ratios are necessary. In contrast, excess alcohol as a substrate resulted in a reduction in enzyme activity and yield. According to M. S. Antczak, A. Kubiak, T. Antczak, and S. Bielecki (2009) increasing the molar excess of alcohol over fatty acids in glycerides has been found to enhance transesterification yield. However, it is important to note that this can also lead to enzyme inactivation, especially when the alcohol is insoluble in the reaction mixture and forms an emulsion. The size of the emulsion droplets depends on the intensity of stirring. Alcohol retards enzyme

activity, resulting in reduced lipase functionality and enzyme deactivation (Matassoli, Corrêa, Portilho, Veloso, & Langone, 2009; Shimada, Watanabe, Sugihara, & Tominaga, 2002). Despite this, ethanol to oil ratios increased further resulting in less esters being produced, and different types of lipases have varying optimal amounts of ethanol for FAEE production, which may be due to ethanol's inhibitory effect on lipase activity. This is consistent in our study that the glycerides removal starts to decrease when increasing the molar ratio from 5:1 to 9:1.

Furthermore, comparable losses to glycerides removal were observed for γ-oryzanol, a component of rice bran oil, with the maximum loss at 47 % (3:1 molar ratio), indicating the potential of reactions during the esterification/transesterification process. The loss of γ-oryzanol may occur as a result of the reaction with the presence of acid in RBAO, which leads to the production of the main products, water and glycerol (Andreas Miller et al., 2004)

In this investigation, 83 % of FAEE was produced, with the remaining % of FFAs not reacting, which is consistent with the predicted stoichiometric reaction. The 3:1 and 5:1 molar ratio have been shown to be the most effective in removing all glycerides. However, it is vital to emphasize the inhibitory impact of ethanol on lipase activity and the need of carefully selecting the ideal ratio for efficient and effective glycerides reduction. So, it was expected that reasons molars of ethanol / oil very high oil would result in low yield. The results show that an increase in ethanol amount when it was completely added at the beginning of the reaction leads to a decrease in glycerides conversion. However, what was observed was a high yield of FFAs for the 7:1 and 9:1 molar ratio. This can be justified by the additions of ethanol in stages, that

minimize the harm done by ethanol on the enzyme (Gomes et al., 2020). There was no significant difference between the ethanol to RBAO ratio of 3:1 and 5:1 ($p > 0.05$, t-test). Therefore, the ethanol to RBAO ratio of 3:1 and 5:1 was chosen in the following experiments.

Several studies have reported different optimum molar ratios for similar reaction systems, which were dependent on the type of lipase and substrate. Corrˆea et al. (2011) studied the effect of methanol and ethanol to FFAs molar ratio during the esterification of palm oil fatty acid distillate (PFAD) from 1:1 to 4:1 molar ratio towards two type of lipases (Novozyme 435 and Lipozyme RM-IM) and showed that different type of alcohol, give different performance. Danta et al. (2013) studied the effect of alcohol: oil molar ratio on the canola oil transesterification reaction in solvent-free medium using free lipase from *Thermomyces lanuginosus* and *Burkholderia cepacia*. Ghosh and Bhattacharyya (1995) esterified acid oils (coconut, soybean, mustard, sunflower and rice bran) with different type of alcohols in 1:1 molar with 10% (by weight of the acid oils) of *M. miehei* lipase. Then the percent conversion of acid oils into esters is directly related to the free fatty acid content and neutral glycerides content of C8 - C18 alcohols. N. Choi et al. (2016) using RBAO was mixed with ethanol at a molar ratio of 1:1 to 1:5 with 5% of lipase (wt. of RBAO) at 300 rpm and 30°C. They evaluate the performance of 3 type of lipases (Novozym 435, Lipozyme RM IM, and Lipozyme TL IM) and indicated that a molar ratio of 1:5 (acid oil to ethanol) was optimum for the transesterification of the acid oil with ethanol in the presence of the three different lipases. These results also consistent with Yomi Watanabe, Nagao, et al. (2007) for the synthesis of biodiesel by the enzymatic transesterification of rapeseed acid oil with methanol (1:1 to 1:10 molar ratio) at 30C,

24 hr with 10% of immobilized *C. antarctica* lipase, produce biodiesel more than78wt% when using 3–7.5 molar amounts of methanol.
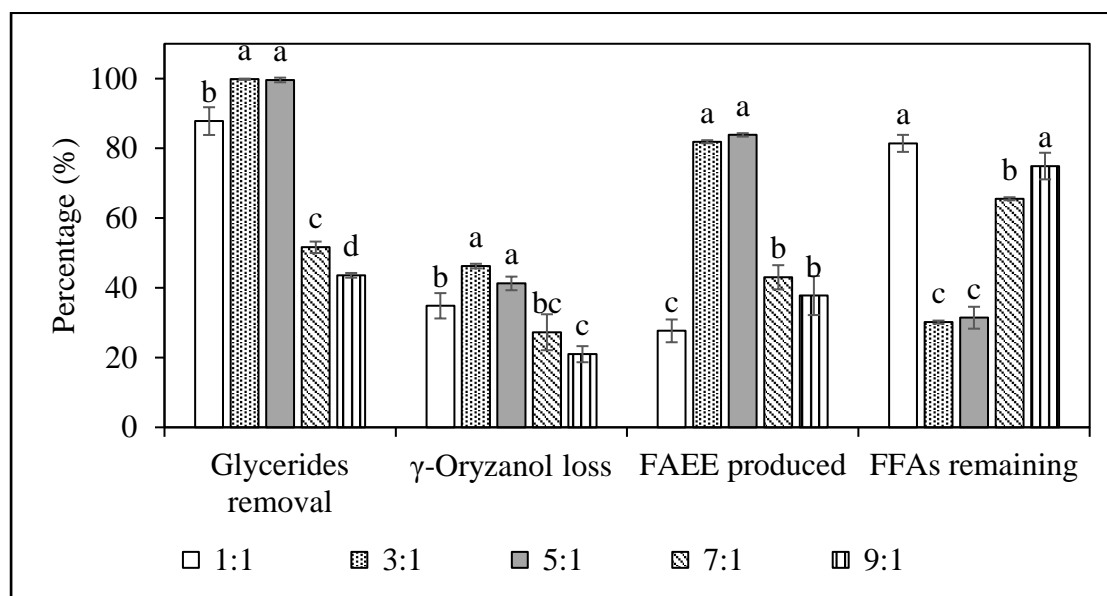
Figure 20: Glycerides removal, γ-oryzanol loss, FAEE produced, and FFAs remaining at different RBAO to water ratio at 40°C, 200 rpm, 10% lipase loading, and 24hr (n=3), there was significant difference in glycerides removal ($p = 6.79 \times 10^{-12}$), γ-oryzanol loss ($p = 7.29 \times 10^{-6}$), FAEE produced ($p = 1.79 \times 10^{-9}$), and FFAs remaining ($p = 5.31 \times 10^{-9}$).

4.3.2   Effects of Temperature

Lipase is an enzyme that catalyses the esterification/transesterification of glycerides, resulting in the formation of FAEE and free fatty acids (FFAs). However, lipase features, notably the ideal temperature, can influence the efficacy of its action. The temperature of the reaction is a significant factor as it affects both the enzymatic

reaction rate and the amount of by-products generated. Temperature enhances the speed and mobility of the enzyme and substrate, leading to increase enzyme activity (M. S. e. Antczak, A. Kubiak, T. Antczak, & S. a. Bielecki, 2009). Consequently, this has an impact on the efficacy of glyceride removal, γ-oryzanol loss, FAEE production and FFAs remaining. According to Figure 21 in this study, the maximum molar conversion (100 %) was archieved at a reaction a reaction temperature of 30 to 50°C for a 3:1 buffer ratio and 40°C for a 5:1 buffer ratio for RBAO. However, It has been discovered that molar conversion decreases beyond a certain temperature due to activity loss and denaturation. The study found a decrease in molar conversion after 50°C and 40°C of 3:1 and 5:1 ratio, respectively, indicating a connection between temperature and enzymatic efficiency in lipase-catalyzed processes. This demonstrates the nonviability of lipase to react at higher temperature, which is supported by the fact that lipase itself cannot sustain high temperatures. According to the manufacturer, this industrial lipase can sustain up to 55°C and start to deactivate after that temperature.

Another trend that has been observed is the loss of γ-oryzanol with increasing temperature. It is believed that the loss of γ-oryzanol might due to its polarity being similar to that of glycerides. It is important to note that different varieties of lipase have varying activation temperatures ranging from 30 to 70°C. Lipase, being a protein-based enzyme, is susceptible to thermal instability at high temperature (Mala & Takeuchi, 2008) which can decrease enzyme stability by disrupting the hydrogen bonds in the enzyme. When the temperature was raised from 50 to 60°C, the amount of reamaining FFAs increase while the other reaction dropped. As lipase is a protein, the high temperature has already denatured it, rendering the FFAs unreacted. This

pattern has observed in previous investigations, emphasizing the importance of using appropriate temperature settings in lipase-catalyzed processes (Fields, 2011).

Finally, several studies have revealed the optimal temperature ranges for lipase-catalyzed processes. The working temperatures for a 3:1 molar ratio range from 30 to 50°C, and for a 5:1 molar ratio, the best condition for this study was found to be 40°C. It is crucial to maintain these temperatures in order to achieve maximal molar conversion and minimise by-product loss. Lipase activation temperature must also be considered, as exceeding the ideal range may result in a reduction in enzyme stability and activity. Overall, while optimising industrial processes for optimal efficiency, the effect of temperature in lipase-catalyzed reactions must be taken into account.

In general, different type of lipase have different activation temperature (Ghaly, Dave, Brooks, & Budge, 2010). Biodiesel yield is enhanced with the increase in reaction temperature, followed by a reduction trend in the yield after reaching the optimum temperature. Operating the reaction above the activation temperature leads to lipase deactivation and low glycerides conversion due to the protein based natural of lipases, which makes them easily destroyed. N. Choi et al. (2016) studied the effect of three type of lipases (Novozym 435, Lipozyme RM IM and Lipozyme TL IM) on RBAO and the tested temperature range of 10 – 60°C. The study showed that the yield increased rapidly during the early stages of the reaction for all the tested temperature, and then the rate of reacease slowed down. Matassoli et al. (2009) studied a range of 30 to 80°C with 3:1 molar ratio of ethanol and crude palm oil, and at 50°C, Lypozyme TL IM produced higher FAEE (25%) with in 4 hours.

Pooja, Anbarasan, Ponnusami, and Arumugam (2021) also studied the range of 20 to 40°C for FAME production of Kapok oil using Porcine Pancreatic lipase and showed that 35°C yielded the the highest FAME (92%). Additionally, A. and V. (2017) studied a range of 25 to 40°C on FAME production of waste sardine oil using *A. Niger* IM and showed that 30°C yieled the highest FAME after 10 hr reaction (92%) Andrade, Errico, and Christensen (2017) evaluates from 30 to 50°C on FAME production of castor oil using Novozyme 435 lipase and showed that 35°C yielded the highest FAME (94.2%).
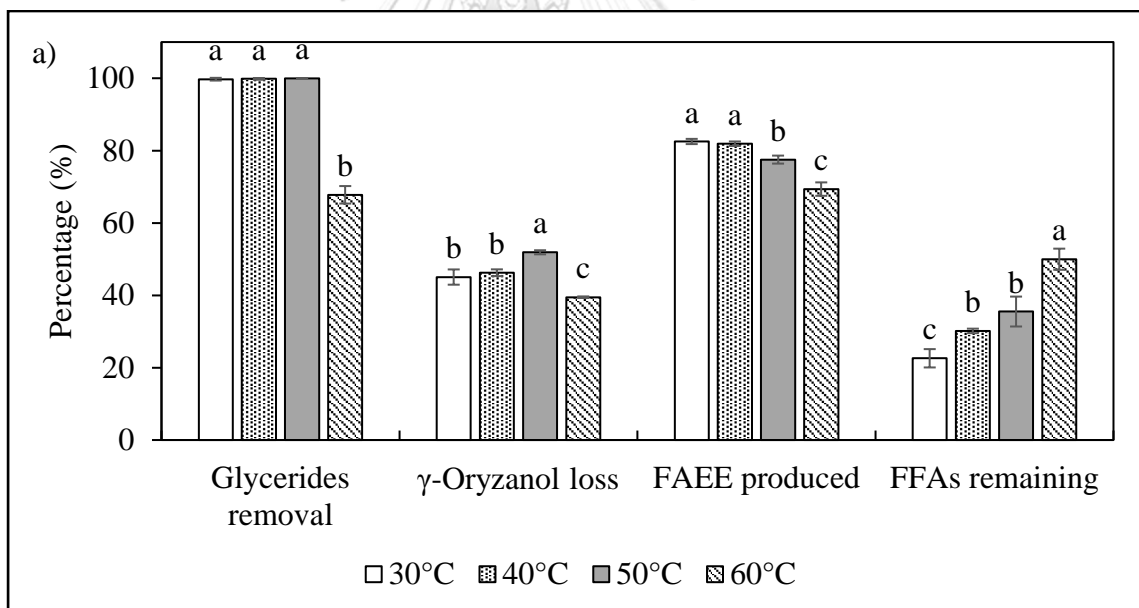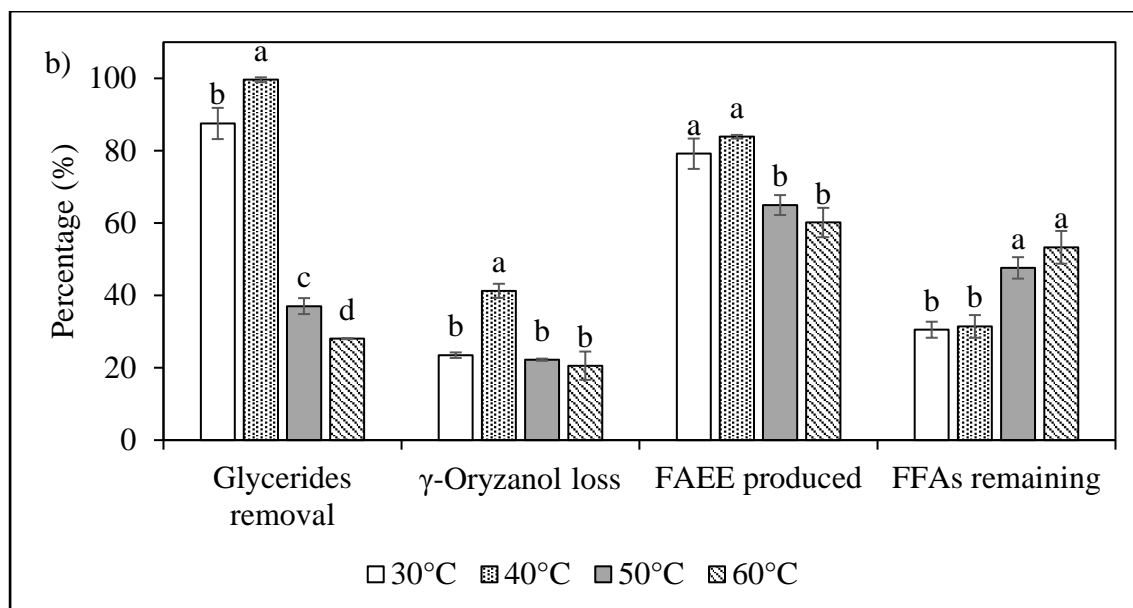
Figure 21: Glycerides removal, γ-oryzanol loss, FAEE produced and FFAs remaining

at different temperature at 200 rpm, 10% lipase (%wt RBAO), 24hr reaction (n=3);

(a) 3:1 molar ratio, there was significant difference in glycerides removal (p =

1.68×10^{-9}), γ-oryzanol loss (p = 1×10^{-6}), FAEE produced (p = 1.98×10^{-6}), and FFAs

remaining (p = 2.89×10^{-7}) and (b) 5:1 molar ratio, there was significant difference in

glycerides removal (p = 3.65×10^{-8}, γ-oryzanol loss (p = 1.54×10^{-4}), FAEE produced

(p = 1.49×10^{-5}) and) FFAs remaining (p = 9.5×10^{-5}).

4.3.3   Effect of reaction time

The main reason to study the effect of time reaction is to determine the optimal time that maximizes production while minimizing energy waste. This allows the enzymatic reaction to reach optimum and achieve high productivity. Figures 22a and b demonstrate that the optimal removal of glycerides (100%) was attained at 18

and 24 hours for both conditions (stationary phase). This is due to the molar ratio, which influences the performance of lipase during the reaction. Higher molar ratios take more time to react, resulting in longer reaction times for glycerides elimination.

Surprisingly, it was discovered that increasing reaction time had no influence on the substance of the responses. In fact, the losses of γ-oryzanol were not significantly different over this reaction period, with approximately 45 % loss for both conditions at 18 hr and 24 hr. This indicated that extending the reaction time beyond the optimum had no impact. This observation demonstrates that γ-oryzanol losses are proportional to glycerides removal. It is evident that if the glycerides do not react, no γ-oryzanol loss occurs. One possible explanation for this phenomenon is that the initial reaction includes converting existing FFAs to FAEE and glycerides, which then convert to mono- and di- before ultimately reacting to form FAEE. This discovery further demonstrates that γ-oryzanol losses parallel glycerides removal. It is obvious that if glycerides do not react, no γ-oryzanol loss occurs.

In general, during enzymatic reaction can be divided into 6 phases (lag, accelerated growth, exponential growth, decelerated growth, stationery and death phase) (Lee, 1992). In the first phase, over 78%of FFAs was converted into FAME within 12 hour, so the ethanol was easy to react with FFAs and glycerides (92%). Possibly, the first reaction utilizes the existing FFAs to convert to FAEE and glycerides to convert to mono- and di- before producing FAEE. In the second phase, the reaction rate slowed, extending from 12 to 18 h, but the conversion of FAEE (78%) and glycerides (9% increase) continues. In the third phase, the reaction

approached equilibrium after 18/24 h, and prolonging the reaction time did not significantly increase the conversion of glycerides removal and FAEE.

In conclusion, the study found that the optimal response time for both buffer ratios of 3:1 and 5:1 is 18 hours and 24 hours, respectively. This is crucial information for companies and researchers working on biofuels and other glycerides transesterification process, as it emphasises the relevance of molar ratio and reaction duration. In industrial practice, prolonging reaction time can be used to complete a batch but usually not favored due to increased cost. The reduction of FFAs is also affected by the methanol-to-FFAs molar ratio (Chai, Tu, Lu, & Yang, 2014). 5:1 molar ratio, takes longer time due to water producing during the reaction, as theoretical only required 3 mol of ethanol is required. This similar with the study by M. S. e. Antczak et al. (2009) which found that the molar ratio affect the enymatic reaction. Others reseachers also obtained semilar results. For example, Chang, Chan, and Song (2021) evaluated the effect of FAME of palm oil using Eversa lipase up to 24 hr of reaction and, showed that the FAME reach the stationary phase at 20hr of reaction, Vipin, Sebastian, Muraleedharan, and Santhiagu (2016) studied the effect of time up to 48hr of the FAME production of Jathropa oil using Rhizopus Oryzae lipase and found that after 30hr, the reaction rate started to decrease, Rahman, Chaibakhsh, and Basri (2011) studied the time reaction of adipic acid and butanol with Novozym 435 up to 600min and showed that the highest FAME was produced at 400min. N. Choi et al. (2016) mentioned the esterification of RBAO with Novozym 435, Lipozyme RM IM, and Lipozyme TL IM reached the optimum time at 18 hr for Novozym 435 and Lipozy me RM IM, while for Lipozyme TL IM, the yield continued to increase after 24hr and Corrˆea et al. (2011) compared Novozym 435,

Lipozyme RM-IM and Lipozyme TL IM up to 2.5h of palm oil fatty acid distillate reaction with ethanol, and found that the optimum time is 2 hr for all types of lipase was 2 hr, resulting 100% conversion for Novozymes, 55% with Lipozyme RM IM and 20% with Lipozyme TL IM.


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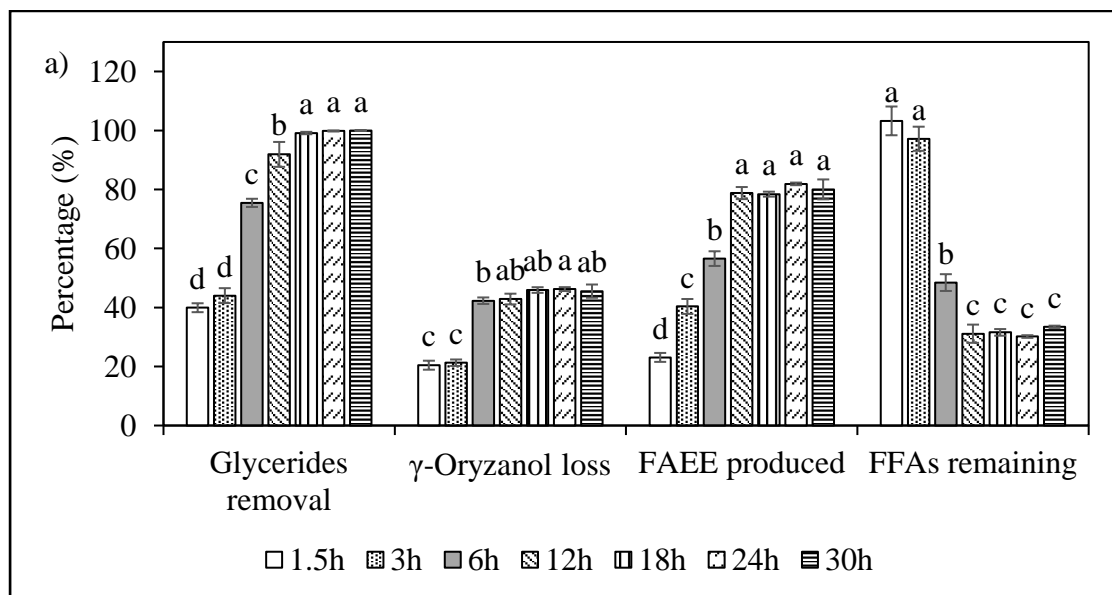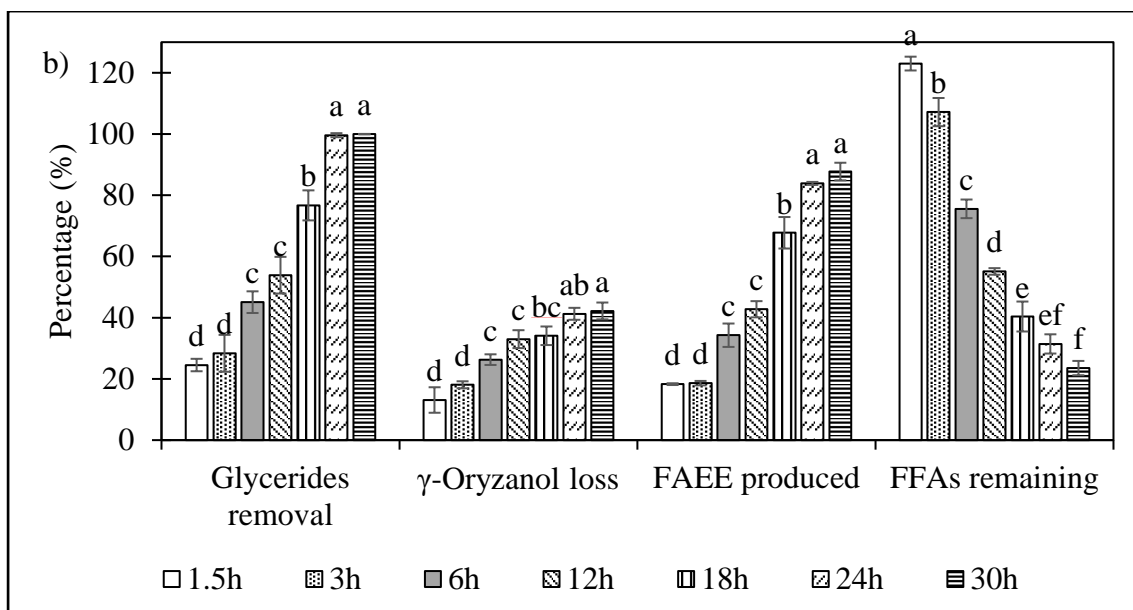
Figure 22 : **G**lycerides removal, γ-oryzanol loss , FAEE produced, and FFAs remaining at different time at 40°C, 200 rpm, 10% lipase (%wt RBAO), (n=3); (a) 3:1 molar ratio, there was significant difference in glycerides removal (p = 1.25×10^{-7}), γ-oryzanol loss (p = 8×10^{-3}), FAEE produced (p = 3.13×10^{-6}), and FFAs remaining (p = 9.47×10^{-6}), and (b) 5:1 molar ratio, there was significant difference in glycerides removal (p = 1.29×10^{-8}), γ-oryzanol loss (p = 9.33×10^{-5}), FAEE produced (p = 6.42×10^{-9}), and FFAs remaining (p = 1.49×10^{-8}).

4.3.4   Effect of Lipase Loading

Another factor that significantly contributes to costs is the price of the enzyme itself, which is higher compared to chemical catalysts. To assess the effect of varying enzyme amounts relative to oil quantity, five different experiments were conducted, using enzyme-to-oil proportions of 5%, 7.5%, 10%, 12.5%, and 15% (w/w) of RBAO

at 3:1 and 5:1 molar ratios. Figure 23 illustrates the results obtained in this study, and except for the experiment using 10% (w/w) of enzyme, all the results were satisfactory. In all cases, the yield of glyceride removal was nearly 100% for both molar ratios (3:1 - Figure 23a and 5:1 - Figure 23b).

This is because the activity of the lipase increased with an increase in the amount of enzyme. This can be attributed to the higher number of available active sites with a higher loading of enzyme, which encourages more reactions to occur (Marangoni, 2003). The study revealed that applying a 10% lipase loading was sufficient to remove nearly all glycerides in both conditions. This research suggests that increasing the lipase loading may not always yield better results. Instead, it may saturate the active site of the lipase, leading to substrate limitation. Furthermore, the researchers discovered that γ-oryzanol loss followed the same pattern as glyceride loss due to their identical polarity. Additionally, the 45% and 40% losses for the 3:1 and 5:1 molar ratios, respectively, were within acceptable limits.

Surprisingly, there was no significant variation in yield between 10% and 15% lipase loading, which is consistent with earlier research. The conversion of glycerides to FAEE and FFAs at a 3:1 molar ratio can be observed by the amount of FAEE produced, which showed no significant difference as the lipase increased from 10% to 15%. However, the amount of FFAs produced continued to increase because the FFAs produced did not react with ethanol to form FAEE. On the other hand, for the 5:1 molar ratio, increasing the lipase loading resulted in an increase in FAEE production and a decrease in remaining FFAs. This indicates that there was still ethanol available to convert FFAs to FAEE.

In a previous study, N. Choi et al. (2016) evaluated the enzyme loading ranging of 2.5 to 15% for esterification of RBAO using Novozym 435, Lipozyme RM IM, and Lipozyme TL IM. The optimal enzyme loadings were determined to be 5% for Lipozyme RM IM and 10% for Novozym 435 and Lipozyme TL IM.. Rahman et al. (2011) studied the reaction between adipic acid and butanol with Novozym 435, using enzyme loadings ranging from 20 to 400 mg, and found that the highest FAME production occurred at 400 mg of lipase. Andrade et al. (2017) investigated FAME production from castor oil using Novozyme 435 with enzyme loadings ranging from 2% to 10%, and showed that 10% lipase loading resulted in the highest FAME production (83%). Corrˆea et al. (2011) compared Novozym 435 and Lipozyme RM-IM for palm oil fatty acid distillate with ethanol, using enzyme loadings from 0.1% to 9%, and found that the optimal loading was 0.5% for Novozymes 435 and 6% for Lipozyme RM IM. These results suggest that using a lower amount of lipase loading can still achieve high yields of FAEE while reducing the cost of the enzyme. Based on this, the researchers decided to utilize a 10% lipase loading in future research to save on enzyme costs.
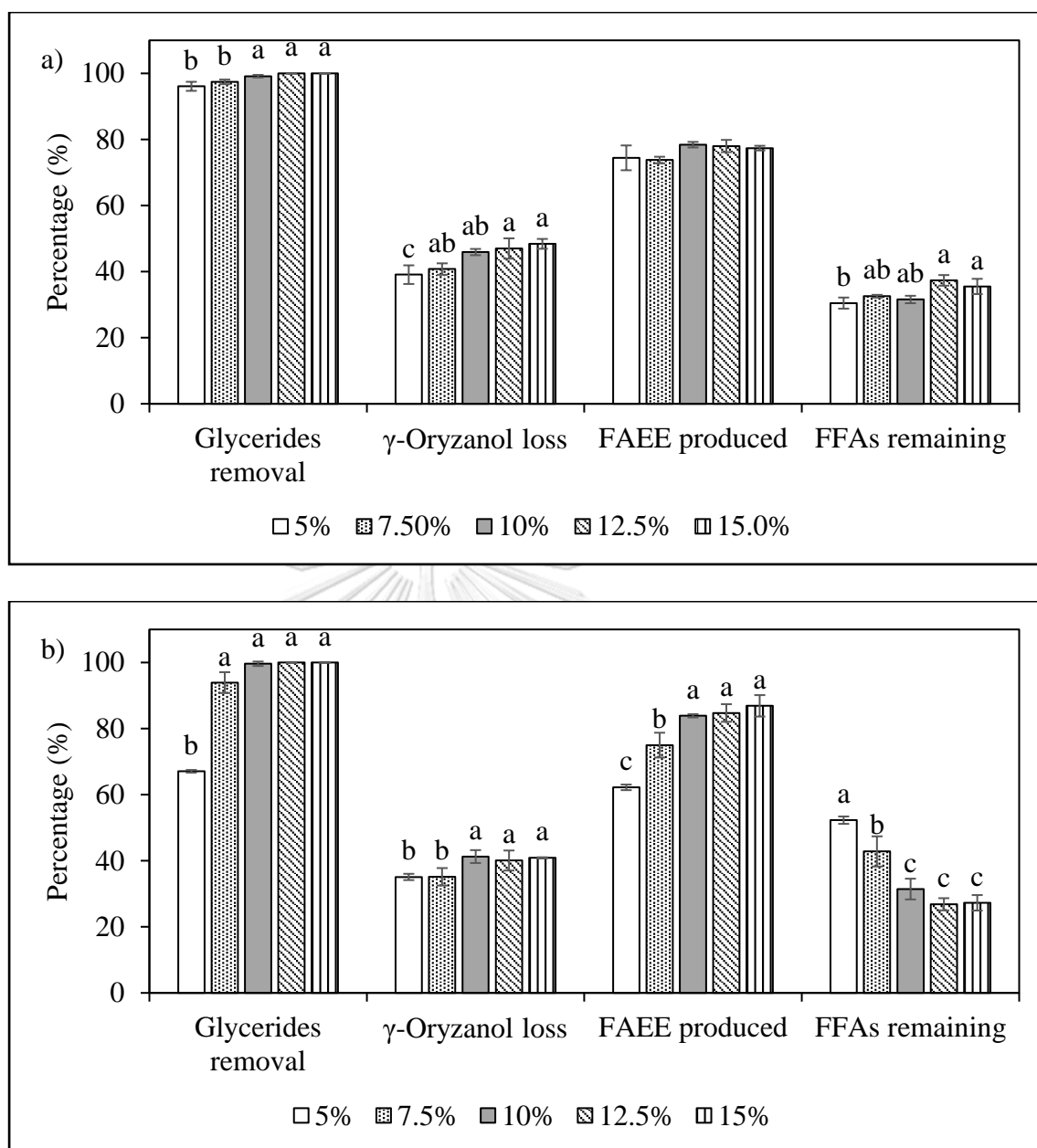
Figure 23: Glycerides removal, γ-oryzanol loss, FAEE produced, and FFAs

remaining at different lipase loading at 40°C, 200 rpm, 24 hr (n=3); (a) 3:1 molar

ratio, there was significant difference in glycerides removal (p = 1.04×10⁻⁵), γ-

oryzanol loss (p = 5.82×10⁻⁴), and FFAs remaining (p = 0.0113), and (b) 5:1 molar

ratio, there was significant difference in glycerides removal (p = 6.08×10⁻⁷), γ-

oryzanol loss (p = 0.0071), FAEE produced (p = 5.71×10^{-7}), and FFAs remaining (p = 5.24×10^{-6}).

4.3.5 Effect of Speed

The last factor to evaluate is the effect of agitation speed during enzymatic esterification/transesterification reactions, which was tested at four different speeds ranging from 200 to 400 rpm using a 4 cm length magnetic stirrer. The results are represented in Figure 24. The study considered two different molar ratios: a) 3:1 and b) 5:1. For both conditions, there was not a significant increase in glyceride removal, although statistical analysis indicated a significant difference with p-values = 0.006 for the 3:1 molar ratio (only a 1% increase in glyceride removal). It was observed that increasing the speed also increased the reaction performance (Phuah et al., 2012). owever, in the 5:1 molar ratio, an increase in agitation speed did not have a significant effect on glyceride removal for both conditions.

Furthermore, an increase in reaction time did not impact the content of all response variables, as reported in previous research. This suggests that the reaction had already reached its maximum yield, and additional reaction time did not lead to any further reactions. In terms of γ-oryzanol losses, both conditions (3:1 and 5:1 molar ratios) showed no significant difference. This indicates that speed does not influence the amount of losses. Therefore, an agitation speed of 200 rpm is suitable for glyceride removal and γ-oryzanol losses.

In addition to glyceride removal and γ-oryzanol loss, two other related responses are the production of FAEE and remaining FFAs. At the 3:1 molar ratio, there was no significant difference in FAEE production, but there was an increase in the amount of FFAs. This may be due to the decrease in droplet size at higher speeds, which improves the interface and triglyceride reduction(Murty et al., 2002a). However, a higher mixing rate subjects the enzyme to shear stress, leading to enzyme denaturation During the esterification/transesterification reaction, the increased interface between oils and the lipase-ethanol solution facilitates the initiation of the reaction(El-Batal, Farrag, Elsayed, & El-Khawaga, 2016). It indicates that no further reaction of FFAs with ethanol to produce FAEE occurs, and the reaction has reached its maximum due to the deactivation of the lipase in ethanol (Danta et al., 2013) at the 3:1 molar ratio. In the 5:1 molar ratio, the decrease in FAEE production may be attributed to the decreased contact surface between the aqueous phase (oil and solvent) and the lipase.

The results showed that at the 3:1 molar ratio, increasing the speed from 200 to 400 rpm resulted in an increase in remaining FFAs from 31% to 40%. However, the speed did not significantly affect the production of FAEE or glyceride loss, although there was a slight increase of 1%. At a speed of 400 rpm, the amount of FAEE decreased from 84% to 81%, which could be attributed to the decreased contact surface between the aqueous phase (oil and solvent) and the lipase. However, the study found that increasing the speeds did not significantly affect the reaction. When scaled up to 20 times, at the same speed of 200 rpm, there was no significant difference in the amount of glyceride removal.

Previous research has also yielded similar results. For example, El-Batal et al. (2016) studied FAME production from waste cooking oil using A. Niger lipase at different speeds (100 to 500 rpm), and 400 rpm yielded the highest FAME production (88%). Lan and Hoa (2015) evaluated Candida rugosa Type VII - L1754 (LCR) and Porcine pancreas (LPP) at speeds ranging from 150 to 400 rpm on coconut oil for FAEE production, and found that 200 rpm yielded the highest FAEE for LPP (39.11%), while 250 rpm yielded the highest FAEE for LCR (33.17%). Meanwhile, Basri, Kassim, Mohamad, and Ariff (2013) studied the production of palm oil esters using Lipozyme TL IM and palm oil with oleyl alcohol at speeds ranging from 250 to 350 rpm, and found that 325 rpm resulted in the highest yield (74.50%). D.Surendhiran and M.Vijay (2014) investigated FAME production from Nannochloropsis oculata oil using Lipozyme TL IM and Burkholderia cepacia MTCC46841 with methanol at speeds ranging from 100 to 500 rpm, and found that 400 rpm yielded the highest yield (95%). Q. Li and Yan (2010) studied biodiesel catalyzed by immobilized Pseudomonas cepacia lipase from Sapium sebiferum oil with methanol at speeds ranging from 100 to 250 rpm, and found that 200 rpm yielded the highest yield (90%).

In conclusion, this study recommends using an agitation speed of 200 rpm due to energy consumption considerations. Overall, the study provides valuable insights into the effects of agitation speed on biodiesel production using lipase-catalyzed transesterification.
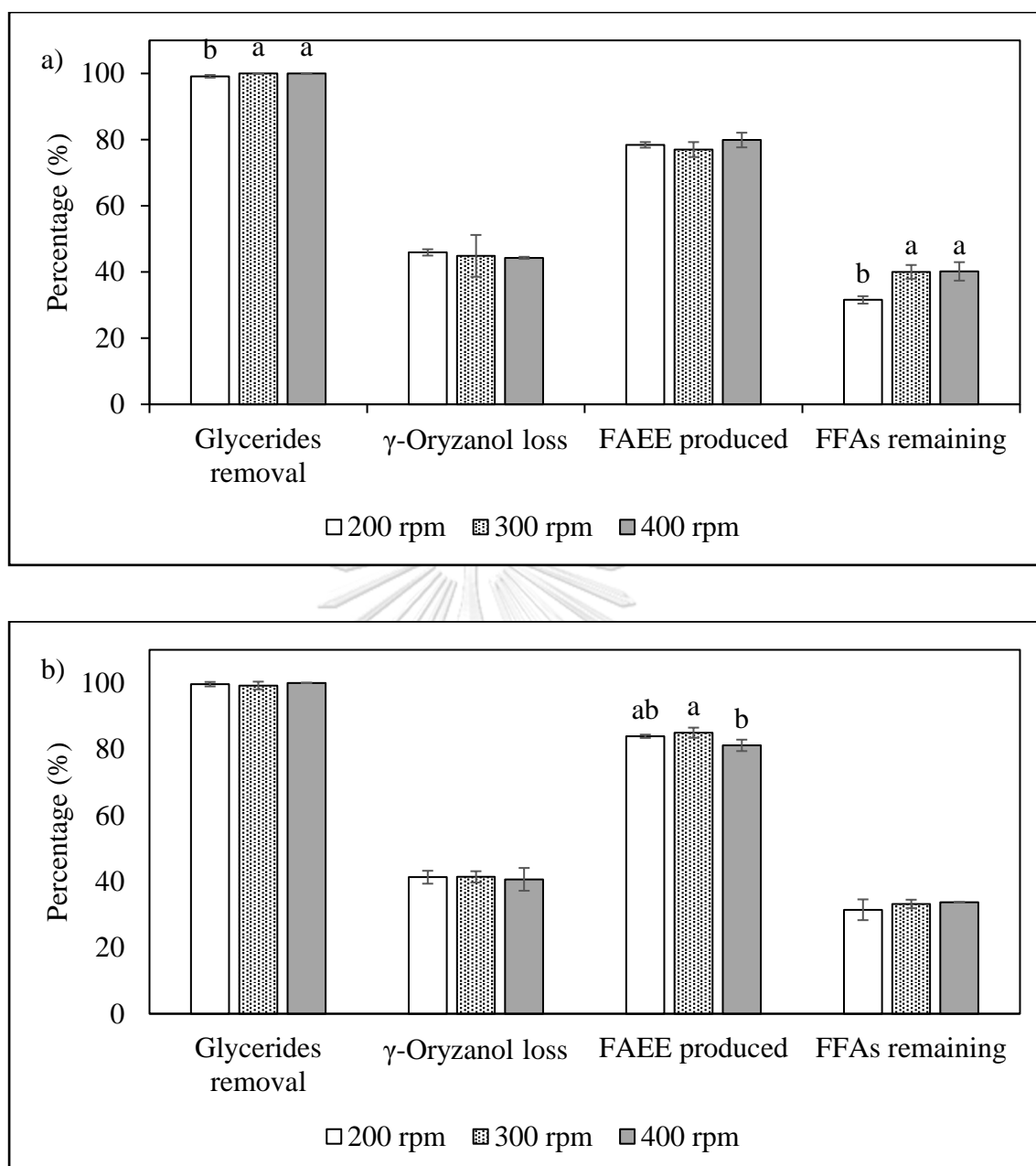
Figure 24: Glycerides removal, γ-oryzanol loss, FAEE produced, and FFAs

remaining at different lipase loading at 40°C, 10% lipase (%wt. RBAO), 24 hr (n=3);

(a) 3:1 molar ratio, there was significant difference in FAEE produced (p = 0.0334),

and (b) 5:1 molar ratio, there was significant difference in FFAs remaining (p =

0.0037), and glycerides removal (p = 0.0059)

In overall, the production of biodiesel from RBAO or RBO has already been studied before and summarized in Table 13 below. It showed that this study can produce more than 75% of biodiesel with almost 100% glycerides removal/conversion at 3:1 and 5:1 molar ratio within acceptable operating conditions.

Table 13: Summary of the biodiesel production from RBAO and RBO

References	Raw material	Catalyst / alcohol	Glycerides Removal	Biodiesel produced (%)	Buffer ratio (Buffer: Raw material)	Time (hr)	enzyme loading	Temp (°C)	Speed (rpm)
(Sombutsuwan et al., 2018)	RBAO	H_2SO_4 Ethanol	-	-	40:1	2	10%	60	-
(Niawanti & Zullaikah, 2017)	RBO	H_2SO_4 Methanol	-	89%	10:1	8	1%	60	300
(N. Choi et al., 2016)	RBAO	Novozym 435 Lipozyme RM IM Lipozyme TL IM Ethanol	-	93% 80%, 88%	5:1	24	10% 5% 10%	40 30 30	300
(Z. Li et al., 2010)	RBO	Candida rugosa Methanol	-	80%	4.058:1	48	6.86%	42	-
(Novy S. Kasim et al., 2007)	RBO	H_2SO_4 Methanol	22%	84%	5:1	2	2%	60	-
(C.-C. Lai et al., 2005)	RBO	Novozym 435 IM 60 Methanol	100% 89.4%	98% 74%	3.6:1	7	5%	50	150
(Zullaikah, Lai, Vali, & *, 2005)	RBO	H_2SO_4 Methanol	-	74% FFAs-96% 63%FFAs-85% 49%FFAs-70%	10:1	24	2%	60	300

References	Raw material	Catalyst / alcohol	Glycerides Removal	Biodiesel produced (%)	Buffer ratio (Buffer: Raw material)	Time (hr)	enzyme loading	Temp (°C)	Speed (rpm)
				24%FFAs- 60% 7%FFAs- 60%					
(Zullaikah, Rakhadima, Rachimoellah, Widjaja, & Sumarno, 2014)	RBO	H_2SO_4 Methanol	-	0.01%- 67% 0.02% - 73% 0.05%-75%	15:1	5	0.01- 0.05%	68	200
(Ghosh & Bhattacharyya, 1995)	RBAO	M.miehei, Different type of Alcohol (C_4 – C_{18})	-	>73%	1:1	4	10%	60	-
This study	RBAO	Aspergillius Niger Ethanol	>98%	>78%	3:1 5:1	18 24	10%	40	200

4.3.6　γ-oryzanol extraction

In recent years, there has been a growing demand for the production of eco-friendly, renewable fuels, such as biodiesel. Biodiesel is obtained from vegetable oils or animal fats and can be used as a replacement for petroleum-based diesel. Numerous studies have focused on improving the efficiency of biodiesel production, including the extraction of γ-oryzanol from rice bran oil. Previous studies have also investigated the extraction of γ-oryzanol from biodiesel from various sources. Figure 25a) and b) in this study demonstrates the extraction of γ-oryzanol using different molarities of aqueous ethanolic NaOH solution from biodiesel for 3:1 and 5:1 molar ratio. The results indicate that the amount of γ-oryzanol extracted increased by up to 15% with an increase in the molarity of the solution. However, the extraction did not show significant differences beyond 2.0 M. This suggests that the protonation and deprotonation of γ-oryzanol had reached their maximum, and further increasing the molarity of the base solution did not have a significant effect on the extraction.

Overall, this study found that the extraction of γ-oryzanol using an aqueous ethanolic NaOH solution was more effective than the conventional method, which involved acid esterification. The use of an aqueous ethanolic NaOH solution allowed for extraction to be conducted at room temperature, reducing production costs. Additionally, the study showed a 7% higher extraction yield compared to the previous acid esterification method. This study supports the findings mentioned by Sombutsuwan et al. (2018) and demonstrates superior results compared to other studies presented in Table 14. These findings represent a significant advancement in improving the efficiency of biodiesel production using a lipase catalyst. The study

also suggests that increasing the molarity of the base solution beyond 2.0 M does not substantially improve the extraction. Therefore, it is important to use the optimal concentration of the base solution to maximize extraction efficiency.

Finally, it is worth noting that this study found a potential 7% increase in biodiesel production using a lipase catalyst through γ-oryzanol extraction using this method. This further underscores the potential of this approach to enhance the efficiency of biodiesel production, making it more cost-effective and environmentally friendly. In conclusion, the study has demonstrated that using an aqueous ethanolic NaOH solution for γ-oryzanol extraction is a more effective and cost-efficient method compared to traditional approaches. The findings of this research have significant implications for biodiesel production, enabling industries to produce high-quality biodiesel in a more sustainable and economically viable manner.
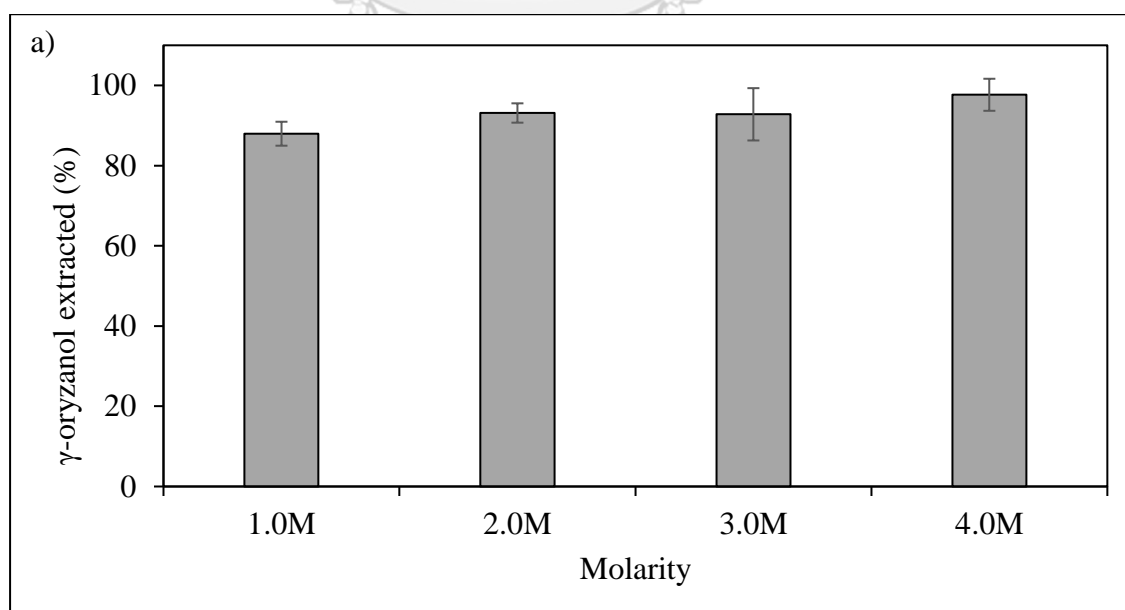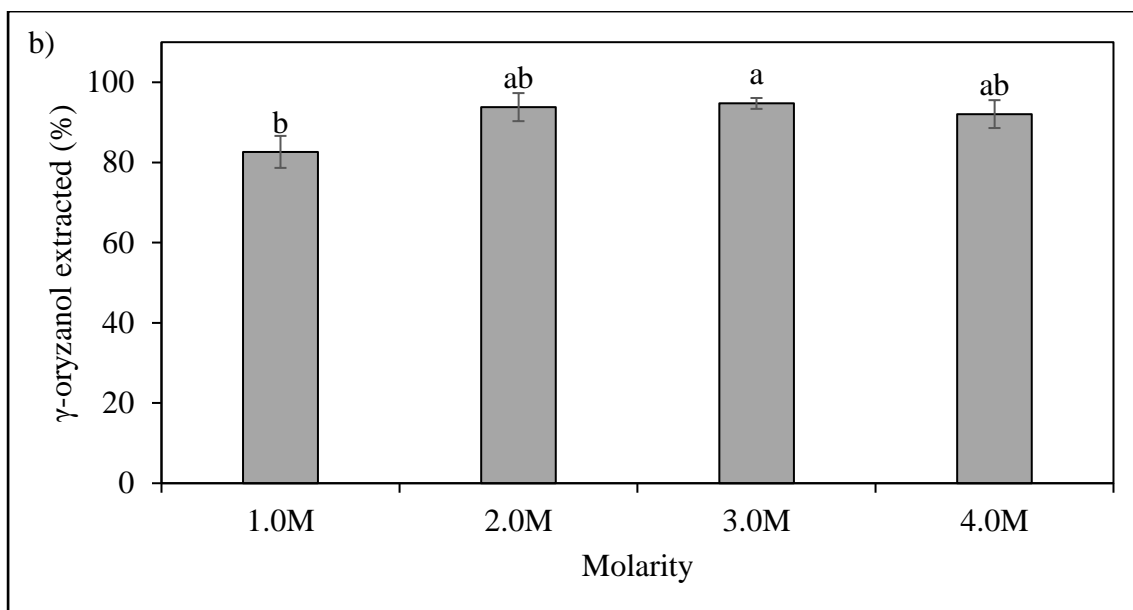
Figure 25 : γ-oryzanol extraction at different molarity of aqueous ethanolic NaOH

solution at room temperature, 1minutes (n=3); a) 3:1 with no significant difference

and b) 5:1 molar ratio, there was a significant difference γ-oryzanol extracted from

FAEE (p = 0.03).

Table 14: γ-oryzanol extraction

References	Extraction Solvent/ solution	Solvent Molarity	Ratio	Time	Temperature (°C)	Speed (rpm)_	γ-oryzanol recovery
(Sombutsuwan et al., 2018)	Aqueous ethanolic NaOH	1.68M AEN + hexane (75:25)	10:1	1min	Room	-	76%
(Niawanti & Zullaikah, 2017)	Deep Eutectic Solvent	choline chloride and ethylene glycol	2:1 4:1	240 min	30	-	98%
(Novy S. Kasim et al., 2007)	Hexane, methanol, acetonitrile	1.Hex + methanol 2.Hex + acetonitrile 3. 3 step Hexane : ethyl acetate Hexane : ethyl acetate Acetone	10ml: 0.65g 10ml: 0.65g 400ml:0.4ml: 0.95g 400 ml: 44ml 400ml	- - 6hr 6hr 30 min	- - - - -	- - 300 300 300	57% 88% after 8times 2.1% 97.6% 0%
This study	Aqueous ethanolic NaOH	AEN +hexane (75:25) 1M 2M -4M	10:1	1min	Room	200	83% 94%

4.4 Conclusion

The main hurdle for the recovery of γ-oryzanol by removing/ converting glycerides to FAEE is the cost of lipase. In this study, the suitable conditions for glycerides removal from RBAO were examined. Glycerides removal can be archived up to 100% with γ-oryzanol loss of 40 and 45% for 3:1 and 5:1 molar ratio respectively. Then, γ-oryzanol extraction with a recovery of a minimum of 80% was successfully recovered from FAEE using an aqueous ethanolic NaOH solution. The application of γ-oryzanol in food, cosmetic and pharmaceutical industries should reduce the cost of FAEE production using waste in the rice bran oil industry. And the use of lipase and base solution (aqueous ethanolic NaOH) are far more effective and efficient for γ-oryzanol extraction. A precise method for glycerides removal and γ–oryzanol extraction from RBAO was established by using a solvent with low toxicity and a low temperature. A high glycerides removal could be obtained using the following extraction conditions: 40° C, 18hr, 10% lipase loading, 200rpm at 3:1 molar ratio and 40° C, 24hr, 10% lipase loading, 200rpm at 5:1 molar ratio. RBAO is one of the richest sources of γ–oryzanol. A two-step process was developed to recover γ-oryzanol from RBAO. In the first step, enzymatic esterification/transesterification was employed to remove the impurities (glycerides and FFAs) into the FAEE phase. In the second step, γ–oryzanol was recovered using base liquid extraction with recovery (>80%).

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1   Overview

RBO has recently gained wide attention as a healthy cooking oil among the consumers. RBAO, a byproduct obtained during the RBO refinery, is an alternative source for γ-oryzanol which is demanded greatly in industries applications. This thesis evaluates the pretreatment step for utilization of RBAO as raw material for γ-oryzanol production. Here, enzymatic reactions were used; hydrolysis and esterification/transesterification, for removal of glycerides in RBAO, which are considered as major impurities for subsequent recovery of γ-oryzanol.

5.2   Conclusions

As a byproduct of the RBO refining, RBAO has been drawing interest due to its high γ-oryzanol content. Nevertheless, due to the presence of other chemicals, subsequent γ-oryzanol separation is not trivial. Due to their comparable physicochemical properties, glycerides are identified as main impurities that must be removed before the γ-oryzanol recovery. The objective of this study is to investigate enzymatic reactions: hydrolysis and esterification/transesterification, with the use of

A. Niger lipase to initially convert glycerides in RBAO into other separable compounds: FFAs and biodiesel, and their reaction effects on γ-oryzanol.

In Chapter 3 describing the 1st part of this study, effects of reaction conditions in enzymatic hydrolysis of RBAO: water to RBAO ratio, time of reaction, temperature, amount of lipase loading, and speed, were first studied on the degrees of glycerides removal, γ-oryzanol loss, FFAs production. Optimal levels of three key reaction conditions: reaction time, temperature and water:RBAO ratio were determined through RSM with FCCD, which provided the maximum glycerides removal with minimum loss of γ-oryzanol. At the selected optimal condition of 22 h, 48.5 °C, and 1:1 water:RBAO ratio, the predicted responses of glycerides removal, γ-oryzanol loss, and FFAs production of 101.40%, 34.82%, and 77.96%, respectively were obtained. They were then compared with the experimental data at this condition which gave the responses of 98.82%, 31.13%, and 73.23%, respectively, showing good agreement within 95% prediction interval. Moreover, the responses from the 10x-scale experiment of 98.52%, 40.29%, and 75.01%, respectively, also in agreement with the prediction and the 1x-scale reaction.

The 2nd part of this study is described in Chapter 4, in which enzymatic esterification/transesterification were initially applied with RBAO for glycerides removal before γ-oryzanol recovery using acid-base extraction method. Effects of reaction conditions: ethanol to RBAO molar ratio, temperature, reaction time, lipase loading and speed were evaluated on the degrees of glycerides removal, γ-oryzanol loss, FAEE production, and FFAs remaining. The best conditions for enzymatic esterification/transesterification were found at 3:1 ethanol to RBAO ratio, 18 h, 10%

lipase loading, 40°C, and 200 rpm, and 5:1 ethanol to RBAO ratio, 24 h, 10% lipase loading, 40°C, and 200 rpm, which gave >98% glycerides removal, ca. 40% γ-oryzanol losses, ca. 78% FAEE produced and around 30% FFAs remaining. Both conditions are suitable to use to remove/ convert glycerides to FAEE and FFAs. To obtained highest γ-oryzanol extraction around 94%, the concentration of aqueus ethanolic NaOH solution required was 2M.

5.3   Recommendations

Based on the results and discussion present in this study, constructive suggestions are proposed for further research:

1) There was observed certain loss of γ-oryzanol during the enzymatic hydrolysis and esterification/transesterification, which should therefore be further minimized. This could be achieved by improving reaction times in the enzymatic pretreatment process. Sonication may be applied to the enzymatic reactions, which could fasten the reaction and that may decrease γ-oryzanol loss and make the easy to recover. On top of that, the addition of ultrasonic can make the reaction shorter.

2) This study used free lipase of industrial grade, nonetheless there are a number of lipases that can be used for this application. Immobilized lipases have shown to enhance the reaction and can be reused. They would thus be interesting to further study on effects of lipase types on the glycerides removal in RBAO during the reactions.

3) Further recovery of γ-oryzanol as well as other existing bioactive compounds in RBAO would be another future work. The recovery/purification techniques include normal phase chromatography, extraction, precipitation, and crystallization, which can be applied after the enzymatic reactions. The future studies could involve evaluating operating conditions for recovery/ purification of bioactive compounds as well as determining optimal conditions.

4) Water and ethanol were used as acyl acceptors in this study for the enzymatic hydrolysis and esterification/transesterification reactions, respectively. It would be interesting to study other solvents which can be acyl acceptors for hydrolysis such as sodium acetate, tris, phosphate buffer, and for esterification/transesterification such as methanol, isopropanol, and butanol. Different solvents could give different effects on the reactions. In addition, these solvents can combine or mix at a certain ratio, which might improve glycerides removal in RBAO.

5) Economic calculation is another factor that can be considered as a future study. This is because the main cost of the proposed process/method is the lipase, which is relatively costly compared to the physicochemical methods for hydrolysis and esterification/transesterification. The advantages of using lipase can be an additional point for commercialization industry in terms of wastewater treatment. As we know, the enzymatic method do not require the neutralization step which involves chemicals.

REFERENCES

A., A., & V., P. (2017). Production of biodiesel by enzymatic transesterification of waste sardine oil and evaluation of its engine performance. *Heliyon, 3*(12), e00486. doi:https://doi.org/10.1016/j.heliyon.2017.e00486

Adachi, D., Hama, S., Nakashima, K., Bogaki, T., Ogino, C., & Kondo, A. (2012). Production of Biodiesel from Plant Oil Hydrolysates using an Aspergillus Oryzae Whole-cell Biocatalyst Highly Expressing Candida Antarctica Lipase B. *Bioresource Technology, 135*, 410-416. doi:https://doi.org/10.1016/j.biortech.2012.06.092

Aguieiras, E. C. G., Cavalcanti-Oliveira, E. D., Castro, A. M. d., Langone, M. A. P., & Freire, D. M. G. (2017). Simultaneous Enzymatic Transesterification and Esterification of an Acid Oil Using Fermented Solid as Biocatalyst. *Journal of the American Oil Chemists' Society, 94*(4), 511-630. doi:https://doi.org/10.1007/s11746-017-2964-4

Aguieiras, E. C. G., Cavalcanti-Oliveira, E. D., Castro, A. M. d., Langonec, M. A. P., & Freire, D. M. G. (2014). Biodiesel Production from Acrocomia Aculeata Acid Oil by (enzyme/enzyme) Hydroesterification Process : Use of Vegetable Lipase and Fermented Solid as Low-Cost Biocatalysts. *Fuel, 135*, 315–321. doi:https://doi.org/10.1016/j.fuel.2014.06.069

Aguilar, R., Ramıírez, J. A., Garrote, G., & M.Vazquez. (2002). Kinetic Study of The Acid Hydrolysis of Sugar Cane Bagasse. *Journal of Food Engineering, 55*, 309-318. doi:https://doi.org/10.1016/S0260-8774(02)00106-1

Ahranjani, P. E., Kazemiini, M., & Arpanaei, A. (2020). Green Biodiesel Production from Various PlantOils Using Nanobiocatalysts Under Different Conditions. *Bioenergy Research, 13*, 10. doi:https://doi.org/https://doi.org/10.1007/s12155-019-10022-9

Aladedunye, F., Przybylski, R., Rudzinska, M., & Klensporf-Pawlik, D. (2013). γ-Oryzanols of North American Wild Rice (Zizania Palustris) . *Journal of the American Oil Chemists' Society, 90*, 1101-1109. doi:https://doi.org/10.1007/s11746-013-2252-x

Andrade, T. A., Errico, M., & Christensen, K. V. (2017). Influence of the reaction conditions on the enzyme catalyzed transesterification of castor oil a possible step in biodiesel production. *Bioresource Technology, 243*, 366-374. doi:https://doi.org/10.1016/j.biortech.2017.06.118

Andrade, T. A., Martín, M., Errico, M., & Christensen, K. V. (2019). Biodiesel production catalyzed by liquid and immobilized enzymes: Optimization and economic analysis. *Chemical Engineering Research and Design, 141*, 1-14. doi:https://doi.org/10.1016/j.cherd.2018.10.026

Andreas Miller, Lina Majauskaite, & Engel, K.-H. (2004). Enzyme-catalyzed hydrolysis of g-oryzanol. *European Food Research and Technology, 218*, 349-354. doi:10.1007/s00217-003-0851-8

Anjinta, A., Usaku, C., Boonnoun, P., Daisuk, P., & Shotipruk, A. (2023). Method Development for Purification of γ-oryzanol from Hydrolyzed Rice Bran Acid Oil by Semi-preparative Chromatography. *Oils and Fats, 72*(1), 39-47. doi:https://doi.org/10.5650/jos.ess22217

Antczak, M. S., Kubiak, A., Antczak, T., & Bielecki, S. (2009). Enzymatic biodiesel

synthesis – Key factors affecting efficiency of the process. *Renewable Energy, 34*(5), 1185-1194. doi:https://doi.org/10.1016/j.renene.2008.11.013

Antczak, M. S. e., Kubiak, A., Antczak, T., & Bielecki, S. a. (2009). Enzymatic biodiesel synthesis – Key factors affecting efficiency of the process. *Renewable Energy, 34*, 1185–1194. doi:doi:10.1016/j.renene.2008.11.013

Arya, M., Rout, A. K., & Samanta, S. (2022). A review on the effect of engine performance and emission characteristics of C.I. Engine using Diesel-Biodiesel-Additives fuel blend. *Materials Today: Proceedings, 51*(8), 2224-2232. doi:https://doi.org/10.1016/j.matpr.2021.11.359

Atadashi, I. M., Aroua, M. K., Aziz, A. R. A., & Sulaiman, N. M. N. (2012). Production of biodiesel using high free fatty acid feedstocks. *Renewable and Sustainable Energy Reviews, 16*, 3275-3285. doi:https://doi.org/10.1016/j.rser.2012.02.063

Avelar, M. H. M., Cassimiro, D. M. J., Santos, K. C., Domingues, R. C. C., Castro, H. F. D., & Mendes, A. A. (2013). Hydrolysis of Vegetable Oils Catalyzed by Lipase Extract Powder from Dormant Castor Bean Seeds. *Industrial Crops and Products, 44*, 452-458. doi:https://doi.org/10.1016/j.indcrop.2012.10.011

Awad, S., Paraschiv, M., Geo, V. E., & Tazerout, M. (2013). Effect of Free Fatty Acids and Short Chain Alcohols on Conversion of Waste Cooking Oil to Biodiesel. *International Journal of Green Energy, 11*(5), 441-453. doi:https://doi.org/10.1080/15435075.2012.727926

Bajaj, A., Lohan, P., Jha, P. N., & Mehrotra, R. (2010). Biodiesel Production Through Lipase Catalyzed Transesterification: An Overview. *Journal of Molecular Catalysis B: Enzymatic, 62*, 9–14. doi:https://doi.org/10.1016/j.molcatb.2009.09.018

Basri, M., Kassim, M. A., Mohamad, R., & Ariff, A. B. (2013). Optimization and kinetic study on the synthesis of palm oil ester using Lipozyme TL IM. *Journal of Molecular Catalysis B: Enzymatic, 85-85*, 214-219. doi:https://doi.org/10.1016/j.molcatb.2012.09.013

Berger, A., Rein, D., Schäfer, A., Monnard, I., Gremaud, G., Lambelet, P., & Bertoli, C. (2005). Similar cholesterol-lowering properties of rice bran oil, with varied gamma-oryzanol, in mildly hypercholesterolemic men. *Eur J Nutr, 44*(3), 163-173. doi:10.1007/s00394-004-0508-9

Boonlai, B. (2019). *Thailand: Rive Bran Oil Production*. Retrieved from In Conference Program of 6th ICRBO: http://seaofindia.com/wp-content/uploads/2019/07/4-Watcharaphon-Boonlai-min.pdf

Borges, M. E., & Díaz, L. (2012). Recent developments on heterogeneous catalysts for biodiesel production by oil esterification and transesterification reactions: A review. *Renewable and Sustainable Energy Reviews, 16*(5), 2839-2849. doi:https://doi.org/10.1016/j.rser.2012.01.071

Brenes, M., García, A., Dobarganes, C., Velasco, J., & Romero, C. (2002). Influence of Thermal Treatments Simulating Cooking Processes on the Polyphenol Content in Virgin Olive Oil. *Journal of Agricultural and Food Chemistry, 50*, 5962−5967. doi:https://doi.org/10.1021/jf020506w

Bressani, A. P. P., Garcia, K. C. A., Hirata, D. B., & Mendes, A. A. (2015). Production of alkyl esters from macaw palm oil by a sequential hydrolysis/esterification process using heterogeneous biocatalysts: optimization by response surface methodology. *Bioprocess Biosyst Eng, 38*(2), 287-297. doi:10.1007/s00449-014-

1267-5

Cardinali, A., Pizzeghello, D., & Zanin, G. (2015). Fatty Acid Methyl Ester (FAME) Succession in Different Substrates as Affected by the CoApplication of Three Pesticides. *PLoS ONE, 10*(12), e0145501. doi: DOI:10.1371/journal.pone.0145501

Carrillo, F., Lis, M. J., Colom, X., M.Lo´pez-Mesas, & Valldeperas, J. (2005). Effect of alkali pretreatment on cellulase hydrolysis of wheat straw: Kinetic study. *Process Biochemistry, 40*(10), 3360-3364. doi:https://doi.org/10.1016/j.procbio.2005.03.003

Cavalcanti-Oliveira, E. d. A., Silva, P. R. d., Ramos, A. P., Aranda, D. A. G., & Freire, D. M. G. (2011). Study of Soybean Oil Hydrolysis Catalyzed by Thermomyces Lanuginosus Lipase and Its Application to Biodiesel Production Via Hydroesterification. *Enzyme Research, 1*, 1-8. doi:https://doi.org/10.4061/2011/618692

Chai, M., Tu, Q., Lu, M., & Yang, Y. J. (2014). Esterification pretreatment of free fatty acid in biodiesel production , from laboratory to industry. *Fuel Processing Technology, 125*, 106-113. doi:https://doi.org/10.1016/j.fuproc.2014.03.025

Chang, M. Y., Chan, E.-S., & Song, C. P. (2021). Biodiesel production catalysed by low-cost liquid enzyme Eversa® Transform 2.0: Effect of free fatty acid content on lipase methanol tolerance and kinetic model. *Fuel, 283*, 119266. doi:https://doi.org/10.1016/j.fuel.2020.119266

Chen, C.-H., Chen, W.-H., Chang, C.-M. J., Lai, S.-M., & Tu, C.-H. (2010). Biodiesel production from supercritical carbon dioxide extracted Jatropha oil using subcritical hydrolysis and supercritical methylation. *The Journal of Supercritical Fluids, 52*, 228-234. doi:https://doi.org/10.1016/j.supflu.2009.12.010

Chew, Y. H., Chua, L. S., Cheng, K. K., Sarmidi, M. R., Aziz, R. A., & Lee, C. T. (2008). Kinetic study on the hydrolysis of palm olein using immobilized lipase. *Biochemical Engineering Journal, 39*(3), 516-520.

Choi, J.-S., Ron, M.-K., Kim, T.-U., Cheon, E. J., Moon, W.-S., & Kim., M.-R. (2015). Characteristics of esterified rice bran oil converted by enzymatic esterification. *Journal of Environmental Biology, 36*(6), 1247-1254.

Choi, N., Lee, J.-S., Kwak, J., Lee, J., & H, I.-H. (2016). Production of Biodiesel from Acid Oil Via a Two-Step Enzymatic Transesterification. *Journal of Oleo Science, 65*(11), 913-921. doi:https://doi.org/10.5650/jos.ess16092

Chotimarkorn, C., & Ushio, H. (2008). The effect of trans-ferulic acid and gamma-oryzanol on ethanol-induced liver injury in C57BL mouse. *Phytomedicine, 15*(11), 951-958. doi:https://doi.org/10.1016/j.phymed.2008.02.014

Chowdhury, A., & Mitra, D. (2015). A kinetic study on the Novozyme 435-catalyzed esterification of free fatty acids with octanol to produce octyl esters. *Biotechnol Prog, 31*(6), 1494-1499. doi:10.1002/btpr.2165

Corrˆea, I. d. S., Souza, S. L. d., Catran, M., Bernardes, O. a. L., FigueiredoPortilho, M. a., & Langone, M. A. P. (2011). Enzymatic Biodiesel Synthesis Using a Byproduct Obtained from Palm Oil Refining. *Enzyme Research, 2011*, 1-8. doi:doi:10.4061/2011/814507

D.Surendhiran, & M.Vijay. (2014). Biodiesel production from Nannochloropsis oculata oil using immobilized lipase with methyl acetate as an acyl acceptor. *BioTechnology:An Indian Journal, 9*(8), 327-334.

Danta, J. H., Paris, L. D. D., Barão, C. E., Arroyo, P. A., Soares, C. M. F., Visentainer, J. V., . . . Zanin, G. M. (2013). Influence of alcohol: oil molar ratio on the production of ethyl esters by enzymatic transesterification of canola oil. *African Journal of Biotechnology, 12*(50), 6968-6979. doi:10.5897/AJB2013.12313

Das, P. K., Chaudhuri, A., Kaimal, T. N. B., & Bhalerao, U. T. (1998). Isolation of γ-Oryzanol through Calcium Ion Induced Precipitation of Anionic Micellar Aggregates†. *Journal of Agricultural and Food Chemistry, 46*(8), 3078-3080. doi:https://doi.org/10.1021/jf9710802

Demirbas, A. (2006). Biodiesel production via non-catalytic SCF method and biodiesel fuel characteristics. *Energy Conversion and Management, 47*(15-16), 2271-2282. doi:Energy Conversion and Management

El-Batal, A. I., Farrag, A. A., Elsayed, M. A., & El-Khawaga, A. M. (2016). Biodiesel production by aspergillus Niger lipase immobilized on barium ferrite magnetic nanoparticles. *Bioengineering, 3*(2), 1-15. doi:10.3390/bioengineering3020014

Encinar, J. M., Gonza´lez, J. F., Rodrı´guez, J. J., & Tejedor, A. (2002). Biodiesel Fuels from Vegetable Oils: Transesterification of Cynara cardunculus L. Oils with Ethanol. *Energy & Fuels, 16*, 443-450. doi:https://doi.org/10.1021/ef010174h

Ferreira, S. C., Fernández, A. M., Bilbao, M. D. d. C., & Fernández, A. M. (2019). New Functional Ingredients From Agroindustrial By-Products for the Development of Healthy Foods. *In Encyclopedia of Food Security and Sustainability, 1*, 351–359. doi:https://doi.org/10.1016/B978-0-08-100596-5.22140-9

Fields, P. A. (2011). Proteins and Temperature. *Encyclopedia of Fish Physiology*, 1703-1708. doi:https://doi.org/10.1016/B978-0-12-374553-8.00192-1

Fjerbaek, L., Christensen, K. V., & Norddahl, B. (2009). A review of the current state of biodiesel production using enzymatic transesterification. *Biotechnology and Bioengineering, 102*, 1298-1315. doi:https://doi.org/10.1002/bit.22256

Francisqueti, F. V., Minatel, I. O., Ferron, A. J. T., Bazan, S. G. Z., Silva, V. d. S., Garcia, J. L., . . . Corrêa, C. R. (2017). Effect of Gamma-Oryzanol as Therapeutic Agent to Prevent Cardiorenal Metabolic Syndrome in Animals Submitted to High Sugar-Fat Diet. *Nutrients, 9*(12), 1299-1309. doi:https://doi.org/10.3390/nu9121299

Freitas, V. O. d., Matte, C. R., Rodrigues, R. C., & Ayub, M. A. Z. (2019). Ultrasound-Assisted Transesterification of Soybean Oil Using Combi-Lipase Biocatalysts. *Brazilian Journal of Chemical Engineering, 36*(02), 995–1005. doi:dx.doi.org/10.1590/0104-6632.20190362s20180455

Garlapati, V., & Banerjee, R. (2013). Solvent-Free Synthesis of Flavour Esters through Immobilized Lipase Mediated Transesterification. *Enzyme Research*, 1-6. doi:http://dx.doi.org/10.1155/2013/367410

Ghaly, A. E., Dave, D., Brooks, M. S., & Budge, S. (2010). Production of Biodiesel by Enzymatic Transesterification: Review. *American Journal of Biochemistry and Biotechnology, 6*(2), 54-76. doi:https://doi.org/10.3844/ajbbsp.2010.54.76

Gharby, S. (2022). Refining Vegetable Oils: Chemical and Physical Refining. *The Scientific World Journal, 2022*, 6627013. doi:https://doi.org/10.1155/2022/6627013

Ghatak, S. B., & Panchal, S. J. (2011). Gamma-Oryzanol - A Multi-Purpose Steryl Ferulate. *Current Nutrition & Food Science, 7*(1), 10-20. doi:10.2174/157340111794941120

Ghosh, S., & Bhattacharyya, D. K. (1995). Utilization of acid oils in making valuable fatty products by microbial lipase technology. *Journal of the American Oil Chemists' Society, 72*, 1541-1544. doi:https://doi.org/10.1007/BF02577851

Gog, A., Roman, M., Tos, M., Paizs, C., & Irimie, F. D. (2012). Biodiesel production using enzymatic transesterification - Current state and perspectives. *Renewable Energy, 39*, 10-16. doi:https://doi.org/10.1016/j.renene.2011.08.007

Gomes, E. d. S., Lemos, T. L. d., Mattos, M. C. d., Filho, E. G., Pascoal, C. V. P., Assuncao, J. C. d. C., & Monte, F. J. Q. (2020). Production of biodiesel by enzymatic catalysis from the castor oil : an analytical chemical study. *Journal of Analytical & Pharmaceutical Research Research, 8*(3), 73-79. doi:https://doi.org/10.15406/japlr.2020.09.00355

GrandViewResearch. (2016). *Gamma Oryzanol Market Size, Analysis*. Retrieved from United States: https://www.grandviewresearch.com/industry-analysis/gamma-oryzanol-market

Guerrand, D. (2017). Lipases Industrial Applications: Focus on Food and Agroindustries. *Oilseeds and Fats Crops and Lipids, 24*(4), 7. doi:DOI: 10.1051/ocl/2017031

Gunasekaran, V., & Das, D. (2005). Lipase fermentation: Progress and prospects. *Indian Journal of Biotechnology, 4*(4), 437-445. Retrieved from http://nopr.niscair.res.in/handle/123456789/5772

Gunawan, S., Maulana, S., Anwar, K., & Widjaja, T. (2011). Rice bran, a potential source of biodiesel production in Indonesia. *Industrial Crops and Products, 33*(3), 624-628. doi:10.1016/j.indcrop.2010.12.027

Gupta, S. (2016). Comparative study on hydrolysis of oils by lipase immobilized biocatalytic PS membranes using biphasic enzyme membrane reactor. *Journal of Environmental Chemical Engineering, 4*(2). doi:https://doi.org/10.1016/j.jece.2016.03.007

Haar, D. v. d., Stäbler, A., Wichmann, R., & Schweiggert-Weisz, U. (2015). Enzymatic esterification of free fatty acids in vegetable oils utilizing different immobilized lipases. *Biotechnol Lett., 37*(1), 164-174. doi:10.1007/s10529-014-1668-1

Hackett, M. J., Zaro, J. L., Shen, W.-C., Guley, P. C., & Cho, M. J. (2013). Fatty acids as therapeutic auxiliaries for oral and parenteral formulations. *Advanced Drug Delivery Reviews, 65*(10), 1331-1339. doi:https://doi.org/10.1016/j.nut.2019.03.008

Hakala, P., Lampi, A.-M., Ollilainen, V., Werner, U., Murkovic, M., Wahala, K., . . . Piironen, V. (2002). Steryl phenolic acid esters in cereals and their milling fractions. *Journal of Agricultural and Food Chemistry, 50*, 5300−5307. doi:https://doi.org/10.1021/jf025637b

Heidtmann-Bemvenuti, R., Nora, N. S., & Badiale-Furlong, E. (2012). Extraction of γ-Oryzanol From Rice Bran. *Ciência e Agrotecnologia, 36*(6), 665-673. doi:https://doi.org/10.1590/S1413-70542012000600008

Hirsch, G. E., Parisi, M. M., Martins, L. A. M., Andrade, C. M. B., Barbé-Tuana, F. M., & Guma, F. T. C. R. (2015). γ-oryzanol reduces caveolin-1 and PCGEM1 expression, markers of aggressiveness in prostate cancer cell lines. *The prostate, 75*(8), 783-797. doi:https://doi.org/10.1002/pros.22960

Hoed, V. V., Ayala, J. V., Czarnowska, M., Greyt, W. D., & Verhé, R. (2010). Optimization of Physical Refining to Produce Rice Bran Oil with Light Color

and High Oryzanol Content. *Journal of the American Oil Chemists' Society, 87*(10), 1227-1234. doi:https://doi.org/10.1007/s11746-010-1606-x

Hoed, V. V., Depaemelaere, G., Ayala, J. V., Santiwattana, P., Verhé, R., & Greyt, W. D. (2006). Influence of chemical refining on the major and minor components of rice bran oil. *Journal of the American Oil Chemists' Society, 83*, 315–321. doi:https://doi.org/10.1007/s11746-006-1206-y

Holliday, R. L., King, J. W., & List, G. R. (1997). Hydrolysis of Vegetable Oils in Sub- And Supercritical Water. *Industrial and Engineering Chemistry Research, 36*, 932-935. doi:https://doi.org/10.1021/ie960668f

I.M. Noor, Hasan, M., & Ramachandran, K. B. (2003). Effect of Operating Variables on the Hydrolysis Rate of Palm Oil by Lipase. *Process Biochemistry, 39*(1), 13-20. doi:https://doi.org/10.1016/S0032-9592(02)00263-7

Islam, M. S., Matsuki, N., Nagasaka, R., Ohara, K., Hosoya, T., Ozaki, H., . . . Hori, M. (2014). Rice Bran Antioxidants in Health and Wellness. *Wheat and Rice in Disease Prevention and Health*, 443–451. doi:https://doi.org/10.1016/B978-0-12-401716-0.00034-9

Ito, J., Sawada, K., Ogura, Y., Xinyi, F., Rahmania, H., Mohri, T., . . . Nakagawa, K. (2019). Definitive evidence of the presence of 24-methylenecycloartanyl ferulate and 24-methylenecycloartanyl caffeate in barley. *Scientific Reports, 9*(12572 (2019)). doi:https://doi.org/10.1038/s41598-019-48985-6

Jahnavi, G., Prashanthi, G. S., Sravanthi, K., & Rao, L. V. (2017). Status of availability of lignocellulosic feed stocks in India: Biotechnological strategies involved in the production of Bioethanol. *Renewable and Sustainable Energy Reviews, 73*, 798–820. doi:https://doi.org/10.1016/j.rser.2017.02.018

Jesus, S. P., Grimaldi, R., & Hense, H. (2010). Recovery of γ-oryzanol from rice bran oil byproduct using supercritical fluid extraction. *The Journal of Supercritical Fluids, 55*(1), 149-155. doi:https://doi.org/10.1016/j.supflu.2010.08.004

Joshi, M., Kaur, R., Kanwar, P., Dhiman, G., Sharma, G., Lata, S., . . . Mishr, T. (2016). To Evaluate Antioxidant Activity of γ-Oryzanol Extracted From Rice Bran Oil. *International Journal of Life Science & Pharma Research,, 6(3), 17–25. Retrieved from*(2), 17-25. Retrieved from http://www.ijlpr.com/admin/php/uploads/281_pdf.pdf

Ju, Y.-H., & Vali, S. R. (2005). Rice bran oil as a potential resource for biodiesel: A review. *Joournal of Scientific & Industrial Research, 64*, 866-882.

Ju, Y.-H., & Zullaikah, S. (2013). Effect of acid-catalyzed methanolysis on the bioactive components of rice bran oil. *Journal of the Taiwan Institute of Chemical Engineers, 44*, 924–928. doi:https://doi.org/10.1016/j.jtice.2013.03.006

Kabbashi, N. A., Mohammed, N. I., Alama, M. Z., & Mirghani, M. E. S. (2015). Hydrolysis of Jatropha curcas oil for biodiesel synthesis using immobilized Candida cylindracea lipase. *Journal of Molecular Catalysis B: Enzymatic, 116*, 95–100. doi:https://doi.org/10.1016/j.molcatb.2015.03.009

Kaewboonnum, P., Vechpanich, J., Santiwattana, P., & Shotipruk, A. (2010). γ-Oryzanol Recovery from Rice Bran Oil Soapstock. *Separation Science and Technology, 45*(9), 1186–1195. doi:https://doi.org/10.1080/01496391003775790

Karis, D., Cain, R., Young, K., Shand, A., Holm, T., & Springer, E. (2022). Non-fuel uses for fatty acid methyl esters. *Biofuel, Bioproduct & Bio refining, 16*, 1893-1908. doi: DOI: 10.1002/bbb.2422

Kasim, N. S., Chen, H., & Ju, Y.-H. (2007). Recovery of γ-oryzanol from biodiesel residue. *Journal of the Chinese Institute of Chemical Engineers, 38*, 229–234. doi:https://doi.org/10.1016/j.jcice.2007.02.002

Kasim, N. S., Tsai, T.-H., Gunawan, S., & Ju, Y.-H. (2009). Biodiesel production from rice bran oil and supercritical methanol. *Bioresource Technology, 100*(8), 2399-2403. doi:https://doi.org/10.1016/j.biortech.2008.11.041

Katdare, A., Thakkar, S., Dhepale, S., Khunt, D., & Misra, M. (2018). Fatty acids as essential adjuvants to treat various ailments and their role in drug delivery: A review. *Nutrition, 65*, 138-157. doi:https://doi.org/10.1016/j.nut.2019.03.008

Keng, P. S., Basri, M., Zakaria, M. R. S., Rahman, M. B. A., Ariff, A. B., Rahman, R. N. Z. A., & Salleh, A. B. (2009). Newly synthesized palm esters for cosmetics industry. *Newly synthesized palm esters for cosmetics industry 29*(1), 37-44. doi:https://doi.org/10.1016/j.indcrop.2008.04.002

Krishna, A. G. G., Khatoon, S., Shiela, P. M., Sarmandal, C. V., Indira, T. N., & Mishra, A. (2001). Effect of refining of crude rice bran oil on the retention of oryzanol in the refined oil. *Journal of the American Oil Chemists' Society, 78*, 127–131. doi:https://doi.org/10.1007/s11746-001-0232-0

Krishna, S. H., & Karanth, N. G. (2002). Lipases and Lipase-Catalyzed Esterification Reactions in Nonaqueous Media. *Catalysis Reviews—Science and Engineering, 44*, 499-591. doi:https://doi.org/10.1081/CR-120015481

Lai, C.-C., Zullaikah, S., Vali, S. R., & Ju, Y.-H. (2005). Lipase-catalyzed production of biodiesel from rice bran oil. *Journal of Chemical Technology & Biotechnology, 80*(3), 331-337. doi:https://doi.org/10.1002/jctb.1208

Lai, O.-M., Jacoby, J. J., Leong, W.-F., & Lai, W.-T. (2019). Nutritional Studies of Rice Bran Oil. *Rice Bran and Rice Bran Oil: Chemistry, Processing and Utilization*, 19-54. doi:https://doi.org/10.1016/B978-0-12-812828-2.00002-0

Laidler, K. J., & Peterman, B. F. (1979). Temperature Effects in Enzyme Kinetics. *Methods in Enzymology, 63*, 1979. doi:https://doi.org/10.1016/0076-6879(79)63012-4

Lan, T. T. B., & Hoa, P. N. (2015). Lipase Catalysis for Transesterification Produces Biodiesel Using Coconut Oil as Main Raw Material Source. *Biological and Chemical Research,, 2015*, 256-267.

Lee, J. M. (1992). *Biochemical Engineering* (2001 ed.). Washington State: Prentice - Hall Inc.

Lemus, C., Angelis, A., Halabalaki, M., & Skaltsounis, A. L. (2014). γ-Oryzanol: An Attractive Bioactive Component from Rice Bran. *Wheat and Rice in Disease Prevention and Health*, 409-430. doi:https://doi.org/10.1016/B978-0-12-401716-0.00032-5

Lerma-García, M. J., Herrero-Martínez, J. M., Simó-Alfonso, E. F., Mendonça, C. R. B., & Ramis-Ramos, G. (2009). Composition, industrial processing and applications of rice bran γ-oryzanol. *Food Chemistry, 115*(2), 389–404. doi:https://doi.org/10.1016/j.foodchem.2009.01.063

Li, D., Zhang, J., Faiza, M., Shi, L., Wang, W., Liu, N., & Wang, Y. (2021). The enhancement of rice bran oil quality through a novel moderate biorefining process. *LWT, 151*(112118). doi:https://doi.org/10.1016/j.lwt.2021.112118

Li, Q., & Yan, Y. (2010). Production of biodiesel catalyzed by immobilized Pseudomonas cepacia lipase from Sapium sebiferum oil in micro-aqueous phase.

Applied Energy, 87(10), 3148-3154. doi:https://doi.org/10.1016/j.apenergy.2010.02.032

Li, W., Li, R.-w., Li, Q., Du, W., & Liu, D. (2010). Acyl Migration and Kinetics Study of 1(3)-positional Specific Lipase of Rhizopus Oryzae-Catalyzed Methanolysis of Triglyceride for Biodiesel Production. *Process Biochemistry, 45*, 1888–1893. doi:https://doi.org/10.1016/j.procbio.2010.03.034

Li, W. N., Chen, B.-Q., & Tan, T.-W. (2011). Esterification Synthesis of Ethyl Oleate in Solvent-Free System Catalyzed by Lipase Membranefrom Fermentation Broth. *Applied Biochemistry and Biotechnology, 163*, 102–111. doi:https://doi.org/10.1007/s12010-010-9020-2

Li, Z., Deng, L., Lu, J., Guo, X., Yang, Z., & Tan, T. (2010). Enzymatic Synthesis of Fatty Acid Methyl Esters from Crude Rice Bran Oil with Immobilized Candida sp. 99–125. *Chinese Journal of Chemical Engineering, 18*(5), 870-875. doi:https://doi.org/10.1016/S1004-9541(09)60141-5

Lim, J.-W., Beh, H.-G., Ching, D. L. C., Ho, Y.-C., Baloo, L., Bashir, M. J. K., & Wee, S.-K. (2016). Central Composite Design (CCD) applied for statistical optimization of glucose and sucrose binary carbon mixture in enhancing the denitrification process. *Applied Water Science, 7*, 3719-3727. doi:https://doi.org/10.1007/s13201-016-0518-9

Lin, L., Ying, D., Chaitep, S., & Vittayapadung, S. (2009). Biodiesel production from crude rice bran oil and properties as fuel. *Applied Energy, 86*(5), 681-688. doi:https://doi.org/10.1016/j.apenergy.2008.06.002

Linfield, W. M., Barauskas, R. A., Sivieri, L., Serota, S., & Sr, R. W. S. (1984). Enzymatic Fat Hydrolysis and Synthesis. *JAOCS, 61*(2), 191-195. doi:https://doi.org/10.1007/BF02678767

López, B. C., Cerdán, L. E., Medina, A. R., López, E. N., Valverde, L. M., Peña, E. H., . . . Grima, E. M. (2015). Production of biodiesel from vegetable oil and microalgae by fatty acid extraction and enzymatic esterification. *Journal of Bioscience and Bioengineering, 119*(6), 706-711. doi:https://doi.org/10.1016/j.jbiosc.2014.11.002

Luković, N., Knežević-Jugović, Z., & Bezbradica, D. (2011). Biodiesel Fuel Production by Enzymatic Transesterification of Oils : Recent Trends , Challenges and Future Perspectives, Alternative Fuel. In D. M. Manzanera (Ed.), *Alternative Fuel* (pp. 47-72).

Machado, S. A., Rós, P. C. M. D., Castro, H. F. d., & Giordani, D. S. (2021). Hydrolysis of vegetable and microbial oils catalyzed by a solid preparation of castor bean lipase. *Biocatalysis and Agricultural Biotechnology, 37*, 102188. doi:https://doi.org/10.1016/j.bcab.2021.102188

Mala, J. G. S., & Takeuchi, S. (2008). Understanding Structural Features of Microbial Lipases—An Overview. *Anal Chem Insights, 3*, 9-19. doi:10.4137/aci.s551

Mannion, D. T., Furey, A., & Kilcawley, K. N. (2016). Free fatty acids quantification in dairy products. International Journal of Dairy Technology. *International Journal of Dairy Technology, 69*(1), 1-12. doi:https://doi.org/10.1111/1471-0307.12301

Marangoni, A. G. (2003). *Enzyme Kinetics: A Modern Approach*

Editor(s):Alejandro G. Marangoni (A. G. Marangoni Ed.): John Wiley & Sons, Inc.

Mardani, M., Farmani, J., & Kenari, R. E. (2015). Efficacy of Some Commercial Lipase in Hydrolysis of Palm Olein for the Production of Free Fatty Acids and

Diacyglycerol Oil. *Journal of Oil Palm Research,, 23*(3), 250 – 260. Retrieved from http://jopr.mpob.gov.my/wp-content/uploads/2015/09/joprv27sept-mohsen1.pdf

Matassoli, A. L. F., Corrêa, I. N. S., Portilho, M. F., Veloso, C. O., & Langone, M. A. P. (2009). Enzymatic synthesis of biodiesel via alcoholysis of palm oil. *Appl Biochem Biotechnol, 155*(1-3), 347-355. doi:10.1007/s12010-008-8424-8

Meedam, A., Usaku, C., Daisuk, P., & Shotipruk, A. (2020). Comparative study on physicochemical hydrolysis methods for glycerides removal from rice bran acid oil for subsequent γ-oryzanol recovery. *Biomass Conversion and Biorefinery*, 1-8. doi:https://doi.org/10.1007/s13399-020-00775-1

Meng, Y., Wang, G., Yang, N., Zhou, Z., Li, Y., Liang, X., . . . Li, J. (2011). Two-step synthesis of fatty acid ethyl ester from soybean oil catalyzed by Yarrowia lipolytica lipase. *Biotechnology for Biofuels, 4*(1), 6. doi:https://doi.org/10.1186/1754-6834-4-6

Michael J. Haas, Michalski, P. J., Runyon, S., Nunez, A., & Scott, K. M. (2003). Production of FAME from acid oil, a by-product of vegetable oil refining. *Journal of the American Oil Chemists' SocietyVolume 80, Issue 1 p. 97-102, 80*(1), 97=102. doi:https://doi.org/10.1007/s11746-003-0658-4

Minatel, I. O., Francisqueti, F. V., Corrêa, C. R., & Lima, G. P. P. (2016). Antioxidant Activity of γ-Oryzanol: A Complex Network of Interactions. *International Journal of Molecular Science, 17*(8), 1107. doi:https://doi.org/10.3390/ijms17081107

Mizuta, K., & Itaya, K. (1978). Effects of gamma-oryzanol and atropine on gastric secretion stimulated by insulin or 2-deoxy-D-glucose. *Folia Pharmacologica Japonica, 74*(4), 517–524. doi:https://doi.org/10.1254/fpj.74.517

Murty, V. R., Bhat, J., & Muniswaran, P. K. A. (2002a). Hydrolysis of oils by using immobilized lipase enzyme: A review. *Biotechnology and Bioprocess Engineering, 7*(2), 57–66. doi:https://doi.org/10.1007/BF02935881

Murty, V. R., Bhat, J., & Muniswaran, P. K. A. (2002b). Hydrolysis of rice bran oil using immobilized lipase in a stirred batch reactor. *Biotechnology and Bioprocess Engineering, 7*, 367-370. doi:https://doi.org/10.1007/BF02933523

Musa, I. A. (2016). The effects of alcohol to oil molar ratios and the type of alcohol on biodiesel production using transesterification process. *Egyptian Journal of Petroleum, 25*, 21-31. doi:http://dx.doi.org/10.1016/j.ejpe.2015.06.007

Myers, R., Montgomery, D., & Anderson-Cook, C. (2016). *Response Surface Methodology: Process and Product Optimization Using Designed Experiments* (Fourth Edition ed.): John Wiley & Sons.

Nagesha, G. K., Manohar, B., & Sankar, K. U. (2004). Enzymatic esterification of free fatty acids of hydrolyzed soy deodorizer distillate in supercritical carbon dioxide. *Journal of Supercritical Fluids, 32*, 137–145. doi:https://doi.org/10.1016/j.supflu.2004.02.001

Najafpour, G. (2015). Enzyme Technology. In Elsevier (Ed.), *Biochemical Engineering and Biotechnology* (pp. 19-49): Elsevier Science.

Narayan, A. V., Barhate, R. S., & Raghavarao, K. S. M. S. (2006). Extraction and Purification of Oryzanol from Rice Bran Oil and Rice Bran Oil Soapstock. *Journal of the American Oil Chemical's Society, 83*(8), 663-770. doi:https://doi.org/https://doi.org/10.1007/s11746-006-5021-2

Nguyen, T. A. V., Le, T. D., Phan, H. N., & Tran, L. B. (2018). Hydrolysis Activity of Virgin Coconut Oil Using Lipase from Different Sources. *Scientifica*, 1-7. doi:https://doi.org/10.1155/2018/9120942

Nguyen, V. T. A., Hoa, P. N., Lam, T. B., & Ai, C. T. D. (2016). Enzymatic hydrolysis of coconut oil using free and immobilized porcine pancreas lipases. *Science and Technology Development Journal, 19*(K3), 70-78. doi:https://doi.org/10.32508/stdj.v19i2.654

Niawanti, H., & Zullaikah, S. (2017). Removal of Bioactive Compound (γ-Oryzanol) from Rice Bran Oil-Based Biodiesel Using Deep Eutectic Solvent. *Chemical Engineering Transactions, 56*, 1513-1518. doi:https://doi.org/10.3303/CET1756253

Nissiotis, M., & Tasioula-Margari, M. (2002). Changes in antioxidant concentration of virgin olive oil during thermal oxidation. *Food Chemistry, 77*, 371-376. doi:https://doi.org/10.1016/S0308-8146(02)00113-9

Norjannah, B., Hwai Chyuan Ong, H. H Masjuki, Juan, J. C., & W.T Chong. (2016). Enzymatic transesterification for biodiesel production: A comprehensive review. *RSC Advances*, 60034-60055. doi:https://doi.org/10.1039/c6ra08062f

Ondul, E., Dizge, N., Keskinler, B., & Albayrak, N. (2015). Biocatalytic Production of Biodiesel from Vegetable Oils. In K. Biernat (Ed.), *Biofuels* (2015 ed., pp. 582). Poland.

Oryza Oil & Fat Chemical Co., L. (2012). *Gamma Oryzanol:Food and Cosmetic Ingredient*. Retrieved from Japan: http://www.oryza.co.jp/html/english/pdf/ORYZANOL 3.1TK.pdf

Patil, S. S., & Mohapatra, A. K. D. (2016). Stabilization of rice bran using microwave: Process optimization and storage studies. *Food and Bioproducts Processing,, 99*, 204–211. doi:https://doi.org/10.1016/j.fbp.2016.05.002

Peanparkdeea, M., & Iwamotoa, S. (2019). Bioactive Compounds from By-products of Rice Cultivation and Rice Processing: Extraction and Application in the Food and Pharmaceutical Industries. *Food Science and Technology, 86*, 109–117. doi:https://doi.org/10.1016/j.tifs.2019.02.041

Pestana-Bauer, V. R., Zambiazi, R. C., Mendonça, C. R. B., Beneito-Cambra, M., & Ramis-Ramos, G. (2012). γ-Oryzanol and tocopherol contents in residues of rice bran oil refining. *Food Chemistry, 134*(3), 1479-1483. doi:https://doi.org/10.1016/j.foodchem.2012.03.059

Pestana, V. R., Zambiazi, R. C., Mendonça, C. R. B., Bruscatto, M. H., Lerma-García, M. J., & Ramis-Ramos, G. (2008). Quality changes and tocopherols and γ-Orizanol concentrations in rice bran oil during the refining process. *Journal of the American Oil Chemists' Society, 85*, 1013–1019. doi:https://doi.org/10.1007/s11746-008-1300-4

Phuah, E.-T., Laia, O.-M., Choong, T. S.-Y., Tan, C.-P., & Lo, S.-K. (2012). Kinetic study on partial hydrolysis of palm oil catalyzed by Rhizomucor miehei lipase. *Journal of Molecular Catalysis B: Enzymatic, 78*, 91– 97. doi:https://doi.org/10.1016/j.molcatb.2012.03.009

Pooja, S., Anbarasan, B., Ponnusami, V., & Arumugam, A. (2021). Efficient production and optimization of biodiesel from kapok (Ceiba pentandra) oil by lipase transesterification process: Addressing positive environmental impact. *Renewable Energy, 165*, 619-631.

doi:https://doi.org/10.1016/j.renene.2020.11.053

Pourali, O., Asghari, F. S., & Yoshida, H. (2009). Sub-critical water treatment of rice bran to produce valuable materials. *Food Chemistry, 115*, 1-7. doi: https://doi.org/10.1016/j.foodchem.2008.11.099

Prasad, R. B. N. (2006). Rachapudi B.N. Prasad. *Lipid Technology, 18*(12), 275-279. doi:https://doi.org/10.1002/lite.200690003

Pugazhenthi, G., & Kumar, A. (2004). Enzyme membrane reactor for hydrolysis of olive oil using lipase immobilized on modified PMMA composite membrane. *Journal of Membrane Science,, 228*(2), 187-197. doi:https://doi.org/10.1016/j.memsci.2003.10.007

Purwanto, M. G. M., Maretha, M. V., Wahyudi, M., & Goeltom, M. T. (2015). Whole Cell Hydrolysis of Sardine (Sardinella Lemuru) Oil Waste Using Mucor Circinelloides NRRL 1405 Immobilized in Poly-urethane Foam. *Procedia Chemistry, 14,*, 256–262. doi:https://doi.org/10.1016/j.proche.2015.03.036

Puthli, M. S., Rathod, V. K., & Pandit, A. B. (2006). Enzymatic hydrolysis of castor oil: Process intensification studies. *Biochemical Engineering Journal, 31*, 31-41. doi:https://doi.org/10.1016/j.bej.2006.05.017

Rahman, M. B. A., Chaibakhsh, N., & Basri, M. (2011). Effect of Alcohol Structure on the Optimum Condition for Novozym 435-Catalyzed Synthesis of Adipate Esters. *Biotechnology Research International, 2011*. doi:https://doi.org/10.4061/2011/162987

Raita, M., Laothanachareon, T., Champreda, V., & Laosiripojan, N. (2011). Biocatalytic esterification of palm oil fatty acids for biodiesel production using glycine-based cross-linked protein coated microcrystalline lipase. *Journal of Molecular Catalysis. B, Enzymatic, 73*(1-4), 74-79. doi:https://doi.org/10.1016/j.molcatb.2011.07.020

Raspe, D. T., Filho, L. C., & Silva, C. d. (2013). Effect of additives and process variables on enzymatic hydrolysis of macauba kernel oil (acrocomia aculeata). *International Journal of Chemical Engineering*, 1–8. doi:https://doi.org/10.1155/2013/438270

Rathod, V. K., & Pandit, A. B. (2009). Effect of various additives on enzymatic hydrolysis of castor oil. *Biochemical Engineering Journal, 47*(1-3), 93-99. doi:https://doi.org/10.1016/j.bej.2009.07.008

Ravn, H. C., Damstrup, M. L., & Meyer, A. S. (2012). Optimization of reaction parameters for enzymatic glyceride synthesis from fish oil: Ethyl esters versus free fatty acids. *Biocatalysis and Agricultural Biotechnology, 1*(4), 273-279. doi:https://doi.org/10.1016/j.bcab.2012.06.002

Reetz, M. T. (2002). Lipases as practical biocatalysts. *Current Opinion in Chemical Biology, 6*, 145-150. doi:https://doi.org/10.1016/S1367-5931(02)00297-1

Ren, H., Li, Y., Du, W., & Liu, D. (2013). Free Lipase-Catalyzed Esterification of Oleic Acid for Fatty Acid Ethyl Ester Preparation with Response Surface Optimization. *J Am Oil Chem Soc, 90*, 73-79. doi:https://doi.org/10.1007/s11746-012-2146-3

Ribeiro, B. D., Castro, A. M. d., Coelho, M. A. Z., & Freire, D. a. (2011). Production and Use of Lipases in Bioenergy: A Review from The Feedstocks to Biodiesel Production. *Enzyme Research*, 1-17. doi:doi:10.4061/2011/615803

Rodrigues, R. C., Volpato, G., Wada, K., & Ayub, M. A. Z. (2008). Enzymatic

synthesis of biodiesel from transesterification reactions of vegetable oils and short chain alcohols. *Journal of the American Oil Chemists' Society, 85*, 925-930. doi:https://doi.org/10.1007/s11746-008-1284-0

S., V. L., Aparna, A. M., & Baskaran, R. (2019). *Production of Biodiesel from Waste Cooking Oil.* Paper presented at the Fifth International Conference on Science Technology Engineering and Mathematics (ICONSTEM),, Chennai, India.

Sahu, S., Ghosh, M., & Bhattacharyya, D. K. (2019). Isolation and Evaluation of Stearin and Olein Fractions from Rice Bran Oil Fatty Acid Distillate by Detergent Fractionation and Conversion into Neutral Glycerides by Autocatalytic Esterification Reaction. *Journal of the American Oil Chemists' Society, 97*(3), 229-327. doi:https://doi.org/10.1002/aocs.12320

Sales, A. (2011). *Production of biodiesel from sunflower oil and ethanol by base catalyzed transesterification.* (Master). Royal Institute of Technology (KTH), Sweeden.

Sandoval, G., Casas-Godoy, L., Bonet-Ragel, K., Rodrigues, J., Ferreira-Dias, S., & Valero, F. (2017). Enzyme-Catalyzed Production of Biodiesel as Alternative to Chemical- Catalyzed Processes: Advantages and Constraints. *Current Biochemical Engineering., 4*, 109-141. doi:https://doi.org/10.2174/2212711904666170615123640

Sapino, S., Ugazio, E., Morel, S., Carlotti, M. E., Berlier, G., Cavalli, R., & Gastaldi, L. (2013). Photochemical and antioxidant properties of gamma-oryzanol in beta-cyclodextrin-based nanosponges. *Journal of Inclusion Phenomena and Macrocyclic Chemistry*. doi:https://doi.org/10.1007/s10847-012-0147-3

Sendzikiene, E., Santaraite, M., & Makareviciene, V. (2020). Lipase-Catalysed In Situ Transesterification of Waste Rapeseed Oil to Produce Diesel-Biodiesel Blends. *Processes, 8*(9), 1118. doi:https://doi.org/10.3390/pr8091118

Serri, N. A., Kamarudin, A. H., & Rahaman, S. N. A. (2008). Preliminary studies for production of fatty acids from hydrolysis of cooking palm oil using C.Rugosa Lipase. *Journal of Physical Science, 19*(1), 79–88.

Shao, Y., & Bao, J. (2019). Rice phenolics and other natural products. *Rice*, 221-271. doi:https://doi.org/https://doi.org/10.1016/B978-0-12-811508-4.00008-3

Sharma, A., Chaurasia, S. P., & Dalaic, A. K. (2013). Enzymatic Hydrolysis of Cod Liver Oil for the Fatty Acids Production. *Catalysis Today, 207*, 93–100. doi:https://doi.org/10.1016/j.cattod.2012.05.006

Sharma, R., Chisti, Y., & Banerjee, U. C. (2001). Production, purification, characterization, and applications of lipases. B. *iotechnology Advances, 19*, 627-662.

Sharma, R., Thakur, V., Sharma, M., & Birkeland, N.-K. (2013). Biocatalysis Through Thermostable Lipases: Adding Flavor to Chemistry. In *Thermophilic Microbes in Environmental and Industrial Biotechnology* (pp. 905-927).

Sharma, R. D., & Rukmini, C. (1986). Rice bran oil and hypocholesterolemia in rats. *Lipids, 21*, 715-717. doi:https://doi.org/10.1007/BF02537246

Shimada, Y., Watanabe, Y., Sugihara, A., & Tominaga, Y. (2002). Enzymatic alcoholysis for biodiesel fuel production and application of the reaction to oil processing. *Journal of Molecular Catalysis B: Enzymatic, 17*(3-5), 133-142. doi:https://doi.org/10.1016/S1381-1177(02)00020-6

Silva, J. R. P. d., Costa, F. P. d., Lerin, L. A., Ninow, J. L., Oliveira, J. V., & Oliveira,

D. b. d. (2019). Application of Different Methodologies to Produce Fatty Acid Esters Using the Waste Chicken Fat Catalyzed by Free NS 40116 Lipase. *Industrial Biotechnology, 15*(5), 193-302. doi:https://doi.org/10.1089/ind.2018.0034

Singanusong, R., & Garba, U. (2019). Micronutrients in rice bran oil. *Rice Bran and Rice Bran Oil: Chemistry, Processing and Utilization*, 125–158. doi:https://doi.org/10.1016/B978-0-12-812828-2.00005-6

Soares, D., Pinto, A. F., Gonçalves, A. G., Mitchell, D. A., & Krieger, N. (2013). Biodiesel production from soybean soapstock acid oil by hydrolysis in subcritical water followed by lipase-catalyzed esterification using a fermented solid in a packed-bed reactor. *Biochemical Engineering Journal*, 1-44. doi:https://doi.org/10.1016/j.bej.2013.09.017

Sohail, M., Rakha, A., But, M. S., Iqbal, M. J., & Rashid, S. (2017). Rice bran nutraceutics: A comprehensive review. *Critical Reviews in Food Science and Nutrition, 57*(17), 3771-3780. doi:https://doi.org/10.1080/10408398.2016.1164120

Sombutsuwan, P., Akepratumchai, S., Krisnangkura, K., Nakornsadet, A., Jeyashoke, N., Aryusuk, K., & Lilitchan, S. (2018). Recovery of γ-Oryzanol from Rice Bran Acid Oil by an Acid-base Extraction Method with the Assistance of Response Surface Methodology. *Journal of Oleo Science, 67*(11), 1405-1415. doi:https://doi.org/10.5650/jos.ess18073

Sousa, J. S. d., Cavalcanti-Oliveira, E. d. A., Aranda, D. A. G., & Freire, D. M. G. (2010). Application of Lipase from the Physic Nut (Jatropha curcas L.) to a New Hybrid (enzyme/chemical) Hydroesterification Process for Biodiesel Production. *Journal of Molecular Catalysis B: Enzymatic*, 133-137. doi:https://doi.org/10.1016/j.molcatb.2010.01.003

Souza, M. S., Aguieiras, E. C. G., Silva, M. A. P. d., & Langone, M. A. P. (2009). Biodiesel Synthesis via Esterification of Feedstock with High Content of Free Fatty Acid. *Applied Biochemistry and Biotechnology, 154*, 253-267. doi:https://doi.org/10.1007/s12010-008-8444-4

Sowcharoensuk, C. (2019). Rice Industry. *Thailand Industry Outlook*.

Srikaeo, K. (2014). Organic Rice Bran Oils in Health. In R. Watson, V. Preedy, & S. Zibadi (Eds.), *Wheat and Rice in Disease Prevention and Health* (1 ed., pp. 453–465): Academic Press.

Stergiou, P.-Y., Foukis, A., Filippou, M., Koukouritaki, M., Parapouli, M., Theodorou, L. G., . . . Papamichael, E. M. (2013). Advances in lipase-catalyzed esterification reactions. *Biotechnology Advances, 31*, 1846-1859. doi:https://doi.org/10.1016/j.biotechadv.2013.08.006

Sun, X., Zhang, L., Yan, J., Song, F., Tian, S., & Xie, J. (2021). Effects of enzymatic free fatty acid reduction process on the composition and phytochemicals of rice bran oil. *Food Chemistry, 337*(127757). doi:https://doi.org/10.1016/j.foodchem.2020.127757

Szcześniak, K. A., Ostaszewski, P., & A. Ciecierska, T. S. (2015). Investigation of nutriactive phytochemical – gamma-oryzanol in experimental animal models. *Journal of Animal Physiology and Animal Nutrition, 100*(4), 601-617. doi:https://doi.org/10.1111/jpn.12428

Talukder, M. M. R., Wu, J. C., & Chua, L. P.-L. (2010). Conversion of waste cooking

oil to biodiesel via enzymatic hydrolysis followed by chemical esterification. *Energy and Fuels, 24*, 2016–2019. doi:https://doi.org/10.1021/ef9011824

Talukder, M. M. R., Wu, J. C., Fen, N. M., & Melissa, Y. L. S. (2010). Two-step lipase catalysis for production of biodiesel. *Biochemical Engineering Journal, 49*(2), 207-212. doi:https://doi.org/10.1016/j.bej.2009.12.015

Tan, T., Nie, K., & Wang, F. (2006). Production of Biodiesel by Immobilized Candida sp . Lipase at High Water Content. *Applied Biochemistry and Biotechnology, 128*, 109-116.

Thein, T. M. L., Jindal, V. K., Jindal, R., & Yoswathana, N. (2019). Acid-Catalyzed Esterification Pretreatment of High Free Fatty Acid Crude Rice Bran Oil for Biodiesel Production. *ENVIRONMENT AND NATURAL RESOURCES JOURNAL, 17*(3), 68-79. doi:DOI: 10.32526/ennrj.17.3.2019.24

Ting, W.-J., Huang, C.-M., Giridhar, N., & Wu, W.-T. (2008). An enzymatic/acid-catalyzed hybrid process for biodiesel production from soybean oil. *Journal of the Chinese Institute of Chemical Engineers, 39*, 203–210. doi:https://doi.org/10.1016/j.jcice.2008.01.004

Tronia, K. L., Silva, S. M., Meirelles, A. J. A., & Ceriani, R. (2013). Study of fatty acid and fatty alcohol formation from hydrolysis of rice bran wax. *Chemical Engineering Transactions, 32*, 1747-1752. doi:https://doi.org/10.3303/CET1332292

USDA. (2019). *Grain : World Markets and Trade*. Retrieved from

Vaysse, L., Ly, A., Moulin, G., & Dubreucq, E. (2002). Chain-length selectivity of various lipases during hydrolysis, esterification and alcoholysis in biphasic aqueous medium. *Enzyme and Microbial Technology, 31*(5).

Vipin, V. C., Sebastian, J., Muraleedharan, C. M., & Santhiagu, A. (2016). Enzymatic transesterification of rubber seed oil using Rhizopus Oryzae Lipase. *Procedia Technology, 25*, 1014–1021. doi:https://doi.org/10.1016/j.protcy.2016.08.201

Wang, M., Nie, K., Cao, H., Deng, L., Wang, F., & Tan, T. (2014). Biodiesel production by combined fatty acids separation and subsequently enzymatic esterification to improve the low temperature properties. *Bioresource Technology, 174*, 302–305. doi:https://doi.org/10.1016/j.biortech.2014.08.011

Wang, X., Wang, X., & Wang, T. (2017). An effective method for reducing free fatty acid content of high-acid rice bran oil by enzymatic amidation. *Journal of Industrial and Engineering Chemistry, 48*(25), 119-124. doi:https://doi.org/10.1016/j.jiec.2016.12.028

Wangdee, K., & Onsaard, E. (2018). Optimization of solid-liquid extraction of γ-oryzanol from rice bran oil soapstock using soxhlet extraction method. *Science and Technology Asia, 23*(1), 1-8. doi:https://doi.org/10.14456/scitechasia.2018.1

Watanabe, Y., Arawaka, T., Kitatamagun, H., & Iwasaki, T. (1969). United States Patent No.: U. S. P. Office.

Watanabe, Y., Nagao, T., Nishida, Y., Takagi, Y., & Shimada, Y. (2007). Enzymatic production of fatty acid methyl esters by hydrolysis of acid oil followed by esterification. *Journal of the American Oil Chemists' Society, 84*, 1015–1021. doi:https://doi.org/10.1007/s11746-007-1143-4

Watanabe, Y., Pinsirodom, P., Nagao, T., Kobayashi, T., Nishida, Y., Takagi, Y., & Shimada, Y. (2005). Production of FAME from Acid Oil Model Using Immobilized Candida antarctica Lipase. *AOCS Press, 82*(11), 821-831.

Watanabe, Y., Pinsirodom, P., Nagao, T., Yamauchi, A., Kobayashi, T., Nishida, Y., . . . Shimadaa, Y. (2007). Conversion of acid oil by-produced in vegetable oil refining to biodiesel fuel by immobilized Candida antarctica lipase. *Journal of Molecular Catalysis B: Enzymatic, 44*, 99–105. doi:doi:10.1016/j.molcatb.2006.09.007

Wongwaiwech, D., Weerawatanakorn, M., Tharatha, S., & Ho, C.-T. (2019). Comparative Study on Amount of Nutraceuticals in By-Products from Solvent and Cold Pressing Methods of Rice Bran Oil Processing. *Journal of Foof and Drug Analysis, 27*, 1-12. doi:https://doi.org/Https://doi.org/10.1016/j.jfda.2018.06.006

Xiao, F., Li, Z., & Pan, L. (2017). Application of Microbial Lipase and Its Research Progress. *Progress in Applied Microbiology*, 8-14.

Xu, Z., & Godber, J. S. (2001). Antioxidant Activity of Tocopherols, Tocotrienols, and γ-oryzanol Components from Rice Bran Against Cholesterol Oxidation Accelerated by 2,2′-azobis(2-methylpropionamidine) Dihydrochloride. *Journal of Agricultural and Food Chemistry, 77*, 547–551. doi:https://doi.org/10.1021/jf0012852

Yasmin, M., Zullaikah, S., Permatasari, A., Marita, I., & Rachimoellah, M. (2019). Effect of acid-catalysed in one step production of biodiesel on total sugars of defatted rice bran. *IOP Conf. Series: Materials Science and Engineering, 588*, 012013. doi:doi:10.1088/1757-899X/588/1/012013

Yılmaz, N. (2016). Middle infrared stabilization of individual rice bran milling fractions. *Food Chemistry, 190*, 179–185. doi:https://doi.org/10.1016/j.foodchem.2015.05.094

Yu, S., Nehus, Z. T., Badger, T. M., & Fang, N. (2007). Quantification of vitamin E and γ-oryzanol components in rice germ and bran. *Journal of Agricultural and Food Chemistry, 55*, 7308-7313. doi:https://doi.org/10.1021/jf071957p

Yusup, S., & Khan, M. A. (2010). Base Catalyzed Transesterification of Acid Treated Vegetable Oil Blend for Biodiesel Production. *Biomass and Bioenergy, 34*(10), 1500-1504. doi:https://doi.org/10.1016/j.biombioe.2010.04.027

Zhuang, X., Yin, T., Han, W., & Zhang, X. (2019). Nutritional ingredients and active compositions of defatted rice bran. In *In Rice Bran and Rice Bran Oil: Chemistry, Processing and Utilization* (pp. 247-270).

Zullaikah, S., Lai, C.-C., Vali, S. R., & *, Y.-H. J. (2005). A two-step acid-catalyzed process for the production of biodiesel from rice bran oil. *Bioresource Technology, 96*(17), 1889–1896. doi:https://doi.org/10.1016/j.biortech.2005.01.028

Zullaikah, S., Rakhadima, Y. T., Rachimoellah, M., Widjaja, T., & Sumarno, S. (2014). An Efficient Method for the Production of Biodiesel from Rice Bran. *In Journal of Proceeding Series (Vol. 1)*.

Zullaikah, S., Wibowo, N. D., Wahyudi, I. M. G. E. D., & Rachimoellah, M. (2019). Deacidification of Crude Rice Bran Oil (CRBO) to Remove FFA and Preserve γ-Oryzanol Using Deep Eutectic Solvents (DES). *Materials Science Forum, 964*, 109-114. doi:https://doi.org/10.4028/www.scientific.net/MSF.964.109
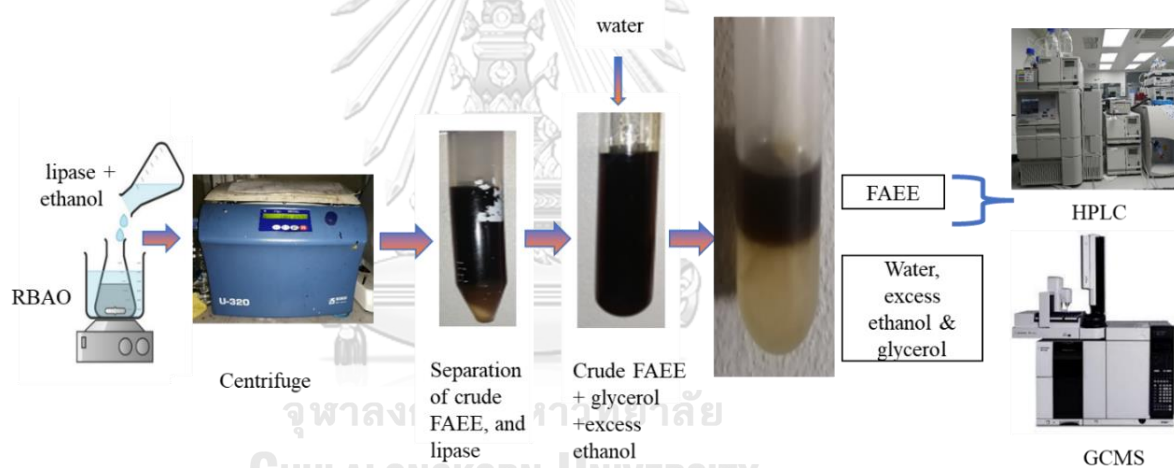
APPENDICES
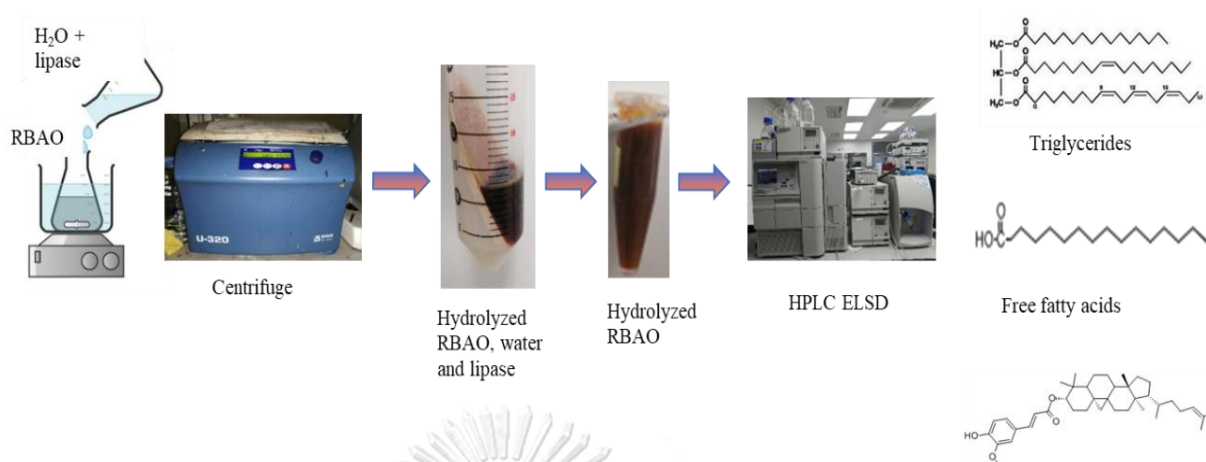
Figure A1: The overall experimental design for enzymatic hydrolysis.

Figure A2:: The overall experimental design for enzymatic
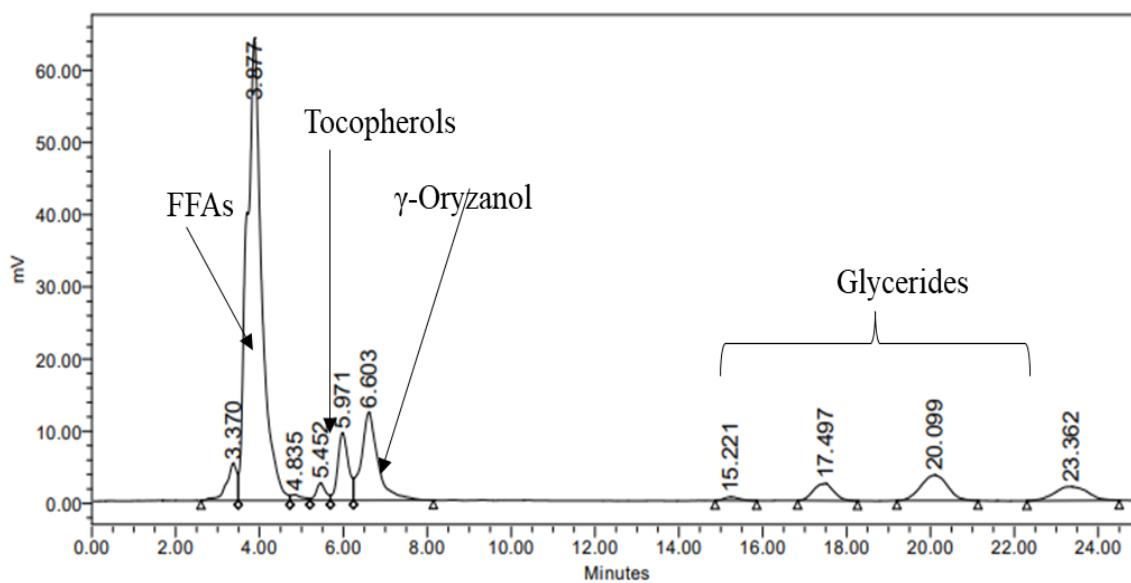esterification/transesterification

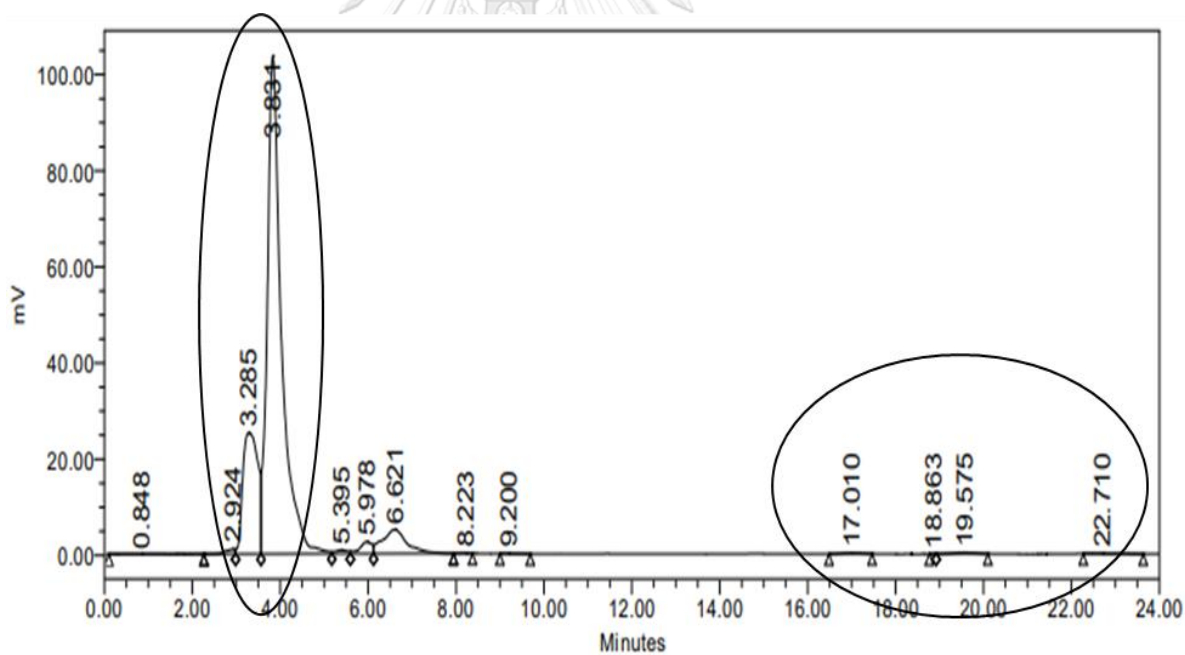**Figure A3**: Chromatogram using HPLC ELSD of RBAO

Figure A4: Chromatogram using HPLC of RBAO after the hydrolysis reaction.

Table A1 : summary of one factor at time (OFAT) on each variables (p-vales)

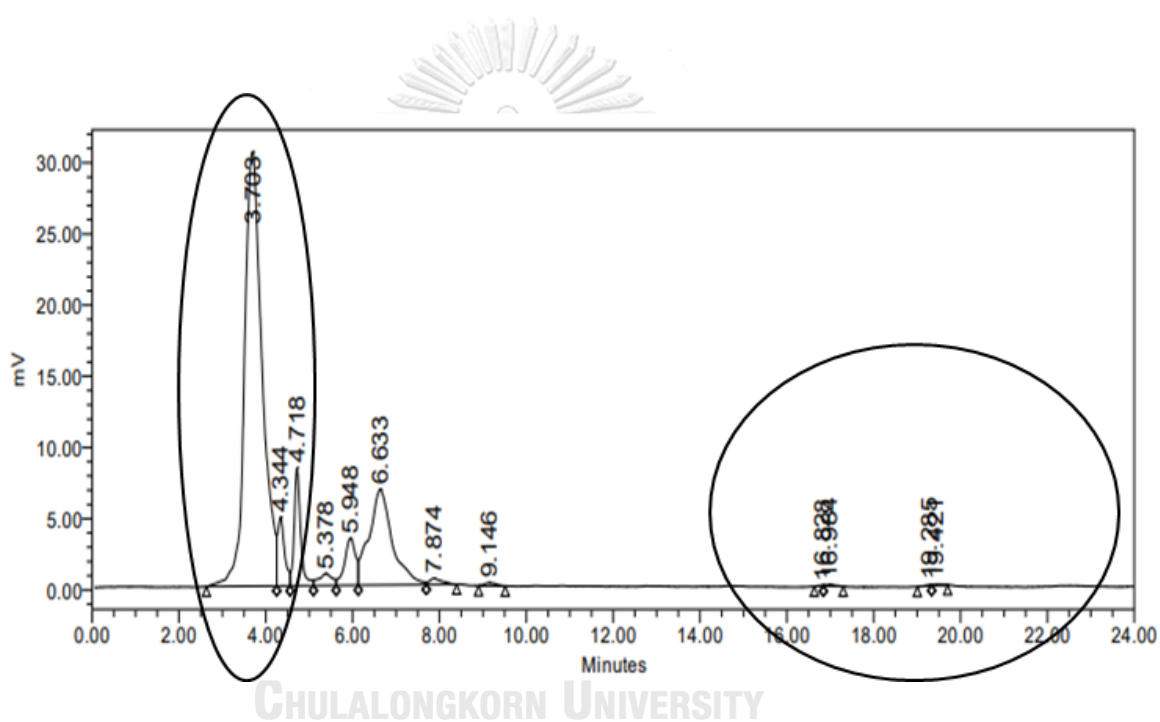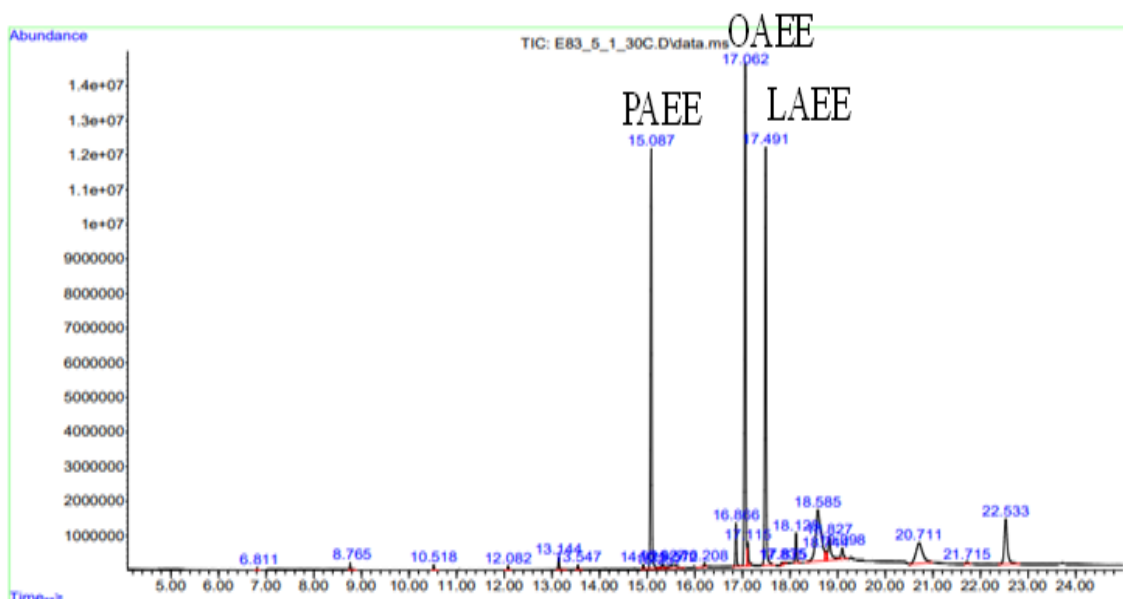| Operating conditions	p-value of glycerides	*p*-value of γ- oryzanol
Time of reaction	1.48 x 10-6	0.10
Temperature	2.07 x 10-7	1.42 x 10-7
Buffer to RBAO ratio	5.37 x 10-5	0.001
Lipase loading	0.003	0.666
Speed	0.003	0.097

Figure A5: Chromatogram using HPLC of RBAO after

esterification/transesterification reaction.

Figure A6: GCMS chromatogram of FAEE produced after the reacti

Table A2: Model selection for Design of Experiments

Type of design		Low	High	Center CRD		Face CRD		Orthogonal QCD		Sepherical CD		Practical CD		Orthogonal BCD	
Name	Units			-1.68	+1.68	-1	+1	-1.57	+1.57	-1.73	+1.73	-1.31	+1.31	-1.77	+1.77
Time	hr	12	36	3.82	44.18	12	36	5.1	42.90	3.22	44.78	8.21	39.79	2.73	45.27
Temperature	C	40	60	33.18	66.2	40	60	34.25	65.75	32.68	67.32	36.84	63.16	32.27	67.73
Buffer ratio	g/g	1	5	-0.3	6.36	1	5	-0.15	6.15	-0.46	6.46	0.37	5.63	-0.55	6.55

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PUBLICATION   1.   Asdarina Binti Yahya, Chonlatep Usaku, Phannipha Daisuk, Artiwan Shotipruk, Enzymatic hydrolysis as a green alternative for glyceride removal from rice bran acid oil before γ-oryzanol recovery: Statistical process optimization,Biocatalysis and Agricultural Biotechnology, Volume 50, 2023, 102727, ISSN 1878-8181, https://doi.org/10.1016/j.bcab.2023.102727.

2. Chonlatep Usaku., Asdarina binti Yahya., Phanipha Daisuk., Artiwan Shotipruk. Enzymatic esterification/transesterification of rice bran acid oil for subsequent γ-oryzanol recovery. Biofuel Research Journal 38 (2023) 1830-1843. https://doi.org/10.18331/BRJ2023.10.2.3.

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