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Enzymatic esterification/transesterification of rice bran acid oil for subsequent γ-oryzanol recovery

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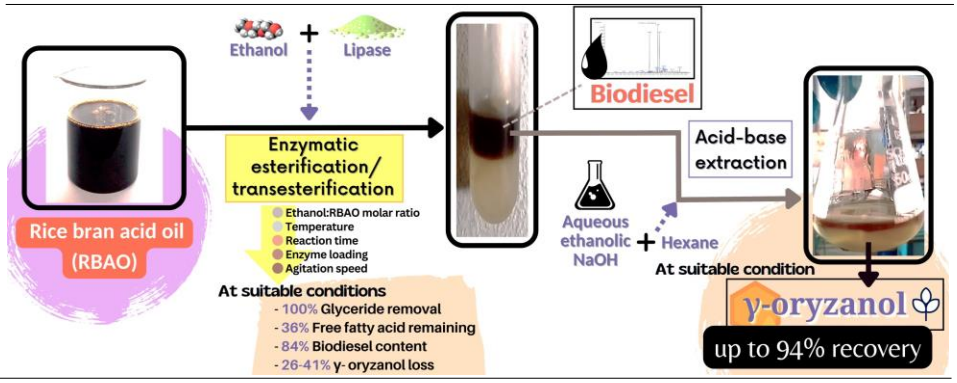
HIGHLIGHTS

- Rice bran acid oil is esterified/transesterified before γ-oryzanol extraction.
- Suitable conditions for enzymatic esterification/transesterification are found.
- 100% glyceride removal is achieved with high biodiesel yield and low γ-oryzanol loss.
- γ-oryzanol in biodiesel was efficiently recovered using acid-base extraction.
- The highest γ-oryzanol recovery (94%) was achieved by a 2-4 M ethanolic NaOH solution.

GRAPHICAL ABSTRACT



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ABSTRACT

This study recovered γ-oryzanol from rice bran acid oil (RBAO), following an initial enzymatic esterification/transesterification to selectively convert its glyceride impurities into fatty acid ethyl esters (FAEEs) or biodiesel. γ-oryzanol was then deprotonated and separated from the biodiesel into the resulting aqueous phase *via* acid-base extraction. Herein, we determine the effects of varying reaction conditions, i.e., ethanol:RBAO molar ratio, temperature, reaction time, enzyme loading, and agitation speed, on the degrees of glyceride removal, γ-oryzanol loss, free fatty acid (FFA) remaining, and biodiesel content. Up to 100% glyceride removal was achieved with a relatively high biodiesel yield (84%) and γ-oryzanol loss as low as 26% under our most suitable reaction conditions (5:1 ethanol:RBAO molar ratio, 40 °C, 24 h reaction time, 10%wt enzyme loading, 200 rpm agitation). Furthermore, of the remaining oryzanol, up to 94% was recovered by the acid-base extraction with 2-4 M ethanolic NaOH solution. Our results suggest that a combination of enzymatic esterification/transesterification with subsequent acid-base extraction offers an efficient alternative approach to the simultaneous production of biodiesel and γ-oryzanol recovery from low-cost RBAO. Based on our analysis of techno-economic and environmental sustainability, integration of the present method into a rice bran oil refinery would make the process profitable, with the minimum use of toxic chemicals and energy.

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Contents

Abbreviations

RBAO	Rice bran acid oil
FFAs	Free fatty acids
FAEEs	Fatty acid ethyl esters
HPLC	High-performance liquid chromatography
GC-MS	Gas chromatography-mass spectrometry
ANOVA	Analysis of variance
GPM	Gross profit margin
COGs	Costs of goods sold

1. Introduction

Esterification/transesterification are reactions between carboxylic acids/esters and an alcohol to produce alkyl esters. They are commonly applied with high-fatty acid vegetable oils and typical short-chain alcohols: methanol or ethanol, to produce fatty acid methyl or ethyl esters (FAMEs or FAEEs), also known as "biodiesel", an alternative to petroleum-based diesel fuels. The reactions can be brought about with the aid of either a typical homogenous acid catalyst (i.e., H_2SO_4, Urrutia et al. (2016)), a heterogeneous solid acid catalyst (Kulkarni et al., 2006), or catalyst-free supercritical conditions (dos Santos et al., 2018). Alternatively, the reactions can be catalyzed by a lipase enzyme under mild conditions with minimum uses of toxic chemicals and energy. Moreover, since methanol rather severely inhibits the activity of lipases (Yusoff et al., 2014), ethanol, which is less toxic and can be renewably sourced, is instead employed. This switch further increases the sustainability and 'green' credentials of the lipase-catalyzed approach for biodiesel production. Furthermore, the substrate specificity of lipase minimizes undesirable side reactions of compounds other than the intended targets (Monteiro et al., 2021).

Currently, the majority of global biodiesel feedstock involves edible oils (Naylor and Higgins, 2017), which are costly due to their applications as foodstuff (Balat, 2011), resulting in inflated retail prices for biodiesel, compared to mineral diesel (Amelia et al., 2023). Utilizing non-edible oils for fuel feedstock is appealing since these resources do not trigger competition with the food industry. One particular low-cost non-edible oil source is rice bran acid oil (RBAO), which is derived from rice bran oil soapstock (RBOS), a major byproduct of the deacidification process of rice bran oil (RBO) chemical refining. During the deacidification step, crude RBO is saponified with a strong base, from which rice bran oil soapstock (RBOS) is generated. Owing to its high water content, RBOS spoils easily. Consequently, its storage life is extended by re-acidulation with H_2SO_4 followed by water removal to obtain RBAO, normally sold as a cheap animal feed. However, RBAO's high content of glycerides (20-50 %) and free fatty acids (FFAs, 40-80 %) (Ghosh and Bhattacharyya, 1995) has attracted recent attention as an alternative low-cost raw material for biodiesel production. While esterification/transesterification of crude/refined RBO (Zaidel et al., 2019; Hoang et al., 2021) for biodiesel production has been extensively investigated (**Table 1**), reports on biodiesel production from RBO refining byproducts, including RBAO, remain scarce. Particularly limited are studies involving lipase-catalyzed esterification and transesterification of RBAO (as in Ghosh and Bhattacharyya (1995) and Choi et al. (2016)).

In addition to glycerides and FFAs, RBAO is also rich in γ-oryzanol, a valuable super-antioxidant with hypocholesterolemic properties (Minatel et al., 2016; Szcześniak et al., 2016). γ-oryzanol is a mixture of ferulic acid esters of triterpene alcohols and plant sterols (Ito et al., 2019) and is naturally found in oil fractions of rice bran. In a chemical RBO refinery, most of the original γ-oryzanol in the crude RBO is lost into RBOS during deacidification (Krishna et al., 2001; Van Hoed et al., 2006). After re-acidulation, the resulting RBAO still contains a relatively high amount of γ-oryzanol (3.8-9%) (Wongwaiwech et al., 2019; Meedam et al., 2022). As a result, RBAO has been investigated as a primary raw material for γ-oryzanol isolation. In this application, glycerides in RBAO must first be removed due to their comparable physicochemical properties to γ-oryzanol to simplify the subsequent γ-oryzanol isolation steps. As reported in several studies (**Table 1**), base-catalyzed hydrolysis is the most common method for glyceride removal in which these tri-esters are converted into more separable FFAs. Although glycerides are completely removed this way, γ-oryzanol losses of up to 60% have been shown to occur during the base-catalyzed reaction.

Two noteworthy studies involved simultaneous acid-catalyzed esterification/transesterification for glycerides removal, and thus biodiesel production, before γ-oryzanol isolation from RBO derivatives (**Table 1**). Ju and Zullaikah (2013) applied acid-catalyzed esterification/transesterification to a liquid byproduct of γ-oryzanol pre-precipitated crude RBO and reported ca. 30-35% loss of γ-oryzanol. Nevertheless, the separation of γ-oryzanol from the resulting biodiesel was not demonstrated. Sombutsuwan et al. (2018) applied acid-catalyzed esterification/transesterification with RBAO, followed by acid-base extraction of γ-oryzanol, in which γ-oryzanol was deprotonated by an alcoholic solution of the strong base to become anionic and in turn phase-separated from the resulting biodiesel (Hakala et al., 2002). γ-oryzanol extraction yields of up to 76% were reported, although this study did not explore the effects of the reaction conditions on γ-oryzanol recovery. Furthermore, neither of these studies probed the effects of reaction conditions on the extent of glyceride removal and biodiesel production. In

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Table 1.
Latest studies on using rice bran oil refinery byproducts for biodiesel production, γ-oryzanol recovery, or both.

Reference	Raw Material	Focus/Result	Reaction				
			Catalyst Wth/Without Alcohol	Glyceride Conversion	FFA Production	Biodiesel Production	γ-Oryzanol Loss
Ghosh and Bhattacharyya (1995)	RBAO*	FFA and Biodiesel production	*Candida cylindracea* and *Mucor miehei* lipase / C_4-C_{18} alcohols	~71-76% of acid oil	>90%	~ 89-151% ester yield	-
Das et al. (1998 and 1999)	RBAO	Hydrolysis + γ-oryzanol extraction	NaOH solution	-	-	-	-
Jesus et al. (2010)	Distilled RBAO	Hydrolysis + γ-oryzanol extraction	NaOH solution	-	-	-	-
Kaewboonnum et al. (2010)	RBOS	Saponification + γ-oryzanol extraction and crystallization	NaOH solution	100%	-	-	40.5%
Ju and Zullaikah (2013)	LP2	Biodiesel production + γ-oryzanol retention	H_2SO_4/ Methanol	-	-	-	~ 35% ~ 30% with N_2 purging
Choi et al. (2016)	RBAO	Biodiesel production	Novozym 435 Lipozyme RM IM Lipozyme TL IM / Ethanol	~90%	-	92%	-
Sombutsuwan et al. (2018)	RBAO	Biodiesel production + γ-oryzanol extraction	HCl/Ethanol	-	-	-	-
Akkarawatkhoosith et al. (2021)	RBO RBOF-AD	Biodiesel production	Supercritical DMC	-	-	97.1% (RBOFAD) 43.6% (RBO)	-
Meedam et al. (2022)	RBAO	Hydrolysis	NaOH solution	100%	-	-	35-44%
Anjinta et al. (2023)	RBAO	Hydrolysis + γ-oryzanol extraction and chromatography	NaOH solution	92%	-	-	60%
This Study	RBAO	Biodiesel production + γ-oryzanol extraction	*Aspergillus niger* lipase/Ethanol			See *Section 3*	

*Abbreviations: FFA: free fatty acid; RBAO: rice bran acid oil; LP2: liquid byproduct from crystallization of crude rice bran oil; RBOFAD: rice bran oil fatty acid distillate; RBO: rice bran oil; DMC: dimethyl carbonate; RBOS: rice bran oil soapstock.

contrast to harsh acid-catalyzed reactions, enzymatic esterification/transesterification is hypothesized to be preferable on account of the enzyme's substrate-binding specificity and mild operating conditions, potentially offering complete glyceride conversion with minimal γ-oryzanol loss. Although Ghosh and Bhattacharyya (1995) and Choi et al. (2016) have previously investigated the lipase-catalyzed esterification/transesterification of RBAO, their studies focused on obtaining high biodiesel yields rather than monitoring γ-oryzanol loss and recovery (**Table 1**).

In this study, we aimed to recover γ-oryzanol from RBAO by first performing enzymatic esterification/transesterification of RBAO with ethanol to convert glycerides into FAEEs, followed by acid-base extraction of γ-oryzanol from the resulting biodiesel. The suitable reaction/extraction conditions that provided complete glyceride removal and minimal γ-oryzanol loss were then determined. Specifically, the effects of key reaction conditions, including ethanol: RBAO molar ratio, temperature, reaction time, enzyme loading, and agitation speed, on the degrees of glycerides removal, FFA remaining, biodiesel content, and γ-oryzanol loss were investigated. For acid-base extraction, the effects of aqueous ethanoic NaOH concentration on the degrees of γ-oryzanol recovery from the biodiesel were studied. The method herein can be further incorporated into industrial RBO refining to simultaneously generate biodiesel and recover high-value compounds from the low-cost waste by-product.

2. Materials and Methods

2.1. Materials and chemicals

RBAO (pH=5) with the composition as shown in **Table 2** was acquired from Thai Edible Oil Co., Ltd Samutprakarn, Thailand. Industrial grade *Aspergillus niger* lipase (E.C. 3.1.1.3) was purchased from Sinobios Imp. & Exp. Co. Ltd, Shanghai, China. The manufacturer's specification of the enzyme activity was 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. 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.

Table 2.
Composition of rice bran acid oil (RBAO).

Components	Amount (%wt)
FFAs[a,b]	46
Glycerides[a]	36
γ-oryzanol[a]	11
Water[c]	1.4
Others	5.3

[a] Determined by HPLC (*Section 2.4.1*).
[b] Components of FFAs were listed in **Supplementary Information** as herein determined by GC-MS (*Section 2.4.2*).
[c] Water content was reported by the supplier.

2.2. Enzymatic esterification/transesterification of rice bran acid oil

The effects of reaction conditions on glycerides conversion, FFAs production, biodiesel content, and γ-oryzanol loss were investigated in ranges specified in **Table 3**, from which suitable reaction

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Table 3.
Reaction parameters and their ranges.

Order	Reaction Parameters	Values Tested
1	Ethanol:RBAO molar ratio*	1:1, 3:1, 5:1, 7:1, 9:1
2	Temperature (°C)	30, 40, 50, 60
3	Reaction time (h)	1.5, 3, 6, 12, 18, 24, 30
4	Enzyme loading (%wt)	5, 7.5, 10, 12.5, 15
5	Stirring speed (rpm)	200, 300, 400

*Molecular weight of acid oil ($M_{w,AO}$) = average molecular weight of fatty acids estimated based on their content in FFAs and glycerides in rice bran acid oil (RBAO), as previously defined by Ghosh and Bhattacharyya (1995) and Choi et al. (2016). The molar ratio was then estimated as follows:

$$\text{Ethanol:RBAO molar ratio} = \frac{\text{Weight of ethanol}}{\text{Molecular weight of ethanol (46.07)}} : \frac{\text{Weight of RBAO (2 g)}}{M_{w,AO}\ (281.57)}$$

conditions are suggested.

The method for enzymatic esterification/transesterification of RBAO was adapted from those described by Choi et al. (2016) and Aguieiras et al. (2017). Briefly, RBAO (2 g) was mixed with ethanol and lipase in a 50 mL Erlenmeyer flask in a temperature-controlled stirring water bath at a specified ethanol:RBAO molar ratio, a specified percentage of enzyme loading (%wt), and a specified stirring speed (rpm). After the mixture was heated to a specified reaction temperature (°C), the reaction was left to proceed for a specified reaction time (h) with magnetic stirring. After the reaction, the upper biodiesel phase was separated by centrifugation (4500 pm, 10 min) and collected. This biodiesel phase was then washed (2 × DI water) to remove any remaining ethanol and glycerol. A biodiesel phase sample (20 µL) was dissolved (either in ethyl acetate or hexane, 3 mL) and filtered (0.45 µm syringe filter) for later analysis by either high-performance liquid chromatography (HPLC) for the analysis of glycerides, FFAs, and γ-oryzanol or gas chromatography-mass spectrometry (GC-MS) for the analysis of biodiesel (*Section 2.4*).

2.3. Extraction of γ-oryzanol

γ-oryzanol residing in the resulting reaction product was recovered using an acid-base extraction method, modified from Sombutsuwan et al. (2018), in which NaOH was used to transform γ-oryzanol to its anionic phenoxide form (deprotonated) to make it separable from the biodiesel phase. Here, the effect of NaOH concentration on γ-oryzanol recovery was investigated to find the most suitable extraction condition as follows: the biodiesel phase (1 g) was first placed in a 50 mL Erlenmeyer flask on a magnetic stirrer and then mixed with hexane (1 mL), followed by addition of aqueous ethanolic NaOH (4 mL) which was composed of 24.09 %v/v ethanol and 75.91 %v/v of NaOH solution at a specified concentration (1, 2, 3, or 4 M). The mixture was stirred at room temperature (200 rpm, 1 min) before phase separation by centrifugation (4500 rpm, 10 min). The lower aqueous phase (pH 13-14) was collected, washed (1 × hexane to remove residual biodiesel), and neutralized (to remove residual NaOH) with dropwise addition of glacial acetic acid to pH 7. For further quantification of γ-oryzanol by HPLC, the sample was prepared by dissolving the neutralized aqueous phase (20 µL) with ethyl acetate (3 mL). The solution was then filtered through a 0.45 µm syringe filter prior to HPLC analysis (*Section 2.4*).

2.4. Quantitative analysis

2.4.1. Quantification of glycerides, FFAs, and γ-oryzanol

The quantification of glycerides, FFAs, and γ-oryzanol in RBAO, the post-reaction biodiesel phase, and the aqueous phase after γ-oryzanol extraction was conducted using HPLC (Waters, USA). The filtered sample in ethyl acetate (5 µL) was injected into a 4.60 mm × 100 mm Sunfire C18 column with a 3.5 µm particle size and 100 Å pore size (Waters, USA). The chromatography was carried out at room temperature using an HPLC system consisting of a pump (Waters, ELSD e2695, USA), used to maintain a constant mobile phase (60:40 methanol:isopropanol) flow rate at 0.5 mL.min^{-1}, and an evaporative light scattering detector (ELSD, SEDEX 80, USA) whose tube temperature and nitrogen gas flow were set at 50 °C and 0.3 L.min^{-1}, respectively, and the impactor was turned off. Concentrations of glycerides, γ-oryzanol, and FFAs were determined by comparing their chromatographic peak areas to those of calibration curves established through analysis of corresponding standards of RBO (for glycerides), oleic acid (for FFAs), and γ-oryzanol.

For the esterification/transesterification of RBAO (*Section 2.2*), quantification of glyceride removal, residual FFAs, and γ-oryzanol loss was done according to the following equations (**Eqs. 1-3**):

$$\text{Glyceride removal (\%)} = \frac{\text{Initial amount of glycerides} - \text{Amount of glycerides after reactions}}{\text{Initial amount of glycerides}} \times 100 \quad \text{Eq. 1}$$

$$\text{FFA remaining (\%)} = \frac{\text{Amount of FFAs after reactions}}{\text{Initial amount of FFAs}} \times 100 \quad \text{Eq. 2}$$

$$\text{γ-oryzanol loss (\%)} = \frac{\text{Initial amount of γ-oryzanol} - \text{Amount of γ-oryzanol after reactions}}{\text{Initial amount of γ-oryzanol}} \times 100 \quad \text{Eq. 3}$$

The extent of γ-oryzanol recovery was calculated by simple comparison with the original amount of γ-oryzanol in RBAO and biodiesel, as follows (**Eqs. 4** and **5**):

$$\text{γ-oryzanol recovery (\%) based on RBAO} = \frac{\text{Amount of extracted γ-oryzanol in ethanol}}{\text{Initial amount of γ-oryzanol in RBAO}} \times 100 \quad \text{Eq. 4}$$

$$\text{γ-oryzanol recovery (\%) based on biodiesel} = \frac{\text{Amount of extracted γ-oryzanol in ethanol}}{\text{Initial amount of γ-oryzanol in biodiesel phase}} \times 100 \quad \text{Eq. 5}$$

2.4.2. Quantification of biodiesel

The content of FAEEs in the biodiesel phase, defined as biodiesel content, was determined using a GC-MS system (Agilent 122-7032, Agilent Technologies, Inc., USA) equipped with a 0.25 mm × 30 m DB-WAX column with 0.25 µm film thickness (Agilent Technologies, Inc., USA). The sample (dissolved in hexane and filtered, 1 µL) was injected into the GC-MS system in a split mode (50:1) at the column oven temperature of 60 °C. In each run, the column temperature was initially started at 60 °C, then raised to 70 °C and finally to 230 °C (8 min hold). The GC conditions were injector temperature of 230 °C, flow control mode of linear velocity, and carrier gas flow rate (helium of 99.9995% purity) of 1.0 mL.min^{-1}. The MS condition employed were: ion source temperature of 200 °C, interface temperature of 240 °C, the scan range of 40–1000 m/z, solvent cut time of 5 min, MS start time of 4 min, end time of 27 min and ionization of EI (70 eV), and a scan speed of 2000. Based on the peak areas of all identified FAEEs (see **Supplementary Information**, ***Section 1***), the biodiesel content was calculated as shown in **Equation 6**:

$$\text{Biodiesel content (\%)} = \frac{\text{Amount of all identified FAEEs}}{\text{Amount of all components}} \times 100 \quad \text{Eq. 6}$$

2.4.3. Statistical analysis

All experiments were performed in triplicate, and the experimental data were expressed as means ± standard deviation (n=3). One-way analysis of variance (ANOVA) with pairwise comparison using a Tukey test at the 95% confidence level (α = 0.05) was carried out in SPSS Statistics 16.0 (IBM Corporation) to determine any statistical difference among/between means of data groups. The significant difference was indicated by different letters or symbols on the data (p<0.05).

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3. Results and Discussion

Being the dominant impurity in RBAO that complicates subsequent γ-oryzanol recovery, glycerides were first removed by transesterifying RBAO with ethanol to convert them into more easily separable FAEEs (or biodiesel). The first part of this study deals with determining the suitable reaction conditions for achieving glyceride removal while minimizing γ-oryzanol loss. However, due to the high FFA content in RBAO, FFAs could simultaneously undergo enzymatic esterification, resulting in biodiesel production. The first part of this study aims to investigate the effects of esterification/transesterification conditions on the percentages of glyceride removal, FFA remaining, biodiesel content, and γ-oryzanol loss and to determine the suitable conditions that attain complete glyceride removal, with minimal loss of γ-oryzanol, and possibly maximal content of biodiesel. Subsequently, acid-base extraction was employed to isolate γ-oryzanol from the resulting biodiesel, and suitable extraction conditions were proposed. In the following sections, the results are presented and discussed concerning the possible reaction/extraction mechanisms. Brief economic and environmental aspects, limitations, and practical implications of the present study are also included.

3.1. Enzymatic esterification/transesterification

In general, any lipase-catalyzed reaction follows a reversible pathway (**Fig. 1**), in which an acyl donor (A1) is cleaved at the ester bond by a free lipase (E) to form an acyl-enzyme intermediate (AE), which then releases a cleaved product (P1) and is then attacked by an acyl acceptor (A2) to obtain a final product (P2) and a free enzyme (Deleuze et al., 1987; Vaysse et al., 2002; Ribeiro et al., 2011). For our esterification/transesterification, A1 corresponds to FFAs/glycerides, A2 is ethanol, and P2 is FAEEs. Nonetheless, with various compounds in the reaction system, other possible undesirable side reactions may occur, including the hydrolysis of glycerides resulting from residual water in the RBAO, which also competes as an A2 acyl acceptor. Other off-target reactions include transesterification and hydrolysis of γ-oryzanol, in which γ-oryzanol is an A1 substrate.

Perhaps even more than other catalytic reactions, the activity of enzymatic reactions depends on operation conditions such as reaction temperature and time. Additionally, its efficacy in biphasic mixtures is affected by aqueous-oil interfacial conditions (Reis et al., 2009b) since lipase becomes activated at the aqueous-oil interface where its hydrophobic region orients in a way that gives the substrate access to the active site (Cheng et al., 2018). The interfacial conditions are subjected to the relative abundance of oil and aqueous components, enzyme loading, as well as extent of phase mixing employed. The effects of these conditions are defined: for the percentages of glyceride removal (**Eq. 1**), FFA remaining (**Eq. 2**), biodiesel content (**Eq. 6**), and for γ-oryzanol loss (**Eq. 3**).

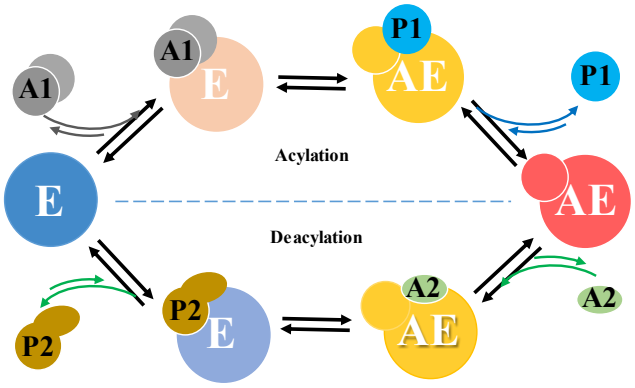

Fig. 1. Reversible pathway of lipase-catalyzed reaction; E is free lipase, A1 is acyl donor, AE is acyl-enzyme intermediate, P1 is the cleaved product, A2 is acyl acceptor, and P2 is the final product.

3.1.1. Effect of ethanol:rice bran acid oil (RBAO) molar ratio

Reactions using the ethanol (aqueous):RBAO (oil) molar ratios of 3:1 and 5:1 gave the best glyceride removal (100%) and biodiesel content (82-84%) with the lowest FFA remaining (ca. 31 %, **Fig. 2**). The highest γ-oryzanol loss (41-46%) was also obtained with these ratios. These ratios were hypothesized to provide the most favorable aqueous-oil interfacial conditions, giving rise to the highest possible yield of the reactions: 1) transesterification of glycerides with ethanol to obtain biodiesel (**Fig. 3a**) in which R1, R2, and R3 can be C16-18 alkyl groups or H for mono/di-glycerides, and 2) hydrolysis with water in RBAO to obtain FFAs, with subsequent conversion to biodiesel *via* esterification (**Fig. 3b** and **c**).

Other than glycerides, γ-oryzanol, comprising esters of ferulic acid (Ito et al., 2019), could become hydrolyzed into ferulic acid (**Fig. 4a** (Bhaskaragoud et al., 2016)) or converted to its ethyl ester (**Fig. 4b** and **c** (Compton et al., 2000; Chigorimbo-Murefu et al., 2009). Although reactions of both ester substrates were observed, the resulting γ-oryzanol loss was considerably lower than that of glycerides (41-46% *vs.* 100%), indicating lower lipase activity with γ-oryzanol. This could be due to the enzyme's binding preference for glycerides over γ-oryzanol. Similar lipase activities for esterification/transesterification of esters with different chain lengths have been reported in a previous study by Vaysse et al. (2002).

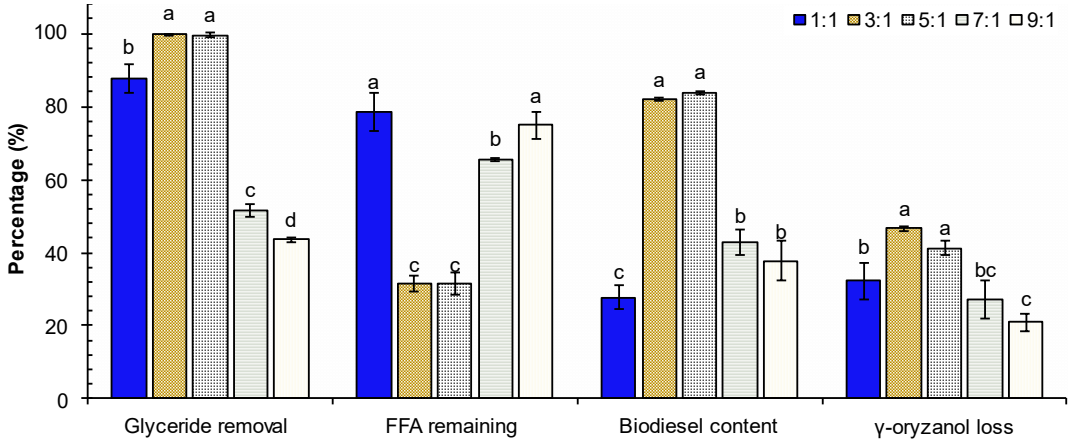

Fig. 2. Effect of ethanol:RBAO molar ratio (1:1-9:1) on percentages (%) of glyceride removal, FFA remaining, biodiesel content, and γ-oryzanol loss, after esterification/transesterification of rice bran acid oil (RBAO) at 40 °C, for 24 h, with 10% enzyme loading, and at 200 rpm. Significant differences are indicated by different letters (a, b, c, d) on the data (p<0.05).

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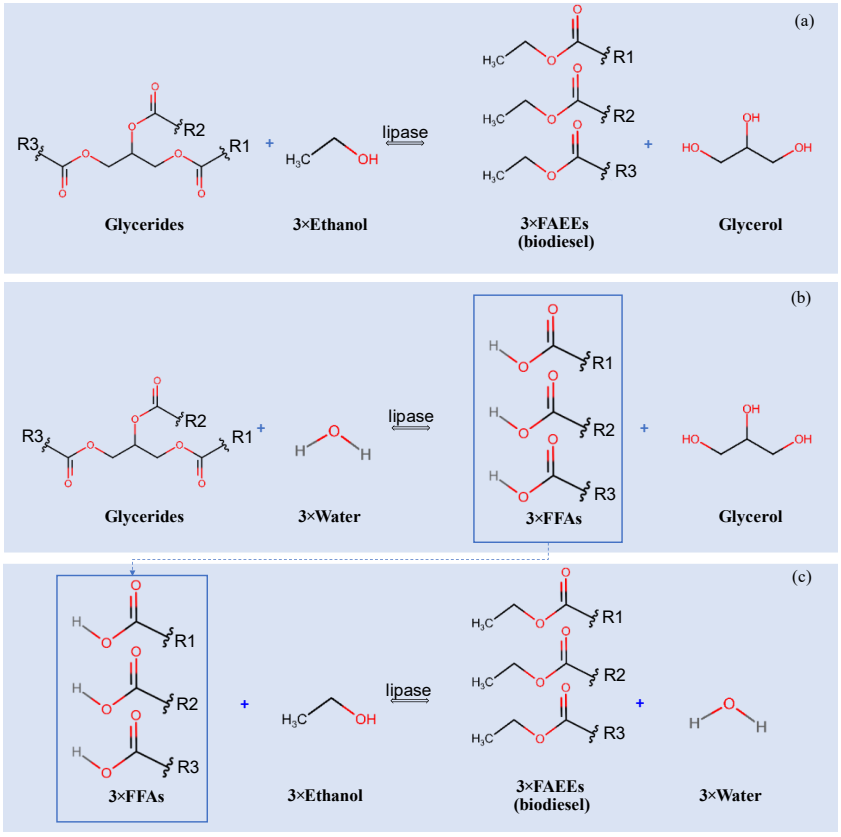

Fig. 3. Transesterification of glycerides (**a**), hydrolysis of glycerides (**b**), and esterification of free fatty acids (FFAs) (**c**). R1, R2, and R3 represent either C16-18 alkyl groups or hydrogen (H).

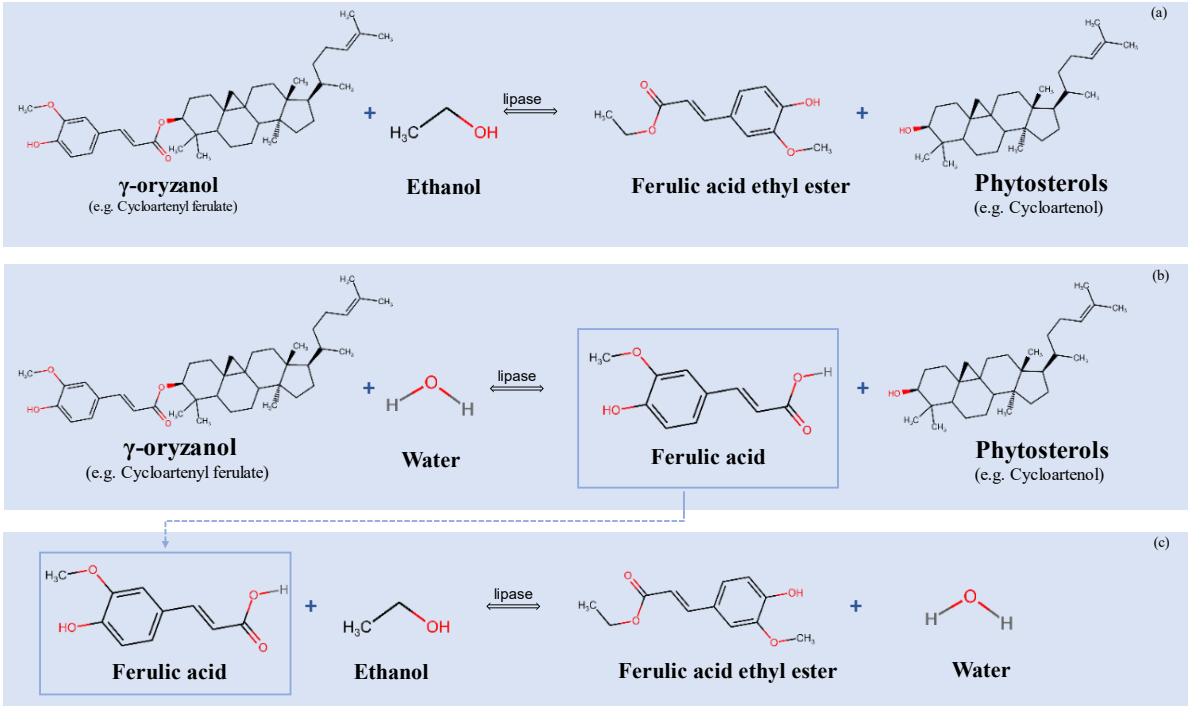

Fig. 4. Possible transesterification of γ-oryzanol (**a**), hydrolysis of γ-oryzanol (**b**), and esterification of ferulic acids (**c**).

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The reactions with the lower ethanol:RBAO molar ratio of 1:1, on the other hand, provided relatively low biodiesel content (ca. 28%) with relatively high FFA remaining (79%). The γ-oryzanol loss was also lower than those with the ethanol-rich ratios of 3:1 and 5:1 (ca. 32% *vs.* 41-46%), indicating low reaction activities. Lower ethanol:RBAO molar ratios could result in diminished interfacial areas, which would, in turn, limit enzyme activation and, thus, reaction activity. Despite low interfacial areas, high glycerides removal (88%) was observed at a 1:1 ratio. Based on the pathway (**Fig. 1**), this high glyceride conversion indicated that glycerides could favorably bind with lipase to form the acyl-enzyme intermediates (AEs). However, at this low ethanol ratio, the amount of ethanol might be insufficient to complete the conversion of all the AEs into FAEEs. In addition, high FFA remaining was observed in the presence of a low ethanol environment at this 1:1 ratio, suggesting that the water present in RBAO could readily gain more access to the AEs, leading to hydrolysis of glycerides to FFAs (Ma et al., 2002).

In ethanol-rich mixtures, the higher ethanol:RBAO molar ratios of 7:1 and 9:1, the higher reaction activities would have been expected due to the resulting higher interfacial areas. Nonetheless, the results (**Fig. 2**) show rather low degrees of glycerides removal (ca. 52 and 44%) and biodiesel formation (ca. 43 and 38%) with relatively high residual FFAs (ca. 66 and 75%). This low reaction activity was also indicated by the observed low γ-oryzanol loss (ca. 32 and 21-27%) at these ratios using excessive amounts of ethanol. This observation is in line with the study by Kamal et al. (2013), who demonstrated that the activity of 6B lipase initially paralleled alcohol content but, after that, dropped off as the alcohol content was increased beyond a certain threshold. This eventual fall in enzyme activity is likely a consequence of disruption of the enzyme active site structure as more alcohol molecules bound onto the vicinity of these sites. This denaturing would lower the enzyme's catalytic efficiency and thus lead to reduced observed activity.

Since ethanol:RBAO molar ratios of 3:1 and 5:1 were found to be suitable by providing complete glyceride removal (**Fig. 2**), these compositions were adopted for all subsequent studies.

3.1.2. Effect of temperature

The effect of temperature variation on the lipase-catalyzed reactions shows that the activity of those reactions did indeed drop off at certain temperatures (**Fig. 5**). At 3:1 ethanol:RBAO ratio (**Fig. 5a**), glyceride removal remained at 100%, and γ-oryzanol loss increased to ca. 52% as the temperature was increased to 50 °C. Beyond 60 °C, sharp decreases in both glyceride removal and γ-oryzanol loss were observed (to ca. 68 and ca. 40%, respectively). The observed steady increase in FFA remaining from 23% at 30 °C to ca. 50% at 60 °C suggests a continual steady decrease in the reaction activity for FFA conversion. As a result of these observed decreases in reaction activities, a drop in biodiesel content at 50 °C was seen, and the biodiesel content peaked instead at lower temperatures of 30-40 °C (ca. 82%).

In the reactions with the 5:1 ethanol:RBAO molar ratio (**Fig. 5b**), the highest glyceride removal (100%), biodiesel content (84%), and γ-oryzanol loss (ca. 41%) were obtained at 40 °C. This lower temperature threshold is likely a consequence of the enzyme's greater temperature sensitivity to denaturing in alcohol-rich media (Kamal et al., 2013). As a consequent precaution, this lower temperature was subsequently employed as our suitable temperature for both reactant ratios.

3.1.3. Effect of reaction time

Our findings revealed when reaction equilibrium was reached, after which the percentages of glyceride removal, FFA remaining, biodiesel content, and γ-oryzanol loss remained unchanged. Overall, 3:1 ethanol:RBAO ratio mixtures showed a leveling-off of all parameters from 18 h onwards; glyceride removal (100%), FFA remaining (ca. 31-33%), biodiesel content (ca. 78-82%), and γ-oryzanol loss (ca. 46-47%) (**Fig. 6a**). The 5:1 ratio required 24 h for the reactions to reach similar equilibration, from which point onwards, the percentages remained roughly constant at their stabilized values (**Fig. 6b**). The longer reaction times required with the 5:1 ratio could be due to the higher ethanol amounts used and the afore-discussed consequent impact on enzyme activity. The lower reaction rates were evident at initial times, as shown in **Figure 6**, in which the percentage of glyceride conversion increased more gradually at the 5:1 ratio compared to the 3:1 ratio. Our collective results suggest 18 and 24 h as suitable reaction times for the reactions at the ratios of 3:1 and 5:1, respectively.

When using short reaction times of 1.5-3 h, the percentages of FFA remaining were observed to be greater than 100% at both ethanol: RBAO molar ratios (**Fig. 6**). This suggested that, at the beginning of the reaction course, FFAs are generated from the hydrolysis of glycerides (**Fig. 3b**), more rapidly than their transesterification into biodiesel (**Fig. 3a**). The results moreover suggested that the initial rate of hydrolysis would be higher than that of esterification of FFAs (**Fig. 3b**). This could be due to the selectivity of lipase, which in the presence of water in the RBAO, naturally favors hydrolysis implying that water is a more active acyl acceptor than ethanol during this period. The finding aligns with a study by Urrutia et al. (2016) in which wet, greasy sewage sludge was used as a feedstock for biodiesel production. The authors showed that, in the lipase-catalyzed process, glycerides were first hydrolyzed to obtain FFAs, which were then esterified to obtain biodiesel.

3.1.4. Effect of enzyme loading

The results of enzyme loading effects (**Fig. 7**) show that, with 10-15% enzyme loading, the percentages of glyceride removal, FFA remaining, biodiesel content, and γ-oryzanol loss were constant in the reactions both at 3:1 (**Fig. 7a**) and 5:1 (**Fig. 7b**) ethanol:RBAO molar ratios, suggesting no further increase in enzyme activities above 10% enzyme loading. This could be due to the limited interfacial area onto which lipase adsorbs for its activation, afforded by the reaction conditions used (Reis et al., 2009a). In other words, the available interfacial area could already be saturated with adsorbed active lipases by 10% enzyme loading. With lower lipase loadings of 5-7.5%, however, there appeared to be a reduction in the reaction activities. The decrease in reaction activities was most noticeable with the 5:1 ratio and 5% lipase loading, in which the glyceride removal and biodiesel content dropped to ca. 67 and 62%, respectively. All in all, 10% enzyme loading was the most effective and adopted for the following studies.

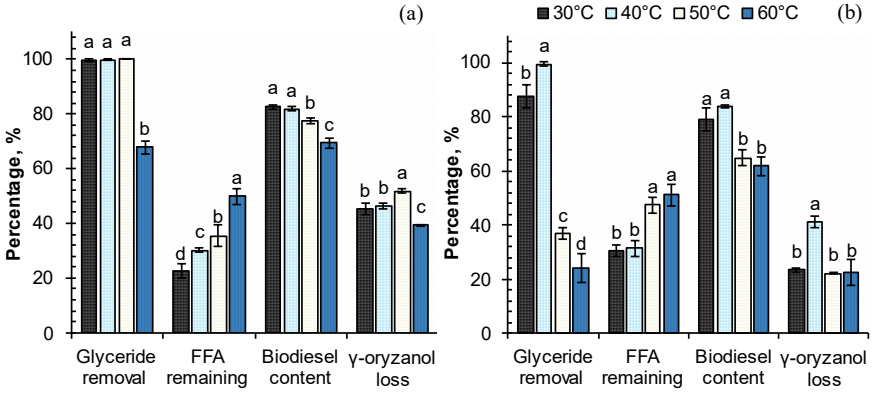

Fig. 5. Effect of temperature (30-60 °C) on percentages (%) of glyceride removal, FFA remaining, biodiesel content, and γ-oryzanol loss after esterification/transesterification of rice bran acid oil (RBAO) at 24 h, 10% enzyme loading, and 200 rpm, with either **(a)** 3:1 or **(b)** 5:1 ethanol:RBAO molar ratio. Significant differences are indicated by different letters (a, b, c, d) on the data (p<0.05).

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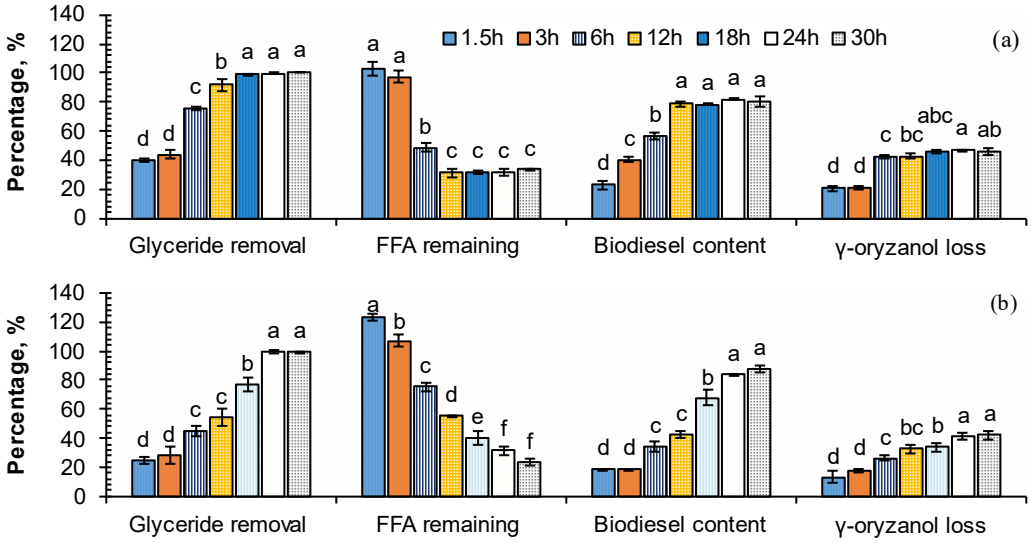

Fig. 6. Effect of reaction time (1.5-30 h) on percentages (%) of glyceride removal, FFA remaining, biodiesel content, and γ-oryzanol loss after esterification/transesterification of RBAO at 40 °C, 10% lipase loading, and 200 rpm, with either **(a)** 3:1 or **(b)** 5:1 ethanol:RBAO molar ratio. Significant differences are indicated by different letters (a, b, c, d, e, f) on the data (p<0.05).

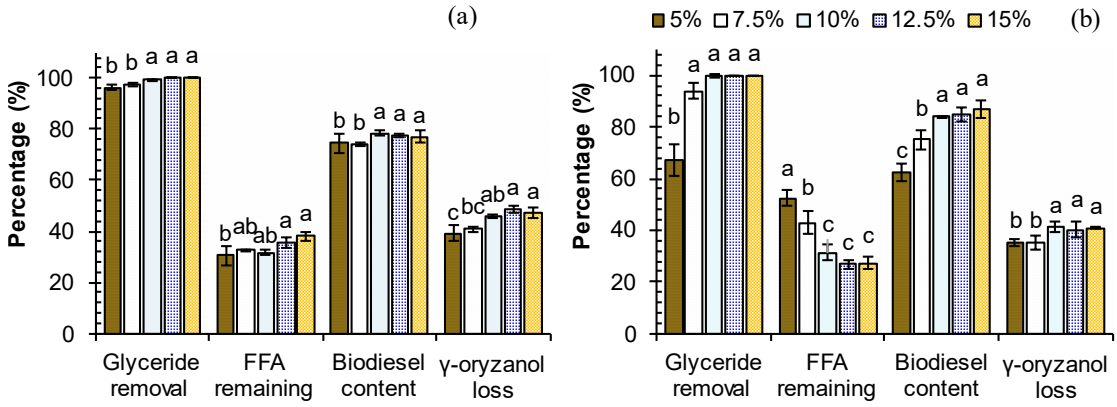

Fig. 7. Effect of enzyme loading (5-15 %wt) on percentages (%) of glyceride removal, FFA remaining, biodiesel content, and γ-oryzanol loss after esterification/transesterification of rice bran acid oil (RBAO) at 40 °C and 200 rpm with either **(a)** 3:1 ethanol:RBAO molar ratio and 18 h reaction time or **(b)** 5:1 ethanol:RBAO molar ratio and 24 h reaction time. Significant differences are indicated by different letters (a, b, c) on the data (p<0.05).

3.1.5. Effect of agitation speed

Improved reaction activities would be expected from increasing agitation speeds since they create smaller micro-droplets of oil phase dispersed in the aqueous phase and increased aqueous-oil interfacial area (Noor et al., 2003; Al-Zuhair et al., 2008). Nevertheless, the overall results (**Fig. 8**) showed relatively little effect on glyceride removal, FFA remaining, biodiesel content, and γ-oryzanol loss in the reactions with 3:1 (**Fig. 8a**) and 5:1 (**Fig. 8b**) ethanol:RBAO molar ratios as the agitation speeds increased from 200 to 400 rpm. This consistency implies that the interfacial areas created by the original speed of 200 rpm were already saturated with the given amount of lipases used (10 %wt). Indeed, there is even a slight decrease in the reactivity seen with higher agitation speeds (300-400 rpm), as shown by a slight increase in FFA remaining in the 3:1 ratio and a slight drop in biodiesel content in the 5:1 ratio (**Fig. 8a**). Rapid agitation could generate high shear stress onto the enzyme with consequent irreversible protein unfolding (Goswami et al., 2012; Piacentini et al., 2021).

Our collected results (**Figs. 2**, **5-8**) together point to suitable reaction conditions for complete glyceride removal involving 3:1 ethanol:RBAO molar ratio, 40 °C, 18 h, 10% enzyme loading, and 200 rpm agitation speed. Using the 5:1 ethanol:RBAO molar ratio for 24 h (with the other conditions unchanged) also proved suitable due to similarly observed 100% glyceride removal. However, the latter conditions provided slightly higher biodiesel content (ca. 84% *vs.* 78%) and lower γ-oryzanol loss (ca. 41% *vs.* 46%) than those at 3:1. This could be due to the effect of higher ethanol amount used in the reactions with 5:1 ratio. Despite the adverse effect of high ethanol content in lowering reaction activities as above-discussed, higher interfacial areas and longer reaction time used in the reactions at the 5:1 ratio would overall allow for the higher glycerides conversion, giving the observed higher biodiesel content. The observed lower γ-oryzanol loss, however, could be related to the change in the binding site structure, which makes reactions with γ-oryzanol unfavorable. Therefore, the suitable 5:1 ratio conditions were chosen to produce the biodiesel phase as the raw material for the subsequent extraction of γ-oryzanol.

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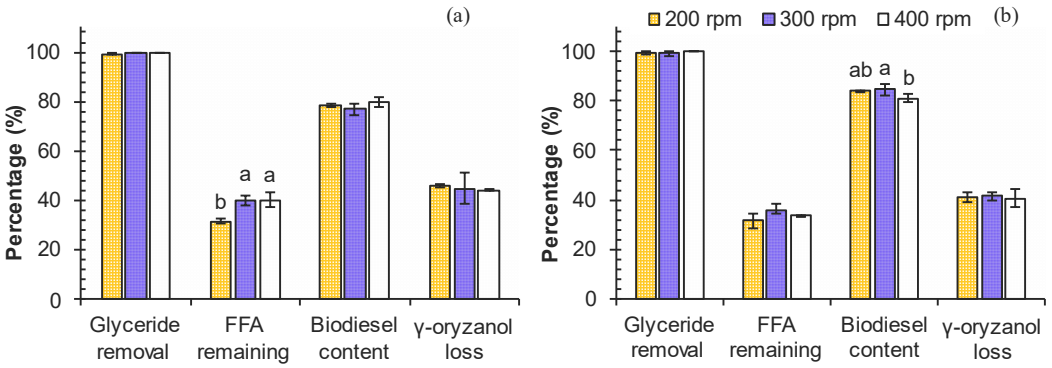

Fig. 8. Effect of agitation speed (200-400 rpm) on percentages (%) of glyceride removal, FFA remaining, biodiesel content, and γ-oryzanol loss after esterification/transesterification of rice bran acid oil (RBAO) at 40 °C and 10% enzyme loading with either **(a)** 3:1 ethanol:RBAO molar ratio and 18 h reaction time or **(b)** 5:1 ethanol:RBAO molar ratio and 24 h reaction time. Significant differences are indicated by different letters (a, b) on the data (p<0.05).

3.2. Extraction of γ-oryzanol

To ensure a sufficient sample for the γ-oryzanol extraction study, a 10× scale-up of the enzymatic esterification/transesterification of RBAO was carried out under our optimized reaction conditions. Complete glyceride conversion was still obtained from this reaction scale (Fig. 9a); nonetheless, the percentages of biodiesel content (76%) and γ-oryzanol (26%) were slightly lower than those with the 1× reaction scale, possibly caused by decreased mass transfer within the larger reaction volume.

From the biodiesel phase obtained, γ-oryzanol recovery was achieved by acid-base extraction. Here, γ-oryzanol was deprotonated by aqueous ethanolic NaOH in hexane and then partitioned between the resulting aqueous and hexane (biodiesel) phases. To find an optimal extraction condition, the effect of NaOH concentration on the percentages of γ-oryzanol recovery estimated based on its original amount in either RBAO or the biodiesel phase (Eqs. 4 and 5) was investigated.

As shown in Figure 9b, as NaOH concentration was increased from 1 to 4 M, γ-oryzanol recovery degrees based on RBAO and biodiesel were found to increase from ca. 55% and ca. 83% to 61-63% and 91-94%, respectively. In theory, increased base concentrations could cause higher degrees of deprotonation, resulting in more γ-oryzanol dissolved in the aqueous phase. Greater γ-oryzanol loss through hydrolysis would be expected with higher base amounts (Meedam et al., 2022). However, only 6-9% γ-oryzanol from the biodiesel was lost during the alkali extraction; our relatively short contact time (1 min) likely helped minimize the extent of hydrolysis. The observed loss could rather be due to the phase equilibria of deprotonated γ-oryzanol between the oil and aqueous phases. 2-4 M concentrations of aqueous ethanolic NaOH were thus suggested as suitable for acid-base extraction of γ-oryzanol from the biodiesel.

3.3. Comparison of results with previous studies

To summarize the results from this study, complete glyceride removal (ca. 97-100%) could be achieved with enzymatic simultaneous esterification/transesterification of RBAO at the most suitable condition (at 5:1 ethanol:RBAO molar ration, 40 °C, 24 h, 10% enzyme loading, and 200 rpm agitation speed). During this process, the loss of γ-oryzanol was as low as 26-41%, while relatively high biodiesel content (ca. 76-84%) was attained. The subsequent acid-base extraction from the biodiesel phase of the reaction product with 2-4 M NaOH concentration provided 94% of γ-oryzanol recovery (based on the amount originally present in the biodiesel phase), which is equivalent to around 61-63% of γ-oryzanol in the original RBAO remained after extraction.

Although this study is the first to report the results on the combined enzymatic biodiesel production and acid-base extraction of γ-oryzanol from RBAO (summarized in Table 4), comparisons are made where possible with the results reported in related literature, most of which fall into three categories: 1) emphasizing esterification/transesterification to produce biodiesel, 2) emphasizing the recovery of γ-oryzanol, and 3) considering both biodiesel production and γ-oryzanol recovery, similar to the current study.

In the first group, Ghosh and Bhattacharyya (1995) applied lipase-catalyzed reactions for biodiesel production and showed 73-78% glyceride conversion, lower than that obtained in this study. Though their biodiesel

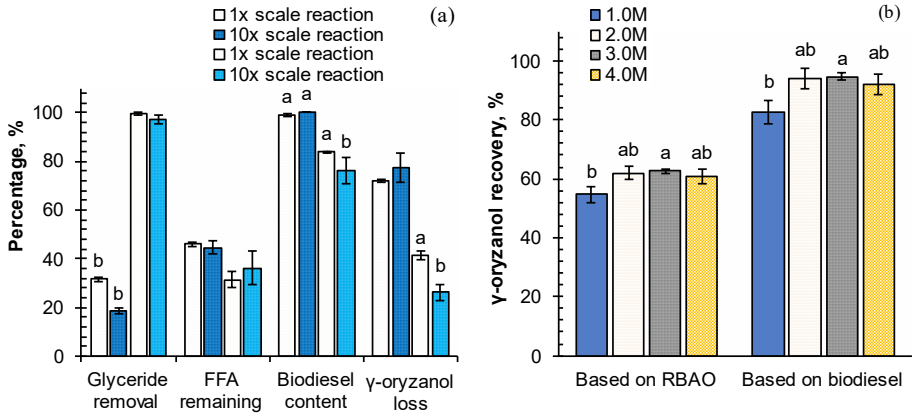

Fig. 9. (**a**) Percentages (%) of glyceride removal, FFA remaining, biodiesel content, and γ-oryzanol loss after esterification/transesterification of rice bran acid oil (RBAO) at selected suitable reaction condition: 5:1 ethanol:RBAO molar ratio, 40 °C, 18 h, 10% enzyme loading, and 200 rpm agitation speed, with one-time (1×) and ten-time (10×) larger amounts of raw materials and (**b**) Effect of aqueous NaOH concentration in ethanol and hexane on percentage (%) of γ-oryzanol recovery via acid-base extraction calculated based on RBAO (Eq. 4) and biodiesel (Eq. 5). Significant differences are indicated by different letters (a, b) on the data (p<0.05).

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Table 4.
Comparison with existing data from previous studies on rice bran acid oil (RBAO) utilization.

Reference	Raw Materials	Method/Goal	Reaction Condition	Glycerides	Biodiesel	γ-Oryzanol	Remark
This Study	-RBAO -Ethanol -*Aspergillus niger* lipase	Esterification/ transesterification with acid-base extraction of γ-oryzanol *Aim*: Glyceride removal for subsequent γ-oryzanol recovery	Reaction - 5:1 ethanol:RBAO molar ratio 40°C, 24 h, 10% enzyme loading 200 rpm agitation speed Extraction - 2-4 M ethanolic NaOH solution; 75.91% ethanol in water, 20.59% hexane, 1 min	>98% conversion	ca. 84% content in the oil phase	26-41% loss* ca. 60% recovery from RBAO** ca. 94% recovery from biodiesel**	*After reactions **From extracted γ-oryzanol
Ghosh and Bhattacharyy, (1995)	-RBAO -C4-C18 saturated alcohols -*Mucor miehei* lipase	Esterification/ transesterification *Aim*: Biodiesel production	Reaction - 1:1 ethanol:RBAO molar ratio 60°C, 4 h, 10% enzyme loading - Not reported agitation speed	73 - 78% conversion*	89.2-151.2% yield**	Not reported	*Estimated based on a fatty acid equivalent **Estimated based on acid oil
Choi et al. (2016)	-RBAO -Ethanol -Lipases: Novozym 435 Lipozyme RM IM Lipozyme TL IM	Two-step Esterification/ transesterification *Aim*: Biodiesel production	1st reaction step - 5:1 ethanol:RBAO molar ratio 40°C (Novozym 435) or 30°C (Lipozyme RM IM), 15 min, 10% Novozym 435 or 5% Lipozyme RM IM, 300 rpm agitation speed 2nd reaction step - 5:1 ethanol:RBAO molar ratio, 30°C (Lipozyme TL IM), 23 h 45 min 10% Lipozyme TL IM, 300 rpm agitation speed	ca. 90% conversion	92% content	Not reported	-
Meedam et al. (2022)	-RBAO -Water -NaOH	Hydrolysis *Aim:* Glyceride removal for subsequent γ-oryzanol recovery	Base-catalyzed reaction - 5:1 water:RBAO ratio (v/v), 90°C, 5-10 min, 2.5 M NaOH solution - Not reported agitation speed Subcritical water reaction - 5:1 water:RBAO ratio (v/v), 220°C, 10 min	100% Conversion* >95% conversion**	Not available	ca. 57–66% remaining* ca. 50% remaining**	*Base-catalyzed **Subcritical water
Sombutsuwan et al. (2018)	-RBAO -Ethanol -H_2SO_4	Esterification/ transesterification with acid-base extraction of γ-oryzanol *Aim:* Biodiesel and γ-oryzanol extraction/ separation	Reaction - 40:1 ethanol:RBAO molar ratio, 60°C, 120 min, 10% HCl - Not reported agitation speed Extraction - 1.855 M ethanolic NaOH solution in hexane, 75.91% ethanol in water, 20.59% hexane, 1 min	Not reported	ca. 55% recovery after acid–base extraction	ca. 76 % recovery from the reaction product	-

* and ** correspond to related information in a given study.

yield with respect to the amount of RBAO used was reportedly relatively high (89.2-151.2%), the effects of reaction conditions on γ-oryzanol were not reported. Choi et al. (2016) employed two consecutive esterification/transesterification of RBAO using rather expensive immobilized lipases. Compared with our study, the percentage of glyceride removal was reported to be lower (90%), while the resulting biodiesel content was higher (92%), possibly as a result of their reaction conditions which allowed more FFA esterification (only ca. 8% FFA remaining).

Among studies where γ-oryzanol recovery is the focus, Meedam et al. (2022) compared base-catalyzed, acid-catalyzed, and non-catalytic hydrolysis under subcritical water for glyceride removal. Based on their results, base-catalyzed reaction and subcritical water hydrolysis were considered suitable, providing complete glyceride removal with 57-66% and 50% γ-oryzanol remaining at the best conditions, respectively. The results are comparable to ours, but the resulting reaction product in their study was soap and FFAs rather than biodiesel. In another study by Sombutsuwan et al. (2018), acid-catalyzed reactions were used for biodiesel production before the acid-base extraction of γ-oryzanol. The authors reported 76% recovery of γ-oryzanol (based on the amount present in the reaction product) after extraction of the aqueous phase with ethyl acetate, followed by drying under N_2. Nonetheless, this study did not report percentages of glycerides conversion, biodiesel yield, and γ-oryzanol recovery based on its original content in RBAO.

3.4. Practical implications and limitations of the present study

In this section, practical implications, including techno-economic and environmental sustainability aspects, as well as potential limitations of the present study, are discussed.

3.4.1. Practical implications

The practicality of the enzymatic esterification/transesterification followed by acid-base extraction can be envisaged as an integrated process in an RBO chemical refinery (**Fig. 10**), in which, in addition to RBO, γ-oryzanol and biodiesel are co-produced from low-cost RBAO. In a standard chemical RBO refinery, approximately 6,000 tons of RBAO annually is generated and is typically sold directly as low-cost animal feed at ca. US$ 0.6 kg^{-1} (Thai Edible Oil Co. Ltd., 2018). This gives the industry an estimated revenue of ca. US$ 3.6 million annually. With the integrated process, it is estimated that ca. 316.8 and 1,130.1 tons of γ-oryzanol and

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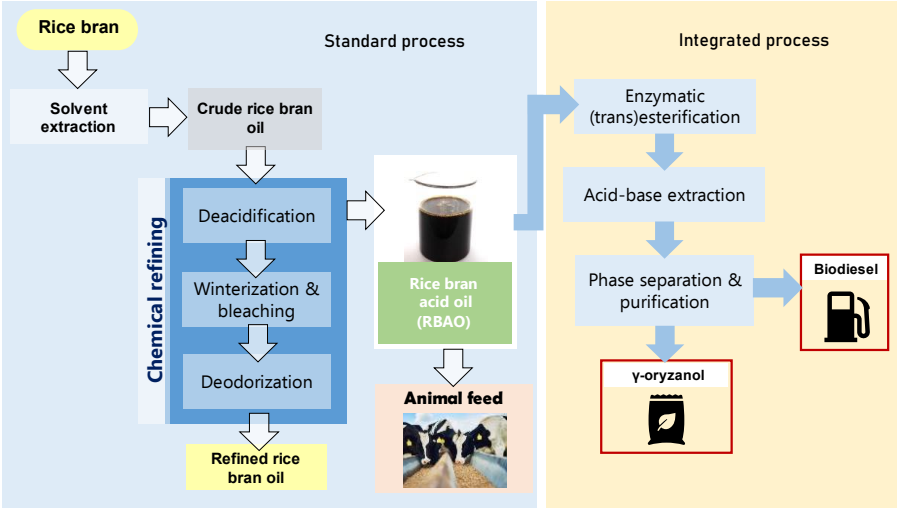

Fig. 10. Schematic representation of refined rice bran oil production process with an integrated process of γ-oryzanol isolation/biodiesel production from rice bran acid oil (RBAO).

biodiesel annually can be produced (see **Supplementary Information**, ***Section 2*** for the detailed calculation). Therefore, with the prices of γ-oryzanol (97% purity) and biodiesel at US$ 220 and US$ 1.0 kg^{-1}, the integrated process gives considerably higher total revenue than the standard process (US$ 70.8 *vs.* US$ 3.6 million annually).

To determine whether or not the process is profitable, the gross profit margin, or GPM (%), can be estimated from the revenue and the costs of goods sold (COGS) as follows (**Eq. 7**) (Murphy, 2022).

$$\text{Gross profit margin, GPM (\%)} = \frac{\text{Revenue - Costs of Goods Sold (COGS)}}{\text{Revenue}} \quad \text{Eq. 7}$$

In general, COGS include costs associated with producing goods, including labor, materials, and manufacturing overhead. However, it should be noted that the COGS herein was calculated only from the cost of all main raw materials required in the integrated process, such as lipase, ethanol, hexane, and NaOH. Based on the estimated consumption of these raw materials from the experimental results of this study and the current raw material prices, the COGS was approximated to be US$ 11.0 million annually (see **Supplementary Information**, ***Section 2***), and the resulting GPM (%) was thus ca. 84.5%. Although this preliminary GMP value may be an overestimate as the direct production costs other than the material costs have not been included, it is projected that the corrected GPM should still lie within the typical range for a healthy business, typically between 50 to 70% (Business Development Bank of Canada, 2023), suggesting that the integrated process would be profitable.

It is also worth noting from **Figure 11** that various factors can greatly influence the GPM (%). These include the uncertainties in market prices and the separation efficiency of the process, which determines the production rate, and, thus, the total sales (revenue). To take these into account, the sensitivity analysis of the GPM (%) was performed for ±50% variations in γ-oryzanol sales, biodiesel sales, and cost of production (raw material). The graphical results (**Fig. 11**) indicated that the variation in the sales of γ-oryzanol, possibly due to changes in the market price and separation efficiency (or production rate), most dramatically affects the GPM. Nonetheless, the smallest GPM (with the 50% reduction in γ-oryzanol sales) is still only marginally lower than 70%. The 50% increase in production cost also greatly decreases the GPM, but the GPM is still higher than 75%. On the other hand, the change in biodiesel sales did not noticeably affect the GPM since its sales account for only 1.6% of the total revenue.

The high glyceride conversion by lipase-catalyzed esterification/transesterification in the present study also implies that glyceride conversion by other lipase-catalyzed reactions with different acyl acceptors can also be brought about with the determination of a suitable reaction condition. Therefore, instead of biodiesel, it would be possible to produce other high-value compounds from the conversion of glycerides/FFAs in RBAO. Aminolysis /amidation, for example, which is the reactions between

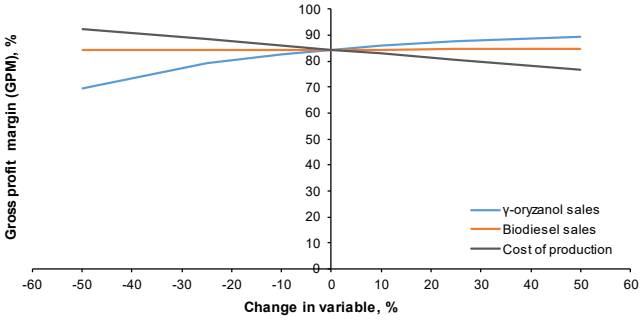

Fig. 11. Sensitivity analysis of gross profit margin, GPM, (%) of the integrated process for γ-oryzanol production from rice bran acid oil (RBAO) with respect to ±50% variations in γ-oryzanol sales, biodiesel sales, and cost of production (raw material).

esters/carboxylic acids as acyl donor and amines as acyl acceptor (van Rantwijk et al., 2000; Manova et al., 2018), have been utilized for the conversion of glycerides/FFAs to produce fatty acid amides (Griffin et al., 2016). Lipase-catalyzed amidation of crude RBO (Wang et al., 2017) and hydrolyzed sunflower oil soapstock (Brenna et al., 2023) was previously demonstrated to produce fatty acid ethanolamides, one of which is oleic acid ethanolamide, a recently FDA-approved pharmaceutical agent for body weight loss (Laleh et al., 2019) with its price up to US$ 4,000 kg^{-1} (see **Supplementary Information**, ***Section 2***). Application of these alternative reactions other than the esterification /transesterification may potentially increase the profitability of the integrated process.

Concerning environmental sustainability, the enzymatic esterification/transesterification for glyceride removal from RBAO proposed here is well accepted as a greener alternative, compared with the conventional acid-catalyzed process using a strong acid, i.e., HCl and H_2SO_4. Specifically, assuming 1-5 wt% of strong acid catalyst typically required for the conventional process (Marchetti and Errazu, 2008; Sombutsuwan et al., 2018), the proposed enzymatic process makes possible the reduction of up to 60-300 tons of the acid catalyst annually, which necessitates a subsequent costly waste treatment process otherwise. Furthermore, compared with the non-catalytic reactions under supercritical conditions (220–350 °C and 100-200 bar (dos Santos et al., 2018)), the enzymatic process occurs at considerably lower temperatures at ambient pressure, thus requiring considerably lower energy input, and thus the operating cost.

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3.4.2. Limitations

Even though the enzymatic approach to glyceride removal from RBAO before γ-oryzanol isolation is technically sound and is potentially profitable, there are two main reasons for the limited commercial application of the process. Firstly, compared to the physicochemical approaches, the lipase-catalyzed reactions tend to require longer reaction times; 18-24 h based on the present study, *vs.* 2 -3 h for acid-catalyzed reactions (Sombutsuwan et al., 2018), which could translate into higher operating cost, i.e., labor and utilities. Secondly, compared with the commonly used acid catalyst, i.e., H_2SO_4, lipase is relatively expensive (US$ 0.26 *vs.* 5.08 kg^{-1}, see **Supplementary Information**, ***Section 2***), and as suggested by the economic analysis addressed earlier, the cost of lipase accounted for ca. 27.6% of the total raw material annual expense, deeming the recovery and reuse of lipase to be necessary (Zuyi and Ward, 1993).

4. Conclusions and Future Perspectives

The results in this study demonstrated the technical, economic, and environmental potential of the herein proposed process as an integral part of the chemical RBO refinery, in which RBAO byproduct is readily converted to biodiesel *via* enzymatic esterification/transesterification followed by γ-oryzanol recovery *via* acid-base extraction. The specificity of the enzymatic reactions was one of the key findings, allowing complete glycerides removal and relatively high biodiesel yields with relatively low loss of γ-oryzanol at rather mild reaction conditions. Furthermore, as high as 94% recovery of γ-oryzanol from the reaction product could be achieved with a relatively simple acid-base extraction at suitable conditions. Future research studies are needed to overcome the limitations discussed earlier. A research area that should be highlighted involves improving lipase activity and selectivity toward the conversion of glyceride/FFAs. Other important future research areas cover the recovery and reuse of lipase, possibly by immobilization of the enzyme onto a solid support, in which reaction performance and enzyme stability after repeated use should be investigated (Raghuvanshi and Gupta, 2010; Chandra et al., 2020). The results of these suggested future investigations will ultimately strengthen the overall benefits of the integrated enzymatic esterification/transesterification process by making it more economically competitive with the conventional acid-catalyzed process.

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Supplementary Information

Section 1. Composition of free fatty acids in rice bran acid oil and resulting fatty acid ethyl esters

The composition of free fatty acids (FFAs) in rice bran acid oil (RBAO) (**Table S1**) was quantified using GC-MS, as described in *Section 2.4.2*.

Table S1.
Composition of FFAs in rice bran acid oil (RBAO).

FFA Components	Chemical formula	Amount (%wt)
Palmitic acid	$C_{16}H_{32}O_2$	23.28
Linoleic acid	$C_{18}H_{32}O_2$	37.53
Oleic acid	$C_{18}H_{34}O_2$	29.71
Eicosanoic acid	$C_{20}H_{40}O_2$	9.48

The composition of biodiesel (fatty acid ethyl esters) is shown in **Figure S1**, which is mainly composed of esters of the main FFAs: palmitic acid, linoleic acid, and oleic acid as indicated by peaks with PAEE (15.087), OAEE (17.062), and LAEE (17.491).

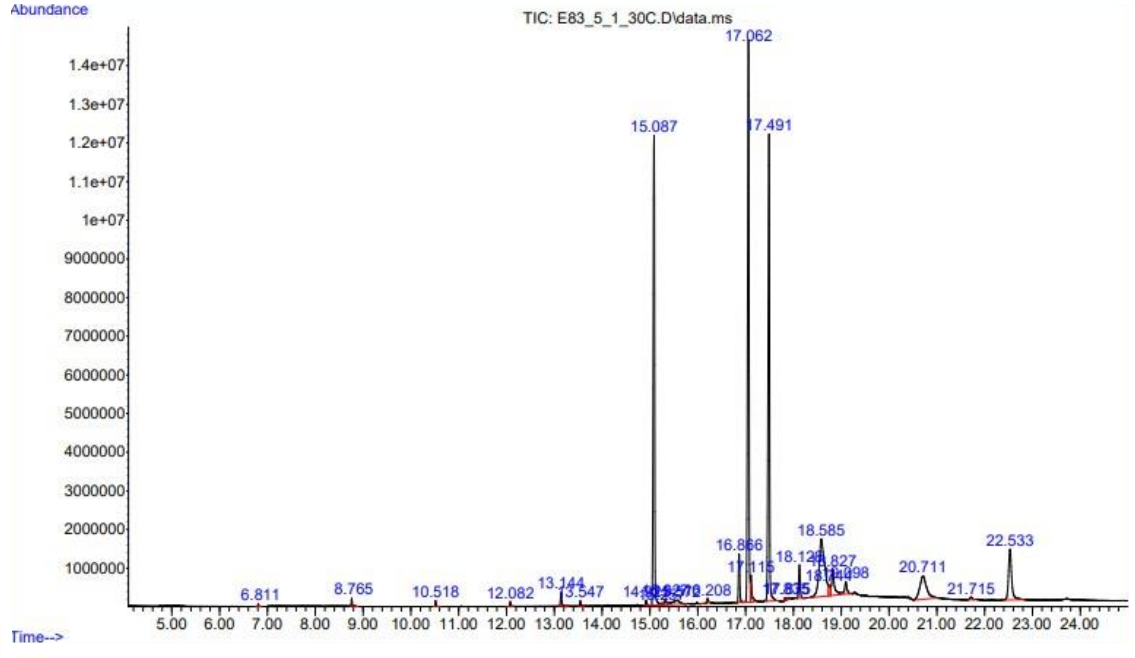

Fig. S1. GC-MS of the resulting fatty acid ethyl esters after enzymatic esterification/transesterification.

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Section 2. Economic analysis of integrated process

Table S2.
Price and cash flow of raw materials and products in the integrated process of γ-oryzanol isolation/biodiesel production from rice bran acid oil (RBAO)*.

Components	**Price** (US$/kg)	**Amount (ton/yr)**	**Cash flow (million** US$ **/yr)**
Rice bran acid oil (RBAO)	0.6	6,000.0	3.60
Products			
γ-oryzanol	220	316.8	69.70
Biodiesel	1.00	1,130.1	1.13
Raw material			
Lipase	5.08	600.0	3.05
Ethanol	0.5	9,872.4	4.94
Hexane	1.5	1,358.1	2.04
NaOH	1.46	657.5	0.96

* RBAO is produced at 6,000 tons/yr. The price of RBAO at 20-25 THB/kg, or ca. US$ 0.6/kg (from Thai Edible Oil Co., Ltd Samutprakarn, Thailand).

As the γ-oryzanol content in RBAO is ca. 11% (as reported in this study), the initial amount in the produced RBAO is ca. 660 tons yr^{-1}. From the reported loss during the esterification/transesterification reactions and the acid-base extraction of 26-41%, a 40% loss was selected and used for the estimation. Thus, after reaction/extraction, the remaining γ-oryzanol was estimated at 396 tons yr^{-1}. With the final separation and purification steps further applied to increase the purity of the γ-oryzanol (assuming an 80% separation factor), 316.8 tons yr^{-1} of the γ-oryzanol production rate was thus obtained. Of the prices of γ-oryzanol (97% purity) between US$ 190-390 kg^{-1} (from https://zebragoherb.en.made-in-china.com/product/mdYtFLOKAGkh/China-Natural-Gamma-Oryzanol-99-From-Rice-Bran-Oil.html, accessed on 25 April 2023), US$ 220 kg^{-1} was used for the calculation.

Of the reported range of 76-84% biodiesel content from the reactions, 80% biodiesel content was used for the calculation. From the enzymatic esterification/transesterification at the most suitable condition, in which 100% conversion of glycerides and ca. 33.5% free fatty acids (FFAs) remaining were resulted, it is calculated that the biodiesel phase contained mainly FFAs and biodiesel, and thus 924 tons yr^{-1} of FFAs and 3,698.4 tons yr^{-1} of biodiesel are produced. After the extraction and the final separation and purification steps with a 55% separation factor for biodiesel, as reported by Sombutsuwan et al. (2018), the production rate of biodiesel was calculated to be 1,130.1 tons yr^{-1}. The price of biodiesel in Thailand at US$ 1.0 kg^{-1} is taken from https://www.krungsri.com/en/research/industry/industry-outlook/Energy-Utilities/Biodiesel/IO/io-biodiesel-21, accessed on 25 April 2023.

Since the reactions used lipase at 10 wt% of RBAO, the consumption rate equals 600 tons yr^{-1}. The price of lipase at US$ 127.00 for 25 kg packs, or US$ 5.08 kg^{-1}, is from https://enzymes.bio/product/lipase-enzyme-powder-enzymatic-activity-100000u-g-lipase-food-grade/, accessed on 25 April 2023.

Ethanol was used both for the reaction and extraction processes. At 5:1 ethanol:RBAO molar ratio, 1.65 g of ethanol was used with 2 g of RBAO for the reactions, and ca. 3 mL (2.4 g) ethanol was used with 1 g of biodiesel phase (biodiesel + FFAs) for the extraction of γ-oryzanol. Therefore, the consumption rate of ethanol was calculated to be 9,872.4 tons yr^{-1} (4950 from the reactions and 4922.4 from the extraction). The price of ethanol at US$ 500 ton^{-1}, or US$ 0.5 kg^{-1}, is obtained from https://www.alibaba.com/product-detail/High-Quality-Chemicals-With-Best-Competitive_1600591809345.html, accessed on 25 April 2023.

Hexane was used to extract 1 mL (0.66 g) for 1 g of the biodiesel phase. Thus, from the biodiesel phase produced per year, the hexane consumption rate was calculated to be 1,358.1 tons yr^{-1}. Its price at US$ 1500 ton^{-1}, or US$ 1.5 kg^{-1}, is from https://www.alibaba.com/product-detail/Price-of-top-quality-liquid-100_1600251049956.html?spm=a2700.galleryofferlist.0.0.4da57181Sv2TC2, accessed on 25 April 2023.

NaOH was used for the extraction process. While 2-4 M NaOH concentration was suggested as suitable, 2 M was selected for use in the process and the calculation. Therefore, 2 moles of NaOH (80 g) was used for 1 L of the ethanolic NaOH. For 1 g of the biodiesel phase, 4 mL of the solution, or 80(0.004) = 0.32 g NaOH was used. Therefore, 657.5 tons yr^{-1} was estimated for the NaOH consumption rate. The price of NaOH in Thailand at 1250 THB for 25 kg packs, or ca. US$ 1.46 kg^{-1}, is obtained from https://www.intiponcheme.com/product/195/%E0%B9%82%E0%B8%8B%E0%B8%94%E0%B8%B2%E0%B9%84%E0%B8%9F-sodium-hydroxide-25-%E0%B8%81%E0%B8%81, accessed on 25 April 2023.

H_2SO_4 price at US$ 0.26 kg^{-1} is from https://langyichem.en.made-in-china.com/product/KZOAJxErgVUC/China-China-Plant-Supply-Industrial-Grade-H2so4-98-7664-93-9-Sulphuric-Acid.html, accessed on 25 April 2023.

Oleic acid ethanolamide price at US$ 0.5-4.0 g^{-1} or US$ 500-4000 kg^{-1} is from https://yiruopharm.en.made-in-china.com/product/edvtcUqOGMWX/China-Nootropics-Weight-Management-Powder-Oleoylethanolamide-Oea-Supplements-CAS-111-58-0.html, accessed on 25 April 2023.

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