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2In-situ (trans)esterification of lipid-dense post-hydrolysis rice bran at ambient pressures with low acid loading

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City, Taiwan c Department of Chemical Engineering, Widya Mandala Surabaya Catholic University, Kalijudan 37, Surabaya, 60114, Indonesia d Taiwan Building Technology Center, National Taiwan University of Science and Technology, Keelung Road, Taipei City, Da'an District, 10607, Taipei City, Taiwan e Department of Chemical Engineering, University of San Carlos, Talamaban Campus, Gov. Cuenco Avenue, Nasipit Talamban, 6000, Cebu City, Philippines ARTICLE INFO ABSTRACT Keywords: Dilute acid hydrolysis

2**In-situ (trans)esterification Lipid-dense** biomass **Rice bran**

Solid acid catalyst Sulfonation Dilute acid hydrolysis was employed to generate lipid-dense post-hydrolyzed rice bran (PHRB) which was uti- lized as feedstock in biodiesel production. Upon drying of the wet PHRB with the entrained dilute acid solution, subsequent carbonization and sulfonation occurred with the material, incorporating significant amounts of sulfur in PHRB as sulfonic acid. The collected dry PHRB was utilized as feedstock in the in-situ (trans)esterification (ISTE) of its lipids to fatty acid methyl esters (FAME), whereby the available acid sites reduced the acid catalyst to be loaded in the reaction system. The optimum reaction conditions under ambient pressures were determined via the Taguchi method and the highest yield achieved was 22.38 ± 0.28 g FAME/100 g PHRB (82.31% con- version or reaction yield), and was achieved at 65 C, SSR of 20 mL methanol/g dried PHRB, 12 h reaction time, ◦ and 5 wt% H2SO4 of the PHRB processed. The post-ISTE PHRB was found to still possess the catalytic activity and could be used

2**for the esterification of oleic acid and methanol and**

appreciable stability. Finally, the FAME-rich product also exhibits free-radical scavenging activity, owing to the presence of bioactive components, which may provide better oxidative stability or could be further recovered as high-value by-products. 1. Introduction

8**Rice is a staple food in** many countries in **Asia, with**

~90% of global rice production (~782 Metric Tons) in 2018 produced in Asia [1]. In the harvesting and processing of paddy rice to produce white rice grains, residues including rice straws, rice husk, and rice bran (RB) are inevi- table [2]. Among the different residues generated in the rice industry, rice bran is the only residue containing significant quantities of lipids (15–20 wt%) [3]. Its lipids make it an interesting agro-industrial residue with various applications as a source or raw material in food, fuel, nutraceuticals, and cosmetics [4–6]. Despite the significant content of lipids in RB, mechanical extraction of RB lipids (RBL) only

allows re- covery of no more than 55% even with an optimized die configuration of a screw press for the continuous extraction and

> 8**recovery of the lipids** [7]. This **is** probably **owing to its** relatively lower **lipid content**

and * Corresponding author. E-mail address: awgo@mail.ntust.edu.tw (A.W. Go). 1 Authors contributed equally to this work. smaller particle size compared to oil-bearing seeds, thus, favoring the use of solvents for lipid recovery from RB [8]. Apart from concerns on how RBL is to be extracted from RB, is the hydrolytic activity of indigenous lipases which rapidly breaks down the available acylglycerides into free fatty acids (FFA), reaching as much as 60 to 80 wt% within 6 months [9–11]. Owing to RBL having high FFA and other non-triglyceride components, refining yield to produce refined rice bran oil, only ranges from 50 to 70% [3], which makes it relatively more expensive to produce when compared to other edible oils. But like other edible oils, RBL has fatty acid distribution, which could be adopted for use as fuel

> 9**in the form of fatty acid methyl ester (FAME**

) [8,12]. Although the presence of FFAs is also a concern in FAME production, these could still be converted to FAME with the use of appropriate catalysts, typically H2SO4 [9–11], or carrying out the (

> 1**trans) esterification** reaction **under sub**- or **supercritical conditions of**

methanol [13–15].

> 2**https://doi.org/10.1016/j.biombioe.2021.106300**

Received 8 March 2021; Received in revised form 13 October 2021; Accepted 7 November 2021 Available online 16 November 2021 List of abbreviation RB Rice Bran RBL Rice bran lipids FFA free fatty acids FAME Fatty acid methyl ester ISTE In-situ (trans)esterifictaion PHRB Post-hydrolysis rice bran SAD Strong acid density FA Fatty acids TFA Total fatty acids SSR solvent-to-solid ratio SLR

> 12**Solvent-to**-lipid **ratio S/N Signal-to-Noise ratio**

DPPH 2,2-Diphenyl-1-picrylhydrazyl Considering that RBL would require the use of solvent for its extraction and recovery, and that methanol serves as both a solvent and an alkyl donor for RBL during (trans)esterification various studies have since explored in-situ (trans)esterification (ISTE) to produce FAME from RBL. During ISTE, conversion of fatty acids and their extraction occur concurrently, which could in principle avoid the use of other solvents than the alkyl donor and eliminates the dedicated extraction step. The earliest attempt to carryout

> 1**ISTE of RBL was by Özgül-Yücel and Türkay**

[9,16,17], back in 1993–2003. From their reported results, it was sug- gestive that acid-catalyzed ISTE is best carried out for RB with high FFA content (~68 wt%) to achieve an overall FA conversion to esters of up

to 86%, or as a pretreatment step to simultaneously deacidify RBL and convert the FFA to FAME

2owing to the better miscibility or solubility of FFA in methanol

. For RB containing lipids with lower FFA (~24 wt%), an optimized acid-catalyzed process reported by Gunawan et al. [18] was only able to achieve a FAME yield of ~50%. Intensifying the acid-catalyzed ISTE,

1Yustianingsih et al. [19] adopted the use of indirect ultrasonication

which allowed higher reaction yields (75–83%) to be achieved even for RB with lipids containing relatively lesser FFA (13–48 wt%). In all reported acid-catalyzed ISTE under ambient pressure con- ditions and temperatures not over the boiling point of methanol in the mixture, the required H_2SO_4 typically ranges from 18 to 28 wt% relative to the weight of RB being processed, with reactions typically carried out in less than 4 h [9,18–20]. Even for (trans)esterification of extracted RBL with high FFA content (~50 wt%) would require the reaction to be carried out for 8–24 h or at least 8 h at elevated temperatures of 100 ◦C to achieve high conversion and products with FAME content of over 95% [11]. Since the generation of FFA in RBL before extraction is unavoid- able, one main challenge in the processing of RBL to FAME is rather the effective conversion of the acylglycerides. In addition, it would also be of interest to reduce the required mineral acid to minimize the waste subsequently generated. In a series of works by Sutanto et al. [14,15,21], a different perspective was presented in the maximized use of the macromolecules present in RB, specifically carbohydrates and lipids. Conventionally, lipids are first extracted from RB and the lipid-free residue is subse- quently hydrolyzed to

2recover the sugars and proteins in the form of hydrolysates to

serve as carbon and or nitrogen source in fermentation processes. In the work by Sutanto et al. [21] these two steps were interchanged, where dilute acid hydrolysis (DAH) using H_2SO_4 was carried out first to obtain sugar-rich hydrolysates (42–51 g glucose/L) and lipid-dense residues (>40 wt%). Generally, most of the available sugars were recovered and lipids remained intact and recoverable from the resulting post-hydrolysis RB (PHRB). The obtained hydrolysates were later successfully

1used in the cultivation of Yarrowia lipolytica [21] and Lipomyces starkeyi [14], for lipid accumulation to also serve as feedstock for biodiesel production

. In addition, the obtained lipid-dense PHRB were subjected to non-isothermal ISTE under subcritical conditions of methanol as the reaction mixture was heated from 30 ◦C to about 165 or 185 ◦C within ~0.5 h, where lipids were successfully converted and recovered in the form of FAME, without the addition of catalyst [14,15]. The successful conversion of the retained lipids to FAME was attributed to the strong acid or sulfonic sites (1–1.6 mmol H /g) present in the obtained PHRB. However, the catalytic activity was + not entirely ruled out since the reaction was carried out under subcritical conditions, which has been previously reported to enable (trans)ester- ifictaion to proceed even without a catalyst for lipids with high FFA [22].

1In a separate work by Go et al. [8], the

proposed direct DAH approach for non-delipidated RB was revisited, but this time around the focus was

3on the extraction of lipids using hexane, which the

authors found that lipids in PHRB were more easily extracted as compared to RB and requires 30 to 80% less solvent. As of writing, no reports have been made on the ISTE of lipids in PHRB

10under ambient pressure and temperatures below 100 C. ◦ Considering that lipids in

PHRB are more easily extracted, the amount of methanol required may be less or perhaps enable the better conversion of the acylglycerides to FAME. Further, if indeed PHRB has its inherent catalytic activity, the required acid during ISTE could be substantially reduced. In line with the above developments and hypothesis, this study is aimed to explore the conversion of lipids PHRB through ISTE

10under ambient pressure and temperatures below 100 C and to verify the

◦ inherent catalytic activity of PHRB. The specific

4objectives of this study are to (i) Investigate the effects of

temperature, SSR, reaction time, and catalyst loading on FAME conversion and yield during ISTE; and (ii) Evaluate the catalytic activity of PHRB, and its residues through the determination of acid sites, and the extent of converting the available lipids during (trans)esterification when no additional mineral acid is added into different reaction systems and through the use of the residues after ISTE

9in the esterification of oleic acid with methanol

.

3**2. Materials and methods Rice bran** (~10 **kg) from a mill in Kaohsiung, Taiwan**

, were collected and stored at − 20 C for further use to avoid changes in the free fatty ◦ acid content. The chemical reagents were acquired through local dis- tributors, which included the following:

4analytical grade ethyl acetate 99.9 vol% (Echo Chemical Co., Ltd, Taiwan), sulfuric acid 95 vol% (Scharlau, Spain), methanol 99

.99 vol% (Aencore, Australia), and so- dium

4chloride 99.9 wt% (Showa, Japan); technical grade hexane (Echo Chemical Co., Ltd, Taiwan) with at least 95 wt% n-hexane; potassium hydroxide 85 wt%

(Acros organics, USA); FAME 37-mix and boron tri- fluoride **methanol complex solution (13–15% BF3) (Sigma, Aldrich, Germany**

). A schematic diagram of the sequence of the experiments to investi- gate the possible use of lipid-dense PHRB as feedstock for FAME pro- duction, optimization of ISTE of lipids in PHRB with methanol near its boiling point and at ambient pressures, and the investigation of inherent catalytic activity of the PHRB's solid matrix is presented in Fig. 1. The details of the experiments are described in the subsections that follow. 2.1. Characterization of RB and PHRB The gathered RB was first determined of its moisture content by freeze-drying ~2 g of the collected samples for 24 h and the loss in weight taken to be the moisture present in the sample. Collected RB

2**was determined of its particle size**

2**by sieving 10 g of samples through**

stan- dard sieves (1,190, 1000, 840, 710, 590, 420, 250 μm) until no changes in the quantity of the particles retained in each sieve. The average particle size was then determined based on the weight fraction of the retained particles between two mesh sizes. Samples were also deter- mined of its lipid content and subsequently its lipid profile, while the lipid-free solids were characterized for their potential acidic functional groups and elemental composition, which are detailed in the following Fig. 1. Schematic flow diagram of producing PHRB, ISTE of lipids in PHRB and subsequent use of post-ISTE PHRB "This figure is to be printed in black and white". subsections. All analyses were done in triplicates. 2.1.1. Lipid content and profile Lipid content (CL) gravimetrically

10**with the aid of a Soxhlet extractor** with **hexane as**

solvent. About 10 g of sample were packed in a cellulosic thimble and subjected to exhaustive extraction for 8 h. The collected extracts were then separated from hexane

4**using a rotary evaporator until constant weight** and **the** obtained extract **was**

taken as the crude lipid contained in the samples. The collected lipid

2**samples were then dissolved in ethyl acetate**

(25 mg/mL) and subjected to gas chromato- graphic (GC) analysis following the conditions previously established [23]. The GC system used was

2**Shimadzu GC-2010 Plus equipped with a split injector, Rxi-5HT column (15 m 0.32 mm x 0.1 μm), and flame × ionization detector. To** aid in **the** identification and quantification **of** the free **fatty**

acids (FFA), monoglycerides, diglycerides, and triglycerides present in the samples, various lipid standards purchased from Sigma-Aldrich were used as references and in the preparation of cali- bration curves. To quantify the saponifiable and unsaponifiable fraction of collected lipids and the total fatty acid (CTFA) that is available for each gram of lipid the procedures

> 3**described in the work of Loyao et al**

. [24] were adopted. After extracting the unsaponifiable matter from the saponified mixture, the saponified fraction was converted back to its free fatty acid form by acidifying the mixture with H_2SO_4 to a pH of 2 and incubated at for 8 h prior to recovery of the total fatty acid via liquid-liquid extraction using hexane as solvent. The determined CTFA served as a reference for the maximum FAME that could be produced, which was multiplied

> 5**by a factor of 1.05 to account for the methyl group attached during**

esterification with methanol. The collected free fatty acids (25 mg) were further subjected to esterification using methanol containing ~14% BF_3 (2 mL) at 85 ◦C for 0.5 h, while placed in a Teflon sealed glass vial, and was subjected to GC analysis for the determination of the fatty acid profile. 2.1.2. Surface characterization and elemental analysis In anticipation of the possible attachment of the residual sulfuric acid onto the RB residues after drying samples of RB and the later PHRB were sent for elemental analysis at the Precision Instrumentation Center, National Taiwan University. Prior to elemental analysis, dried samples were delipidated, elemental (C, H, O, N, S) analysis was carried out & using an elemental analyzer (Elementar Vario EL cube, Germany) with sulfanilic acid and benzoic acid used as reference materials. To evaluate the possible acidic groups that are present in the samples' solid matrixes, delipidated RB and PHRB, were subjected to FTIR spectral scan using Shimadzu IRTracer-100. Samples were ground mixed with pre-dried KBr using mortar and pestle. The resulting powder mixture for each sample was pelletized using a mechanical press, before scanning the samples, a pure KBr pellet was used as a blank.

> 2**To have a more quantitative basis** of **the acid** groups attached, these **were** estimated **via titration**

following previously reported procedures [25], with some modification. Briefly, for total acid density (TAD), standardized 0.1 M NaOH solution (30 mL) was added to 0.5 g of lipid-free and moisture-free sample, in a 50-mL scintillation vial and was continuously stirred for 6 h to facilitate the neutralization of the acid sites. The liquid was separated by vacuum filtration, with the filtrate analyzed of the residual NaOH via

> 2**titration with a 0.1 M standardized HCl solution until equivalence point**

. The moles of NaOH consumed were taken as the number of moles of acid present on the solid. The strong acid density (SAD) was determined similarly but instead, 2 M NaCl solution was mixed with the sample to induce ion exchange with the strong acid sites. During the ion-exchange process, HCl is released, which was then quantified by titrating the liquid phase (free of solids)

> 2**with a 0.1 M standardized NaOH solution until equivalence point. The**

amount of HCl released during ion exchange corresponds to the strong acid sites on the solid samples. To estimate the week acid density (WAD), this was taken to be the difference between TAD and SAD.

> 2**All analyses were carried out in duplicates and the acid densities are** reported **as mmol H /g of**

lipid-free dried biomass. + 2.2. Preparation of PHRB via dilute acid hydrolysis The PHRB was prepared following the procedure described previ- ously in the work of Sutanto et al. [21]. About 67 g of RB (containing 16.3 wt% lipids on a dry basis) were placed in 500-mL Teflon-lined

> 8**screw-capped** bottles **and mixed with sulfuric acid**

solution (3 vol% of concentrated H2SO4)

> 3**at an SSR of 8 mL/g (on** a **dry and lipid-free**

basis). The mixture was then incubated at 95 C to commence the DAH and was ◦ intermittently shaken at 30-min intervals, for a duration of 6 h. The residual solid after DAH was separated from the hydrolysates by filtra- tion, with the collected solids dried in an oven (50 ± 5 ◦C) for ~5–7 days to achieve constant weight and reduce the moisture to less than 10 wt%. The obtained solids after drying tend to clump together, and were sub- ject to grinding using a food processor, which was then stored in poly- propylene bottles for further use and are then referred to as PHRB. 2.3. In-situ transesterification optimized via Taguchi method Post-hydrolysis rice bran was weighed into a 100-mL glass bottle with Teflon-lined screw caps while achieving a predetermined SSR of 10, 15, and 20 mL/g with a constant amount of methanol (80 mL). The ISTE was carried out at constant stirring (200 rpm) at predetermined reaction times of 4, 8, and 12 h, at various temperatures of 55, 65, and 75 ◦C, and acid (H2SO4) concentrations of 0, 5, and 10 wt%, relative to the solid. In-situ (trans)esterification

> 7**experiments were carried out** adopting **the Taguchi method** and using **the**

L9 orthogonal array with each run carried out duplicates, with the coded experimental runs and responses summarized in Table 1. For a given reaction condition the reaction mixture was

> 5**removed from the water bath after** the set reaction **time and immediately**

> 8**filtered to separate the solid** residues from **the**

crude extracts and methanol. The filter cake was washed with methanol (30 mL) to maximize the recovery of the products entrained. The filtrate was then transferred into a pre-weighed and pre-dried flask for methanol removal and recovery in a rotary evaporator. To recover the lipid frac- tion and the FAME produced from the reaction the resulting concentrate was suspended in hexane (~15 mL) subjected to ultrasonication in an ultrasonic bath to facilitate the extraction and recovery. The extraction and recovery with hexane as solvent was repeated 4 times to maximize recovery of the hexane soluble materials and were pooled into a sepa-

ratory funnel. The pooled hexane extracts were then washed 4 times Table 1 Summary of coded experiments and responses

12**for L9** (33) **orthogonal array experiments on in-situ (trans)esterification of**

post-hydrolysis rice bran (PHRB) obtained after dilute acid hydrolysis (DAH). Expt. Run Trial Variables Varied (Taguchi Levels) Yield, g FAME/100 g PHRB Average Yield ± SD S/N T, ◦C (A) SSR, mL·g− 1 (B) T, h (C) H2SO4, wt.% relative to the solid (D) 1 1 2 2 1 2 3 1 2 4 1 2 5 1 2 6 1 2 7 1 2 8 1 2 9 1 2 10* 1 2 3 55 10 (1) (1) 55 15 (1) (2) 55 20 (1) (3) 65 10 (2) (1) 65 15 (2) (2) 65 20 (2) (3) 75 10 (3) (1) 75 15 (3) (2) 75 20 (3) (3) 65 20 (2) (3) 4 0 (1) (1) 8 5 (2) (2) 12 10 (3) (3) 8 10 (2) (3) 12 0 (3) (1) 4 5 (1) (2) 12 5 (3) (2) 4 10 (1) (3) 8 0 (2) (1) 12 5 (3) (2) 0.0966 8.15 ± 2.14 0.0664 0.1085 14.73 ± 5.50 0.1862 0.2052 21.02 ± 0.71 0.2153 0.1581 14.52 ± 1.82 0.1323 0.1911 16.80 ± 3.27 0.1449 0.1985 18.89 ± 1.36 0.1793 0.1666 18.31 ± 2.34 0.1997 0.1215 15.24 ± 4.37 0.1833 0.1107 12.91 ± 2.60 0.1475 0.2271 22.38 ± 0.28 0.2220 0.2224 17.77 22.45 26.45 23.14 24.26 25.49 25.15 23.12 21.95 26.99 * Confirmatory test runs based on Taguchi method (Run 10). with salt solution (~15 mL) to remove hydrophilic components and residual acids. The crude product (mcrude) was then obtained by concentrating the hexane phase in a pre-weighed boiling flask until constant weight with the aid of a rotary evaporator. Gas chromatog- raphy was adopted as the method of quantifying the amount of FAME (CAE) in the crude product, following a procedure previously mentioned. Equations (1) and (2) were then used to calculate process yields (YS) relative to the mass of solid or PHRB (mPHRB) used in each reaction and apparent conversions or reaction yields (YP) based on the maximum attainable product or FAME (YS max), respectively. Further, the result- ing yields are expressed in terms of signal-to-noise (S/N) ratios using the “the-higher-the-better” function (3) of the Taguchi method and are used as responses for subsequent optimization and statistical analysis. YS = mcrumdePH×RBCAE × 100 (1) YP = YS × 100 = YS CL × CTFA × 1.05 × 100 YS max (2) ( ) S

7**N = − 10 × log 1 ∑n 1 n i=1**

yi2 (3) From the obtained S/N ratios for each experimental the overall average S/N ratio (S/Nave) and along with the average S/N ratio for a given variable at a specified level (S/Ni) to generate the response chart and for predicting the optimum S/N response (Equation (4)). In aid of a more objective assessment, analysis of variance (ANOVA) of the resulting responses was also carried out. The predicted optimum response was

5**confirmed by running a confirmatory test employing the optimum factor levels as analyzed**

using the Taguchi method and the confidence interval of the predicted response was calculated using equation (5). ( ) S = S + ∑q S S Npred Nave i=1 Ni Nave − (4) then characterized using FT-IR analysis, determined of its SAD, and used as the catalyst for the esterification of oleic acid with methanol. For the esterification reaction, post-ISTE PHRB (0.5 g) was added to a mixture of oleic acid (5 g) and methanol (14 mL) in a 50-mL scintillation vial, with the content continuously stirred at 200 rpm over a period of 24 h at 60 ◦C. An aliquot (100 µL) was taken at predetermined time intervals over the 24 h reaction period to monitor the FAME content in the product. The obtained aliquots were centrifuged to remove the solids and the supernatant was

2**placed in a vacuum oven to remove the** unreacted **methanol. The** bulk product **collected**

after the 24 h reaction was then separated from the catalyst, washed, and concentrated, as previously described before subjecting analysis. The collected products, free of methanol,

> 2**were then weighed and dissolved in ethyl acetate for GC analysis**

. As for the separated and recovered solids, these were washed with methanol and dried to test for possible reuse. 3. Results and discussions Table 2 is

> 5**a summary of the** characteristics **and** lipid **composition of the collected**

RB and generated PHRB. Similar to previous reports [8, 21], the lipid content of PHRB (~31 wt%)

> 4**is higher than the native** RB (~**16 wt%). The**

observed increase in lipid content could be attributed to the dissolution of the solid fraction [26]. In the current work, ~45 wt% of the original mass from the native RB with the originally available lipid remaining in the residual solids after hydrolysis. In addition, lipids were also hydrolyzed increasing the FFA content in the lipids obtained from PHRB (~21 wt%) as compared to RB (~15 wt%). If lipids from RB or PHRB are to be further processed, both could not adopt the use of a base as the catalyst. The higher FFA content need not always translate to a disadvantage since RBL with higher FFA content are easier to convert to FAME, especially with an acid catalyst [11]. The increase in FFA is not only owing to the hydrolysis of the existing acylglycerides but also the release of fatty acids (FA) initially bound to the solid matrix, which is supported by the increase in the TFA content by 8% when compared based on the native RB (15.06–16.31 g TFA/100 g RB). This increase also translates to higher FAME that could be potentially produced for a given amount of RB processed. With the current PHRB generated 27.19 g FAME can be produced from 100 g PHRB (~29.39 g/100 g, dry basis). √ (1) CI = Fα:1,v2 × ve × + 1

> 7**total number of experiments** neff r ; neff = **sum of** degress **of freedom used in estimating the response + 1**

(

> 5**5) where Fa:1,v2 is the F-ratio of the significant level a, a is the significant level, 1-a is the confidence level** (95%), **v2 is the degree of freedom of pooled error variance, ve is the pooled error variance, r is the number of repeated trials**, and neff is **the**

number of effective measured results. 2.4. Catalytic activity of PHRB and its residues after ISTE The catalytic activity of the PHRB obtained after drying the post- hydrolysis residue was tested by extracting the lipids with methanol in a Soxhlet extractor or by suspending the PHRB in a reactor with meth- anol over a predesignated time without the addition of a catalyst. Near the boiling point of methanol and under ambient pressure conditions, no FAME should be generated, if the PHRB does not have any catalytic activity, otherwise, the PHRB itself catalyzes the ISTE reaction. For the catalytic activity of residues after ISTE, some preparations and charac- terization were made before the actual test. After the ISTE of PHRB lipids under

the predicted conditions that would yield the highest FAME, the remaining solids after filtration were collected, dried, and delipidated (hexane as solvent). The resulting delipidated post-ISTE residues were Not reported in earlier works on the generation of PHRB is its sulfur content. Compared to native RB which did not contain any quantifiable amount of sulfur, the resulting PHRB contains as much as 4.8 wt% sul- fur. The presence of sulfur in the PHRB is indicative of the attachment of the sulfuric acid to the solid matrix of the residue and may provide certain catalytic activity. Further, extraction using methanol as solvent was also carried out with the aid of a Soxhlet extractor for 8 h. For RB, the yield in hexane soluble material (13.99 g/100 g moisture-free RB) is only ~85% of the available lipids. However, the yield in hexane soluble material (30.82 g/100 g moisture-free PHRB) for PHRB is similar to the available lipids, which supports the fact

4**that lipids are more easily extracted from** PHRB as compared **to**

RB. More interestingly, hexane soluble fraction from the methanol extraction of PHRB was found to contain significant quantities of FAME (~83 wt%), which was not observed from extracts obtained from RB. The following section further details some findings to better support the catalytic activity of the ob- tained PHRB and preliminary optimization of the ISTE of lipids in PHRB.

3**Table 2 Characteristics of collected** rice bran (RB) **and**

post-hydrolysis RB (PHRB) and lipid profile. Biomass RB PHRB* Particle size (μm) Composition (wt.%) Moisture Lipidsc Free fatty acide Monoglyceridee Diglyceridee Triglyceridee Unsaponifiable Mattere Total Fatty Acid Content Theoretical Maximum FAME Yieldf Sulfur Contentc 534.79 ± 14.86 10.33 ± 0.36a 16.33 ± 0.93 (16.33 ± 0.93)d 15.32 ± 0.20 3.60 ± 0.13 14.32 ± 0.88 57.33 ± 2.00 7.90 ± 0.59 84.70 ± 0.65e (14.34 ± 0.65)d ~88.94 (~15.06)c,d not detected 643.07 ± 6.89 7.46 ± 0.11b 31.35 ± 2.20 (17.39 ± 2.71)d 21.13 ± 2.56 2.84 ± 0.310 7.86 ± 2.95 45.26 ± 0.17 7.52 ± 1.35 89.29 ± 1.78e (15.53 ± 2.44)d ~93.75 (~29.39c/ ~16.31d) ~4.8742 (~2.7042)d *

3**Obtained after subjecting RB to dilute acid hydrolysis with 3 %v/v** acid (95% H_2SO_4) **at an SSR of 8 mL/g (based on dry-lipid-free biomass) for 6 h at** 95 ◦**C with intermittent shaking (30 min interval). a Moisture as received** or obtained (**expressed in wet basis). b Moisture** content determined **after oven drying (expressed in wet basis). c Expressed in dry basis. d Expressed relative to the native dry biomass and in dry basis (dry matter yield after hydrolysis**

= 55.48 ± 7.73 wt%).

3**e Expressed relative to the extracted lipids. f**

Theoretical maximum FAME

5**estimated by multiplying a factor of** 1.05 **to the**

determined

3**total fatty acid content to** account for **the**

incorporated methyl group. 3.1. Catalytic activity and yield of FAME from PHRB during ISTE The conversion of RBL would typically require the addition of an acid catalyst during ISTE. In a related work by Zullaikah et al. [27] where a Soxhlet extractor was used to facilitate ISTE, H_2SO_4 at ~9.3 wt% rela- tive to the RB being processed had to be added to the receiving/boiling flask together with the methanol to enable the reaction to occur while achieving a yield of ~17 g FAME/100 g RB, with the crude product containing only ~75 wt% FAME after 5 h. With the use of PHRB, a FAME yield of 24.00 0.26 g FAME/100 g PHRB (~14 g FAME/100 g native ± RB) with a purity of ~83% FAME after 8 h of extraction and reaction time, without the need of adding a catalyst. These results further support the catalytic activity that PHRB possesses. For ISTE of lipids in PHRB conducted using a Soxhlet extractor, FAME yields and purity did not significantly change beyond 8 h of processing time (Fig. 2a). One means to enhance the yield without the addition of a catalyst is to increase the SSR by adding more methanol into the system. However, for a Soxhlet extractor, the true SSR is limited by the extractor volume, even if more solvent is introduced in the boiling flask. Thus, for ISTE conducted in a Soxhlet extractor, the actual SSR could not be effectively and practically varied. In addition, with Soxhlet extractor being a laboratory apparatus used to analytically determine extractable components from a given sample, its practical scale-up for industrialization may also impose another challenge. To see whether an increase in SSR would result in better yields and for later practical application in the industry, a batch reactor was used in subsequent re- actions for comparison, with results of the ISTE presented in Fig. 2b.

13**As could be observed from Fig**. 2b, the **increase in** SSR **from** 10 **to**

15 mL/g improved the yield, but further increase resulted in poorer yields and subsequently, the conversion achieved. Increasing the SSR would mean more methanol is added to facilitate both the reaction and the extraction. However, considering that PHRB is at the same time the catalyst, increasing the methanol added into the system, inevitably di- lutes the system, and thus, resulting in poorer yield and conversion beyond a certain point. In addition, compared to the system where a Soxhlet extractor was used, lower conversion and yields were achieved in a batch reactor. These results are observed because the

3**extraction process involved in a Soxhlet extractor is**

not limited by equilibrium, since fresh

6**solvent comes in contact with the solids** in **the**

extraction chamber, while typical batch-type extraction is limited by equilibrium and solubility. Further, although the

3**extraction process in a Soxhlet extractor** occurs at **a**

temperature (~68

1**C) near the boiling point of the**

◦ solvent, the temperature of the boiling flask varies from 90 to 150 ◦C depending on the contents of the flasks during the duration of the extraction process. The elevated temperature may have also influenced the reaction and thus, resulting in higher overall FAME conversion and yield. Interestingly, for ISTE in a batch reactor, higher product purity (~91%) could be achieved. In addition, unlike the ISTE carried out in the Soxhlet extractor which achieved a high yield (28.67 g/100 g PHRB) in hexane soluble material, the yield in hexane soluble material achieved using a batch reactor was only 22.82 g/100 g PHRB at an SSR of 15 mL/g after 8 h, and significantly decreased to 16.59 g/100 g PHRB when SSR was increased to 20 mL/g. These results indicate that the ISTE of RBL in PHRB probably occurs in a manner where lipids that have limited sol- ubility in methanol are primarily reacted first within the solid matrix before diffusing out to the bulk liquid in the form of FAME and partial glycerides. Although the batch reactor system is not as good in facili- tating the extraction of the lipids as compared to a Soxhlet extractor, the equilibrium limited extraction process facilitates the release of FAME Fig. 2. The catalytic activity of PHRB during ISTE without the addition of catalyst for the reactions carried out in a Soxhlet extractor with an SSR of ~17 mL/g and under open reflux for 8 h (a) and in a batch reactor with different SSR of 10, 15, and 20 mL/g at 75 ◦C, for 8 h with constant stirring at 200 rpm (b). “This figure is

> 6**to be printed in black and white”. Fig**

. 3.

> 5**Response graph of the-higher-the-better S/N ratios of** FAME yield (S/**Nave** = 23.31). **A**

: temperature (55, 65, 75 ◦C); B: solvent-to- solid ratio (SSR: 10, 15, 20 mL/g); C: time (4, 8, 12 h); D: H2SO4 (0, 5 10 wt% relative to the solids). “This figure is to be printed in black and white”. formed within the solid matrix, minimizing unreacted products found in the final product during ISTE of RBL from PHRB without the addition of a catalyst. 3.2. Optimization of FAME yield during ISTE of lipids in PHRB with a batch reactor

> 3**To better understand the influence of** main factors (**temperature, SSR**

, time, and catalyst loading) on the FAME yield during ISTE of RBL from PHRB in a batch reactor, Taguchi’s L9 orthogonal array was adopted. Presented in Fig. 3 is the response graph based on the average of the S/N ratios summarized in Table 1.

> 4**As could be observed, increasing the temperature** (A) **from** 55 **to** 65 **C**

results in an increase in ◦ the S/NA and correspondingly, the yield. However, the yield tend to decrease

> 15**as the temperature** was further **increased to 75 C. At** tem- ◦ peratures over **the**

boiling point of methanol (~65 ◦C), the

> 15**decrease in the FAME yield** is often attributed **to**

the vaporization of methanol [28, 29]. However, it should be noted that the system is not only of pure methanol but a mixture of various materials and that the system is a closed system. Thus, the actual boiling point of the mixture is expected to be higher than 65 C, and if there is an increase in the quantity of ◦ methanol in the vapor phase, this should be minimal as there was no apparent change in the volume of the reaction mixture and boiling was not also observed. Another possibility would be the degradation of fatty acid chains into hydrocarbons as observed by Kasim et al. [13] during ISTE of lipids in RB under supercritical methanol conditions (T = 300 ◦C, P = 30 MPa) for just 5 min. However, no significant hydrocarbon peaks are observed from the chromatographic analysis made with the products obtained from the ISTE of lipids in PHRB. A plausible explanation for the observed decrease would be the competing use of methanol during ISTE of lipids in PHRB with other reacting components and the extraction & solubilization of other constituents of PHRB at an elevated temperature of 75 ◦C. Nevertheless, the best operating temperature from the response graph to maximize the conversion of available lipids to FAME would be at 65 ◦C. For SSR (B), an

> 6**increase in SSR from** 10 **to** 20 **mL/g** increased **the** S/ NB indicating **the**

positive main effect of SSR. This observation is similar to the observations made by Shiu et al. [20] during the acid-catalyzed ISTE of lipids in RB, where FAME conversions were improved as the SSR was increased from 2.5 to 20 mL/g. Considering that the amount of methanol added into the system during ISTE even at the lowest SSR is already more than enough for the reacting fatty acid chains and to ensure that the forward reaction is favored, the addition of more methanol into the system aids the reaction through some other mecha- nism. During ISTE, apart from (trans)esterification reaction, it also in- volves the diffusion of methanol and the reacted & unreacted fatty acids into and out of the solid matrix, as well as solubilization of the different components part of the solid phase. In addition, higher SSR also results in better suspension and/or dispersion of the PHRB. Thus, the addition of more methanol or an increase in SSR improves the overall mass transfer of the system. In a related work where

> 3**lipids from** PHRB **were extracted with hexane**

as the solvent, the

> 6**increase in SSR from 4 to 12 mL/g** resulted **in** better **diffusivity**

of the lipids [8]. In view of reaction time (C), the prolonged

> 14**reaction time** of **up to** 12 **h** positively influences **the yield**

as indicated by the increasing S/NC. The average S/NC at 12 h translates to a yield of 20.7 g FAME/100 g PHRB or a reaction yield (~conversion) of ~76% even with low catalyst loading (0–10 wt%). This is comparable to the reported reaction yields (~75%) achieved by Shiu et al. [20] and Yustianingsih et al. [19] after 4 h of ISTE

> 1**with a catalyst loading of** 27.6 **wt**%. Compared **to**

base-catalyzed transesterification reactions, acid-catalyzed systems are generally slower.

1**As reported by Zullaikah et al**

. [11], extracted, dewaxed, and degummed RBL requires long reaction times of over 12 h to achieve products containing a high methyl ester content of over 50 wt% specially for lipids with low FFA content (<25 wt%), and would require at least 12 h for lipids with high FFA (~75 wt%) to achieve ~95 wt% FAME content in the product. Thus, prolonging the reaction time up to 12 h is necessary to ensure that the reaction reaches equilibrium, thereby maximizing the conversion. Moreover, the use of PHRB allows RB lipids with relatively lower FFA content (~21 wt%) to still be effectively converted to FAME within a reasonable time scale. In relation to the reaction rate, the addition of H2SO4 (D) generally aids the ISTE and shifts the equilibrium towards the formation of the products, but the addition of more acid, beyond 5 wt%, did not significantly change the average S/ND. Compared to earlier works on acid-catalyzed ISTE of lipids in RB, the use of PHRB as a starting material requires a much lesser catalyst to be added, which is attributed to the inherent catalytic property of the PHRB matrix.

7**In the Taguchi design of experiments, the main effect is determined**

via a Pareto analysis by taking the difference (Δi) between the maximum and minimum S/Ni of a given variable. Within the levels of the variables investigated in the ISTE of lipid in PHRB, the order as to the factors giving the biggest influence on the achieved yields is as follows, Time (C), catalyst loading (D), SSR (B), and temperature (a) (Table 3). Among the 4 factors, the temperature range explored exhibited the least influ- ence with a contribution of only ~12% based on the sum of square Summary of Pareto analysis and analysis of variance (ANOVA) for the FAME yields expressed in terms of signal-to-noise ratios obtained from employing Taguchi L9 (33) orthogonal array design of experiment. Parameters Variables and Levels Average Signal-to-Noise Ratio (S/Ni) Error Total Temperature (A) Solvent-to-solid Ratio (B) Time (C) H2SO4 (D) 1 2 3 Average Δi Rank DOFa SSb MSc F d p-valuee Contribution (%) DOFa SSb MSc Fd p-valuee Contribution (%) 22.2210 22.0187 24.2959 23.2754 23.4069 24.6297 23.3079 23.3079 2.0749 2.6110 4 3 2 2 6.5022 10.2310 – – – – – – 12.4430 19.5788 0 2 0.0000 10.2310 0.0000 3.7289 0.1434 0.8746 7.1358 22.1268 21.3252 22.5123 24.3636 25.2847 24.2350 23.3079 23.3079 3.1579 3.0384 1 2 2 2 17.8075 17.7150 – – – – – – 34.0776 33.9007 2 2 17.8075 17.7150 11.3053 11.2129 0.4347 0.4311 0.6970 0.6988 21.6346 21.4577 – – – – – – 0 0.0000 – – – – 2 6.502 26.0086 – – 49.7719 – – – – – – 8 52.2557 – – – 100.0000 8 52.2557 – – – 100 a

5**DOF: degrees of freedom**. b **SS: sum of squares**. c **MS: sum of square error**. d **F**: F-**ratio**. e **p value (Fcrit**

= 39; two-tailed,19; one-tailed at p = 0.05). errors. Considering that its contribution is the least if the influence of temperature will be taken as the error or the unaccounted interaction effects there seems a significant contribution (~49%) owing to the interaction of the factors investigated apart from its main effects. To have a more objective assessment,

11**analysis of variance (ANOVA) was** conducted and **the**

11**results are summarized in Table 3**. Despite **the** changes in **the** levels **of**

all three of the main variables being insignificant ($p > 0.05$), the contribution of time (C), catalyst loading (D), and SSR (B) were 21.63%, 21.46%, and 7.14%, respectively. Although the adopted experimental design does not enable the determination of interaction effects, its main advantage is its optimi- zation process solely based on the main effects. Based on the response graph (Fig. 3), an improved or optimized response could be achieved from the combination of reaction parameters at levels with the highest S/N for a given variable investigated, which in this case are A2, B3, C3, and D2. Using equations (4) and (5), the predicted optimum S/N at 95% confidence level is 28.65 7.85 dB. The predicted value for S/N is ± within the limits of the theoretical maximum of 28.68 dB. Triplicate runs employing the optimum conditions from the above analysis resulted in an average S/N of 27 dB, which coincides well with the predicted value. Under such optimum conditions, the actual FAME yield achieved during ISTE of PHRB is 22.38 ± 0.28 g/100 PHRB (24.19 ± 0.46 g/100 g moisture-free PHRB), which translates to a conversion of 82.31%. Although the resulting yield and conversion did not reach the theoretical maximum, it is still the highest response obtained from the combination of the variables (Table 1). Thus, with Taguchi L9 orthogonal design, the variables contributing to the FAME yield during the ISTE of PHRB could be easily determined along with the combination of variable levels required to achieve the highest FAME yield. After ISTE under the optimum conditions, ~24.5 wt% of the original mass of PHRB was recoverable during separation, hereinafter referred to as the post-ISTE residue. The residue contained ~20 wt% hexane soluble material after subjecting it to Soxhlet extraction. Accounting for the FAME (~44 wt%) in the hexane soluble material, the overall FAME yield is 23.60 0.47 g/100 PHRB (25.50 0.62 g/100 g moisture-free ± ± PHRB), which is equivalent to an overall conversion of 86.8% of the available fatty acid chains. The overall conversion is comparable to the results obtained via the use of the Soxhlet extractor as the reaction vessel (Fig. 2a). Although the apparent yield and conversion based on the extracted material by methanol after ISTE in a batch reactor, it has higher FAME purity (~84%) as compared to the results from ISTE conducted in a Soxhlet extractor (~82%, Fig. 2a). The non-FAME component in the product after ISTE of PHRB in a batch reactor at op- timum conditions are mainly partial glycerides (~13–15 wt %) and phytosterols and their esters (~3–4 wt% as oryzanol equivalent), while the hexane soluble extract from the post-ISTE residue mainly contained 10.31 wt% MG, 15.45 wt% DG, and 14.95 wt% phytosterols apart from FAME. Again, these results further indicate that the ISTE process con- ducted in the reactor is favorable as it minimizes the extraction of unreacted components, facilitating the formation of the FAME in the solid matrix and is thereafter diffused out to the bulk liquid. 3.3. Surface characterization and catalytic activity of Functionalized-RB The elemental composition of RB and PHRB are similar in magnitude in terms of C, H, O, and N as summarized in Table 4. It is noteworthy that slightly lower carbon and nitrogen are contained in the PHRB owing to the hydrolysis of the carbohydrates and proteins [14] is accompanied by the detection of significant amounts of sulfur, which is not previously detected in the native RB. The acid densities, total, strong, and weak are much greater in PHRB than RB (Table 4). The attachment of sulfur-containing groups on the residue could be in the form of sulfonic acid sites, which have resulted in the increase in the strong acid sites of the residue from 0.10 ± 0.02 to 1.24 ± 0.01 mmol H /g dried lipid-free + residue after acid hydrolysis. This value is comparable to that reported by Sutanto et al. [15] which is at 1.63 ± 0.05 mmol SO3H/g dried PHRB, where they also explored the catalytic activity of PHRB at subcritical conditions. In this study, the apparent catalytic activity of PHRB was observed earliest in the Soxhlet extraction with methanol as solvent, and later in a stirred batch reactor, as previously discussed. The darkening of color to the point of charring of the post-hydrolysis residue after drying to con- stant weight is an indicator of the subsequent co-synthesis of solid acid catalyst of the lipid-densified RB. To confirm the sulfur-containing group attachment to the residue, notable peaks (1055, and 1219 cm− 1) in the FT-IR spectra in Fig. 4a, corresponding to the sulfonic group found in PHRB, not previously found in native RB. This is also in agreement with the peaks found by Sutanto et al. [15] at 1134 and 1088 cm− 1 which Elemental composition and acid densities of lipid-free rice bran (RB) and post-hydrolysis RB (PHRB). Material Ê %Ha %Oa %Na %Sa Sulfur Content b (mmol S/g solid) SAD c, (mmol H+/g solid) TAD d, (mmol H+/g solid) WAD e, (mmol –OH/ g solid) RB 40.88 ± 0.08 5.95 ± 0.02 44.46 ± 0.22 2.87 ± 0.15 Not Detected Not Detected 0.10 ± 0.02f (0.0831) g 1.78 ± 0.10f (1.4786) g 1.68 ± 0.10f (1.3956) g PHRB 43.62 ± 0.13 5.22 ± 0.07 41.66 ± 1.57 3.49 ± 0.00 7.10 ± 0.07 2.22 ± 0.02 (1.5240) 1.24 ± 0.01 (0.8513) 5.06 ± 0.10 (3.4737) 4.02 ± 0.10 (2.7597) a Results obtained from elemental analysis of pre-dried, lipid-free samples. b

Calculated from sulfur weight percentage from elemental analysis. c Strong Acid Density (SAD) determined from a 6 h reaction time of 0.5 g of pre-dried lipid-free samples with 2 M NaCl solution. d Total Acid Density (TAD) determined from a 6 h reaction time of 0.5 g of pre-dried lipid-free samples with 0.1 M NaOH solution. e Weak Acid Density (WAD) calculated as the difference between TAD and SAD. f Values expressed in terms lipid-free solids and in dry or moisture-free basis. g Values expressed in termed of solid samples containing lipids and in dry or moisture-free basis. Fig. 4. Fourier transform infrared spectra (a) and strong acid cites (b) of delipidated RB, PHRB, and residual PHRB. “This figure is to be printed in black and white”. they attributed to sulfonic acid stretching. Nazir et al. [30] also attrib- uted peaks at 1213 cm when sulfonating carbonized lignin from − 1 sugarcane bagasse, while Wang et al. [31] also observed sulfonic acid peaks at 1377 and 1040 cm− 1 for sulfated chars. In addition, peaks corresponding to the vibrations of –OH (1606 cm− 1) and C–O (1715 cm− 1) in sulfated chars [31] are also present in RB, PHRB, and Post-ISTE PHRB. Moreover, characteristic peaks corresponding to

> 16**C–H aliphatic axial deformation in CH2 and CH3 groups** from **cellulose, hemicellulose, and lignin** [32] **at**

2922, 2924, 2920 cm are observed in all three − 1 materials for RB, PHRB, and post- ISTE PHRB, respectively. After ISTE (trans)esterification, the PHRB residue is still observed to have a characteristic peak corresponding to the sulfonic acid group at 1058 cm− 1. In view of the strong acid density as seen in Fig. 4b, despite the decrease by ~91% after the (trans)esterification reaction, post- ISTE PHRB still has 0.11 mmol H+/g dried lipid-free residue. The post-ISTE residue suspected to still have sulfonic sites owing to the residual strong acid sites was further utilized

> 2**in the esterification of oleic acid and methanol**, which achieved **a** high **conversion**

with a product purity of ~90% after 24 h of reaction time at 60 C (Fig. 5a). Furthermore, the ◦ stability of the catalytic activity of the post-ISTE PHRB was investigated in a reusability study of up to 5 cycles of

> 9**esterification. It can be observed** from Fig. 5b, **that the decrease in** FAME purity in **the**

product over time decreases as well with the increase in the number of cycles. The per- centage decrease in the conversion of oleic acid into methyl oleate at the fifth cycle, when compared to the first cycle at 24 h reaction time, is ~62%. The

> 13**observed decrease in conversion** is comparable to **the** ~64% **decrease in** the **acid**

density of the post-ISTE PHRB from 0.11 to 0.04 mmol H /g dried lipid-free residue after the fifth esterification cycle. + This is consistent with observation previously reported on the use of carbon-based solid acid catalyst derived from biomass via direct sulfo- nation with sulfuric acid [33,34], where the decrease in activity is attributed to the leaching of loosely bound components with sulfonic acid moieties, as evidenced by the decrease in the acid sites. However, these are also observed to eventually achieve a stable activity over the continued use of the catalyst. Dilute acid hydrolysis as pre-treatment for densifying the lipids in the

native material also imparted an important function or feature to the remaining solid matrix during the post-processing of the residue after Fig. 5.

2**Esterification of oleic acid** with **methanol** (1st Cycle) **at a solvent-to-oil ratio of 20**

mol/mol for 24 h at 60 ◦C with constant stirring at 200 rpm (a) and reusability of PHRB residues after ISTE as catalyst (b). “This figure is to be printed in black and white”. hydrolysis. Due to its acquired sulfonic sites during the drying step after DAH, utilizing post-hydrolysis residue as the lipid-containing feedstock for in-situ biodiesel production minimizes the needed external acid catalyst addition as in this study. Further, post-ISTE residue may be optimistically adopted in esterification reactions with the right catalyst loading to process oils high in free fatty acids. 3.4. Process comparison and evaluation In this study, post-hydrolyzed RB was utilized in in-situ FAME pro- duction as lipid-containing biomass whereby its acquired acid catalytic sites contributed to the reduction of sulfuric acid addition. From the Taguchi method discussed in earlier sections, the optimum conditions in a stirred batch reactor at ambient pressure which gave the best con- version of 82% with a purity of 85% FAME in the product was at 65

14**C, ◦ with a reaction time of** 12 **h**. Summarized **in** Table 5 are **the**

reported studies adopting RB and PHRB as feedstock for FAME production. To achieve comparable conversion or higher than those observed in this study at a short reaction time (1–4 h) and under ambient pressures was only possible for RB with lipids having significantly high FFA content (48–68%) while requiring high amounts of H_2SO_4 at 18.4 and 27.6% of the solid [9,19,35]. In view of SSR, RB with lipids having higher FFA (48–68%) required lower SSR (≤10 mL/g) [9,19] to achieve their best conversion performances as compared to those with low FFA (<40%), which required an SSR of over 10 mL/g to achieve high conversion [20], but higher SSR does not ensure complete conversion [35]. For RB with lipids having high acylglycerides would require the use of ultrasound irradiation to achieve an appreciable conversion of ~75% while keeping the SSR at 10 mL/g [19]. At first glance it may seem that the required SSR of 20 mL/g by PHRB is a disadvantage, however, it should be noted that the lipid content of PHRB is at least 1.9 times as much. When the solvent-to-lipid ratio (SLR) is taken into consideration, the required SLR is comparably less (<65 mL/g), especially considering that PHRB con- tains less FFA. Further, despite the high amounts of acylglycerides in PHRB the equivalent catalyst loading is less than 10 wt% of the solid and does not require the use of ultrasonication to achieve a conversion of over 75%. Among the studies summarized for in-situ biodiesel production, the best conversion (~95 and 97%) was achieved under subcritical condi- tions of methanol. In these studies, the least SSR of 4 mL/g was employed, with no acid added relying on the acquired catalytic activity by the PHRB [14,15]. These experiments were also performed at shorter non-isothermal durations (~0.5 h), but at high temperatures and pressures of 185 and 165 C at 2.5 and 1.6 MPa, respectively. In this ◦ study, the best conversion was achieved at 12 h where the long reaction time was required to compensate for the lower operating temperature at ambient pressure, and low FFA content of the PHRB lipids. With the H_2SO_4 added, the equivalent total catalyst loading of 8.4 wt%, relative to the solid, is only slightly higher than the PHRB used in the study operated at subcritical conditions. Without the additional H_2SO_4, the inherent strong acid sites when expressed as H_2SO_4 are only 3.8 wt% of the available solid. Without the addition of H_2SO_4, the highest product purity of 91%

1**FAME in the product could be achieved**

at 75 C after 8 h. ◦ The lower SSR of 15 mL/g, when compared to the run under optimum condition (SSR of 20 mL/g), could have given a better purity due to minimized extraction of unreacted lipids. Despite the

advantages of the subcritical conditions in obtaining the best performance, one disadvantage is the exposure of the more valuable components of interest to high temperature and pressure. In the lipids from RB, oryzanol and phytosterols are bioactive compounds available for recovery or could serve as antioxidant agents in the fuel. The crude product from the best ambient condition from the Taguchi method, PHRB, and RB lipids were tested for their DPPH (2,2-Diphenyl-1-pic- rylhydrazyl) radical scavenging activity with the results presented in Fig. 6. It can be observed that at a concentration of 50 µL/mL, the in- hibition by all 3 lipid samples (Crude FAME, PHRB lipids, and RB lipids are higher than 90% and are not significantly different. In view of their IC50, RB lipids have the highest activity requiring only 0.54 0.02 µL/ ± mL, as compared to PHRB lipids (1.37 0.14 µL/mL) and the crude ± FAME (3.17 ± 0.13 µL/mL). For comparison, their respective inhibitory activities are higher than β-sitosterols but lower than γ-oryzanol (IC50 = 73.81 4.67 µg/mL) and are comparable to previously reported free- ± radical scavenging activity of methanolic extract from rice bran with 93.91% inhibition at a concentration of 50 mg/mL [36]. The high radical scavenging activity may be attributed to the presence of γ-ory- zanol, and trace amounts of tocopherols present in the product, where pure tocopherols were observed to exhibit a radical scavenging activity of at least 90% inhibition even at a concentration of 0.05 µL/mL. It must be noted that FAME (methyl oleate) was also tested and did not exhibit any free-radical scavenging activity even at high concentrations of 150 µL/mL. Despite the observed reduction in the

11**free-radical scavenging** activity **of the** crude product **and**

the PHRB lipids as compared to the native RB lipids, the observed activity is still significantly high and could well be taken advantage of as part of the additives in the fuel to avoid oxidation of unsaturated FAME. Comparison of results from acid-catalyzed

1**in-situ (trans)esterification of rice bran lipids**

#. Material [Ref.] PHRB [This Study] PHRB [This Study] RB [9] RB [19] RB [20] PHRB [14,15] Feedstock Quality b

1**Moisture Content, M (wt**.%): 7.46 **Lipid Content, L (wt**.%): 31.33 **Free Fatty Acid, F (wt**.%): 21.13 **Particle Size, P (mm**): 0.643 **Alkyl Donor and Co-solvent**

(mL/g) c Methanol: 20 Solvent Loading c SFRn (n/n): 611 SLR (mL/g): 63.8 SSR (mL/g): 20 Catalyst Loading Acid Type Strong acid Sites and H2SO4 Concentration in Solvent (vol%) 0.28 (H2SO4 equivalent) Concentration relative to the 8.4 (H2SO4 solid (wt.%) equivalent) Mixing/Irradiation/Heating Mixer Type Magnetic Stirrer Mixing Rate 200 rpm Heater Type Hotplate/Water Bath Irradiation Rate n.a.f Reaction Temperature, Pressure, and time Temperature (◦C): 65 Pressure (MPa): Ambient Time (h): 12 Space Loading/Reactor Loading d SL (mL/g): ~30 RL (vol%): ~65 Yields and Purity (%) e Process Yield Relative to Solids, 22 YS: Process Yield Relative to Lipids, 77 YL: Reaction Yield, YP: 82 FAME Purity, CF: 85 7.46 31.33 21.13 0.643 15 458 48 15 Strong acid Sites: 0.78 mmol/g solidi 0.14 (H2SO4 equivalent) 3.8 (H2SO4 equivalent) Magnetic Stirrer 200 rpm Hotplate/Water Bath n.a. 75 Ambient 8 ~22 ~66 21 72 76 91 12 n.s. 19 n.s. 19 or 68 13 or 48 n.s.f n.s 4 10 -g – ~21* – 4 10 H2SO4 H2SO4 2.5 1.5 18.4 27.6 Magnetic Ultrasound Stirrer Bath n.s.f – n.s. Water Bath n.a. 35 kHz, 500 W ~64* 60 Ambient Ambient 1 4 – 25* – ~45* -g – – – 24/86 75/83 – 71/82 4 17 3 n.s. 15 – ~88* 15 H2SO4 1.0 27.6 Magnetic Stirrer n.s. n.s. n.a. 60 Ambient 4 25* ~65* – – 75 90 4/9 41 or 48 33/- n.s. 5 91/80 12/10 5 Strong acid Sites: 1.08/1.63 mmol/g solidi 0.6/0.9 (H2SO4 equivalent) 5/7 (H2SO4 equivalent) No stirring n.a.f Subcritical Reactor 4/5 ◦C/min 185/165 2.5/1.6 0/0 (~0.5)h 9.7 61 36/43 88/89 95/97 -/- aTemperature (

1**T), pressure (P), and time (t**

). b

1**Rice bran quality in terms of moisture content (M, wt**

.% in as determined basis),

1**lipid content (L, wt**.% in dry basis), **free fatty acid content (F, wt**

.% relative to the lipids), and

1**particle size (P, mm**). c **Alkyl donor and co-solvent relative to the mass of dry solid** (mL/**g), solvent loading as expressed in terms of solvent-to**-total fatty acid **molar ratio** (SFRn), **solvent-to- lipid volume to mass ratio (SLR**, mL/**g), and solvent-to-solid volume to dry biomass ratio (SSR**, mL/**g**

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1**d Space loading (expressed as the reactor volume to the amount of biomass loaded**, mL/**g), and reactor loading (percentage of the reactor volume occupied by the reaction mixture). e Yields expressed as the amount of FAAE with respect to** 100 g of **the solid** (YS), **with respect to** 100 g of **the** available **lipids** (YL), **and** percentage **with respect to the theoretical maximum amount of alkyl ester** (YP), **with purity** (CF) **as the** fatty acid **ester content**

in the product (wt.%). f Not applicable (n.a.) or

1**not specified (n.s.). g Incomplete information to allow estimation**

. h Time required to reach desired temperature before reaction was stopped. i Acid sites relative to solid (including lipid and moisture). # Nomenclature and method for the estimations for the responses or entries for acid catalyzed ISTE of RB lipids or PHRB are adopted from a previously published review [35] by the same group of authors, with the original source of the relevant information cited in the table. *

1**Entries to the table are calculated based on available information to facilitate comparison**

. 4. Conclusions Dilute acid hydrolysis is a beneficial pretreatment for RB which generates PHRB, a lipid-densified residue with an acquired catalytic activity for (trans)esterification reaction and is a viable feedstock for in- situ biodiesel production. During DAH, part of the solid matrix was dissolved in the filtrate increasing

the lipid content from 16.33 to 31.35%. Upon drying of the post-hydrolysis residue, the remaining lipid-containing solid matrix underwent carbonization and sulfonation, resulting in residues functionalized with sulfonic acid groups. The available strong acid sites increased 12.4 times (0.10 0.02 to 1.24 ± ± 0.01 mmol H /g dried lipid-free residue). This functionalization enables + lesser amounts of required sulfuric acid for the conversion of lipids to FAME. Taguchi method was successfully employed to determine the optimum ambient conditions for the ISTE of PHRB lipids in a stirred batch reactor. From the ANOVA of the responses, it was found that re- action time and catalyst loading had the highest contribution of 21.6% and 21.4%, respectively, in improving the FAME yield. The highest FAME yield, 22.38 0.28 g/100 PHRB, corresponding to an 82.31% ± conversion, could be achieved at 65 C, SSR of 20 mL methanol/g dried ◦ PHRB, 12 h reaction time, and 5 wt% H2SO4 to the solid in the system. The post-ISTE residue still possesses residual catalytic activity which and

2**was successfully** utilized **in the esterification of oleic acid and methanol**

up to 5 cycles with good stability. Lastly, post- ISTE FAME- containing crude yield still contained bioactive components which have an IC50 of 3.17 0.13 µL/mL in view of DPPH radical scavenging ± activity.

4**Dilute acid hydrolysis** not only **allows the generation of**

sugar- rich hydrolysates, but also lipid-dense residues for biodiesel production, and a functionalized solid matrix to serve as acid catalyst. Moreover, the resulting FAME-rich crude product contains bioactive compounds which Fig. 6. DPPH radical scavenging activity of RB and PHRB lipids, products, and possible components (gamma oryzanol concentration in µg/mL). “This figure is

1**to be printed in black and white**

”. may be recovered as high-value by-products or keeping them in the FAME mixture to improve potentially oxidative stability. Authors

1**contribution Alchris Woo Go – Writing Original Draft, Writing-Review & Editing**

, Supervision, Project Administration, Formal Analysis, Funding Acqui- sition, Conceptualization, Methodology, Visualization. Kristelle L. Qui- jote – Writing Original Draft, Writing-Review & Editing, Formal Analysis, Investigation, Data Curation. Chintya Gunarto – Writing- Review & Editing. Investigation, Formal Analysis, Visualization. Yi- Hsu Ju – Writing-Review & Editing, Conceptualization, Resources, Su- pervision. Artik Elisa Angkawijaya – Writing-Review & Editing, Re- sources, Supervision. Shella Permatasari Santoso – Writing-Review & Editing. Ramelito C. Agapay –

4**Writing-Review & Editing**. Declarations **of competing interest None**. Acknowledgments **Authors would like to thank the Ministry of Science and Technology, Taiwan, for the** financial **support provided through the grant MOST 108- 2218-E-011-032-MY3**

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basic facilities. References [1] Food and Agriculture Organization of the United Nations - Statistics Division, Crop Production and Trade Data, FAOSTAT, 2019. http://www.fao. org/faostat/en/#data. (Accessed 7 July 2021). [2] A.W. Go, A.T. Conag, R.M.B. Igdon, A.S. Toledo, J.S. Malila, Potentials of agricultural and agro-industrial crop residues for the displacement of fossil fuels: a Philippine context, Energy Strateg. Rev. 23 (2019) 100–113, https://doi.org/ 10.1016/j.esr.2018.12.010. [3] B.S. Luh, in: Rice (Ed.), Springer US, Boston, MA, 1991, https://doi.org/10.1007/ 978-1-4899-3754-4. [4] Y. Wang, Applications of rice bran oil, in: L.-Z. Cheong, X. Xu (Eds.), Rice Bran Rice Bran Oil, Elsevier, 2019, pp. 159–168, https://doi.org/10.1016/B978-0-12- 812828-2.00006-8. [5] K. Gul, B. Yousuf, A.K. Singh, P. Singh, A.A. Wani, Rice bran: nutritional values and its emerging potential for development of functional food - a review, Bioact. Carbohydrates Diet. Fibre. 6 (2015) 24–30, https://doi.org/10.1016/j. bcdf.2015.06.002. [6] U. Garba, R. Singanusong, S. Jiamyangyeun, T. Thongsook, Extraction and utilisation of rice bran oil. A review, Riv. Ital. Delle Sostanze Grasse. 96 (2019) 161–170. [7] C. In Prasit, B. Thumkote, A study to determine an optimal die configuration for rice bran oil extraction using a screw press machine, J. Sci. Technol. Kasetsart Univ. 6 (2017) 32–47. http://kuojs.lib.ku.ac.th/index.php/jstku/article/view/ 3446. [8] A.W. Go, T.Y.N. Pham, C.T. Truong, K.L. Quijote, A.E. Angkawijaya, R.C. Agapay, C. Gunarto, Y.-H. Ju, S.P. Santoso, Improved solvent economy and rate of rice bran lipid extraction using hydrolyzed rice bran with hexane as solvent, Biomass Bioenergy 142 (2020) 105773, https://doi.org/10.1016/j.biombioe.2020.105773. [9] S. Özgül, S. Türkay, In situ esterification of rice bran oil with methanol and ethanol, J. Am. Oil Chem. Soc. 70 (1993) 145–147, https://doi.org/10.1007/ BF02542617. [10] A.W. Go, S. Sutanto, L.K. Ong, P.L. Tran-Nguyen, S. Ismadji, Y.H. Ju, Developments in in-situ (trans) esterification for biodiesel production: a critical review, Renew. Sustain. Energy Rev. 60 (2016) 284–305, https://doi.org/10.1016/j. rser.2016.01.070. [11] S. Zullaikah, C.-C. Lai, S.R. Vali, Y.-H. Ju, A two-step acid-catalyzed process for the production of biodiesel from rice bran oil, Bioresour. Technol. 96 (2005) 1889–1896, https://doi.org/10.1016/j.biortech.2005.01.028. [12] L. Lin, D. Ying, S. Chaitep, S. Vittayapadung, Biodiesel production from crude rice bran oil and properties as fuel, Appl. Energy 86 (2009) 681–688, https://doi.org/ 10.1016/j.apenergy.2008.06.002. [13] N.S. Kasim, T.H. Tsai, S. Gunawan, Y.H. Ju, Biodiesel production from rice bran oil and supercritical methanol, Bioresour. Technol. 100 (2009) 2399–2403, https:// doi.org/10.1016/j.biortech.2008.11.041. [14] S. Sutanto, A.W. Go, K.H. Chen, P.L.T. Nguyen, S. Ismadji, Y.H. Ju, Release of sugar by acid hydrolysis from rice bran for single cell oil production and subsequent in- situ transesterification for biodiesel preparation, Fuel Process. Technol. 167 (2017) 281–291, https://doi.org/10.1016/j.fuproc.2017.07.014. [15] S. Sutanto, A.W. Go, S. Ismadji, Y.-H.H. Ju, Hydrolyzed rice bran as source of lipids and solid acid catalyst during in situ (trans)esterification, Biofuels 11 (2020) 221–227, https://doi.org/10.1080/17597269.2017.1348190. [16] S. Özgül-Yücel, S. Türkay, FA monoalkylesters from rice bran oil by in situ esterification, JAOCS, J. Am. Oil Chem. Soc. 80 (2003) 81–84, https://doi.org/ 10.1007/s11746-003-0655-7. [17] S. Özgül-Yücel, S. Türkay, Variables affecting the yields of methyl esters derived from in situ esterification of rice bran oil, JAOCS, J. Am. Oil Chem. Soc. 79 (2002) 611–614, https://doi.org/10.1007/s11746-002-0531-5. [18] S. Gunawan, S. Maulana, K. Anwar, T. Widjaja, Rice bran, a potential source of biodiesel production in Indonesia, Ind. Crop. Prod. 33 (2011) 624–628, https:// doi.org/10.1016/j.indcrop.2010.12.027. [19] L. Yustianingsih, S. Zullaikah, Y.H. Ju, Ultrasound assisted in situ production of biodiesel from rice bran, J. Energy Inst. 82 (2009) 133–137, https://doi.org/ 10.1179/014426009X12448168550064. [20] P.J. Shiu, S. Gunawan, W.H. Hsieh, N.S. Kasim, Y.H. Ju, Biodiesel production from rice bran by a two-step in-situ process, Bioresour. Technol. 101 (2010) 984–989, https://doi.org/10.1016/j.biortech.2009.09.011. [21] S. Sutanto, A.W. Go, K.-H.H. Chen, S. Ismadji, Y.-H.H.

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