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5Biodiesel production under subcritical solvent condition using subcritical water treated whole *Jatropha curcas* seed kernels and possible use of hydrolysates to grow *Yarrowia lipolytica*

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Institut Teknologi Sepuluh Nopember, Kampus ITS Keputih Sukolilo, Surabaya 60111, Indonesia highlights Subcritical water treatment of *Jatropha curcas* kernels to ease lipid extraction. Direct utilization of whole JCL kernels for in situ transesterification. Size reduction is not required prior to transesterification. Subcritical solvents (methanol, acetic acid and water) transesterification. Possible use of JCL kernel hydrolysates as yeast culture media.

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Biodiesel *Jatropha curcas* L. Subcritical water *Yarrowia lipolytica* abstract In this work, whole *Jatropha curcas* L. seed kernels were firstly treated in subcritical water (448 K, 2.0 MPa initial N₂, 15 min, kernel to water ratio 0.5 g 1) and then the treated kernels were used

5in the in situ production of biodiesel using a solvent mixture

of 75% methanol and 25% acetic acid. It was found that hydrolysate collected from subcritical water treatment of seed kernels contained reducing sugars and can be used to grow *Yarrowia lipolytica* without the need of detoxification. The in-situ (trans)esterification was successfully optimized using Taguchi design of experiments and a high yield of 101.7 and 65.1 g FAME per 100 g of extractable lipid and dry kernel, respectively could be achieved under optimized conditions (523 K, 3.0 MPa initial CO₂ and 7.5 cm³ g⁻¹ solvent to solid ratio). The developed process can tolerate high FFA and moisture content in feedstock. © 2013 Elsevier Ltd. All rights reserved. 1. Introduction Subcritical water (SCW) technology has been successfully applied in enhancing and maximizing lipid extraction from microbial cells [1–3]. Hydrolysis of soybean oil is one of the early works on catalyst-free hydrolysis under SCW condition [4]. Later applications of SCW hydrolysis of various edible and non-edible oils were Abbreviations: AG, acylglyceride; AOCS, American Oil Chemist Society; FA, fatty

2acid; FAME, fatty acid methyl ester; FFA, free fatty acid; HTGC, high temperature gas chromatography;

JCL, *Jatropha curcas* L; SCW, subcritical water; SSR, solvent to solid ratio; S/N, signal-to-noise ratio; TRS, total reducing sugars; DNS, 3,5-dinitrosalicylic acid. †

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11.066 also reported [5–8]. SCW hydrolysis of phospholipids was successfully carried out without catalyst [3,9]. The driving force for the reaction is the existence of ionic species of SCW. However free fatty acids (FFAs) and phosphoric acid released during hydrolysis of acylglycerides (AGs) and phospholipids were also observed to have catalytic activities in recent studies [3,7,8]. Minami and Saka [7] utilized SCW to hydrolyze triglycerides in rapeseed oil and then carried out esterification of the FFAs generated under supercritical methanol conditions. Chen et al. [6] using *Jatropha curcas* L. (JCL) seed oil adopted a similar concept. Ju et al. [10] utilized SCW for the transesterification reaction of soybean oil. Although most of the SCW treatments and reactions were carried out at 473–623 K, the use of strong mineral acids and corrosive bases can be avoided as ions of SCW exhibit both acidic and basic properties. The application of acetic acid in the field of biodiesel was first reported by Saka et al. [11] to produce triacetin and FFA. The use of acetic acid and CO₂ was later adopted in a one-step transesterification process [12]. These approaches had a common goal of reducing the severity of the process and reducing or avoiding the production of low value glycerol to improve the overall economics of the process. In-situ transesterification can avoid the long extraction time required [13], thus improving the process productivity.

10 In-situ transesterification of wet activated sludge under subcritical

conditions (448 K, 8 h) was studied by Huynh et al. [14], with

10a fatty acid methyl ester (FAME) yield comparable to that of

the conventional acid catalyzed reaction which required 24 h. The same process was applied utilizing wet oleaginous yeast (*Yarrowia lipolytica*) cells [15] and wet microalgae (*Chlorella vulgaris*) cells [16]. Levin et al. [17] utilized wet microalgal biomass to carry out carbonization at 523 K. Through carbonization, algal biomass was easily separated from water (culture media) thus resulting in lower moisture for subsequent transesterification step. The culture media was then reused for algal cultivations without the need of sterilization [17,18]. JCL seed is widely studied as feedstock in in situ transesterification to produce biodiesel. Lim and Lee [19] optimized the production of FAME from JCL seeds using supercritical methanol process. Other organics in the product were characterized with aims to recover high value by-products. In this study a two-step in situ transesterification of JCL seed kernels was explored employing SCW treatment of whole kernels and direct (trans)esterification of wet SCW treated JCL kernels in solvent mixture (methanol, acetic acid and water) under subcritical condition. Taguchi design of experiments (DOE) was employed for finding optimum operation conditions. The increasing interest in biodiesel production has also led to the exploration of microbial lipids. *Y. lipolytica*, an oleaginous yeast, has been tapped as a promising source of microbial lipid. Low cost agricultural waste hydrolysates like those from bagasse [20], rice bran [21], rice hulls [22] have been studied. Reducing sugars recovered from hydrolysates of SCW treated JCL kernels, might be utilized as a new source of low cost media. The recovered hydrolysate from SCW treatment of JCL seed kernels were analyzed of its reducing sugar content and inhibitors (furfural and 5-hydroxymethylfurfural (5-HMF)) and explored for possible utilization as media for growing oleaginous yeast *Y. lipolytica*.

2. Materials and methods

2.1. Materials JCL seeds were obtained from Muhammadiyah University (Malang, Indonesia). Seeds obtained were sun dried and kept at 253 K prior to use. Standards of fatty acid (FA), AG such as monoolein, diolein and triolein and fatty acid methyl esters (FAMES) were obtained from Supelco (Bellfonte, PA).

2.2. Sample preparation and characterization After being deshelled, seed kernels were kept at 253 K prior to use. Water content of the kernels was measured by putting grounded kernels (5 g) in a pre-dried glass tube. The kernel loaded tube was put into a freeze drier (Labconco FreeZone 2.5 dm³ Model: 7670520, Kansas City, MO) operated at 229 K and 11.0 Pa for 48 h. Water content of the kernel was calculated based on difference in weight of kernel sample before and after freeze-drying. Soxhlet extraction was used for determining the amount of extractable crude lipid in dry seed kernels (5 g), weighed to the nearest four decimal places with an analytical balance (Shimadzu, ATX224), using n-hexane (150 cm³) as solvent for 12 h. FFA content in the crude lipid was determined by the titrimetric method following AOCS official methods (Method Ca 5a-40) and by high temperature gas chromatography (HTGC); a detailed analysis condition is mentioned elsewhere [3]. Saponified lipids obtained after determination of unsaponifiable matter were collected and acidified to pH 2 with concentrated sulfuric acid at 333 K until the solution was clear. The solution was then allowed to settle until 2 phases were formed. The upper layer was extracted with hexane and further reacted with BF₃-methanol for later analysis of FA profile using HTGC. Before the AG composition of the extracted lipids was determined, dewaxing and degumming was carried out following the method described by Rajam et al. [23]. To estimate the total reducing sugars (TRS) that could be released from constituent carbohydrates, 100 mg of defatted ground kernels were placed in a tube, HCl (2.5 N, 5 cm³) was added and hydrolysis was carried out at 368 K for 3 h. The tube was then cooled to room temperature and its content was neutralized with sodium carbonate until the effervescence ceased. The reaction mixture was filtered and rinsed with deionized water. The collected filtrate was then made up to 100 cm³ using deionized water. TRS released during the

hydrolysis was analyzed using dinitrosalicylic acid (DNS) method and was expressed in terms of glucose concentration. 2.3. SCW treatment Whole kernels was

2loaded into a glass chamber (190 cm³) and placed in a high-pressure reactor

(290 cm³). A detailed description of the reactor is mentioned elsewhere [3]. The reactor was sealed and flushed with nitrogen prior to heating. The reaction chamber was initially pressurized to 2.0 MPa using nitrogen to ensure water was at subcritical state at 448 K carried out in this study. After SCW treatment, the reactor was rapidly cooled, pressure was released and product in the reactor was collected. Hydrolysate was decanted and filtered. The filtrate and the treated whole kernels were collected. The filtrate was analyzed for TRS and inhibitors (5-HMF and furfural) using DNS method and high performance liquid chromatography (HPLC) as described elsewhere [24]. SCW treated kernels were then freeze-dried and crude lipid in the dried kernels was extracted and characterized. 2.4. In-situ transesterification SCW treated whole kernels (7.5 g) containing 59% water by mass was loaded into the reactor. Kernels were mixed and suspended in a mixture of methanol (15–30 cm³) and acetic acid (0–15 cm³). The reactor was sealed and purged with nitrogen. Prior to heating, the reactor was pressurized using nitrogen or CO₂. Stirring

2was provided by an external magnetic stirrer.

It took 40–45 min to reach the reaction temperature (523 K).

2After the reaction the reactor was cooled to room temperature. The cooled reaction mixture was

filtered to separate the spent solid from liquid products. The retained

2solid was washed thrice each with 30 cm³ methanol.

Methanol and acetic acid in the filtrate were removed using a rotary evaporator (313 K and 13.3 kPa). The concentrated organic extract was then extracted thrice each with 30 cm³ hexane to recover FAME produced and washed thrice each using 20 cm³ salt solution (5% NaCl) in a separation funnel to remove non-lipid products co-extracted. Solution was allowed to clarify in between washings. The upper hexane phase, which contained FAME, was withdrawn. Hexane from the combined extract was removed. The recovered product was weighed and analyzed for its FAME, FA and AG contents. 2.5. Experimental design Experiments based on Taguchi method were carried out and analyzed statistically using analysis of variance (ANOVA). In the Taguchi method it utilizes a signal-to-noise ratio (S/N) to evaluate the response of the experimental trials [25]. In this study biodiesel produced with higher yield were preferred and thus the S/N ratio with the higher the better function is defined as: $S/N = 10 \log_{10} \frac{1}{n} \sum_{i=1}^n y_i^2$

4where y_i is the i th quality parameter (yield) and n is the number of trials. A

factorial design of 34 was carried out using Taguchi L₉ orthogonal array. In this study, two sets of experiments were carried out (Table 1), the first was to investigate the effects of solvents and nitrogen pressure at 523 K. The second was to lower down the severity of the reaction by utilizing CO₂ as the pressurizing gas. In Taguchi method the average S/N ratios are plotted in a response graph. With the optimum factor levels identified, it then assumes that the contribution of each factor to the overall response is additive. $y_{pred} = \mu + \sum_{i=1}^q X_i \delta_i$ where y_{pred} is the predicted response with the optimum factor level,

1 q is the number of significant processing parameters, μ is the overall mean of

all responses and y_i

1is the mean response value of the i th level of the significant processing

parameter. The predicted response (in this study the yield) is confirmed by running a confirmatory test employing the optimum factor levels as analyzed. A confidence interval

1 can be calculated using the equation:

ff fff

Cl $\frac{1}{4}$ Fa :1;

v2 me neff 1 p 1 r δ 3p

1 where Fa:1, v2 is the F-ratio of significant level a, a is the significant level,

1 a is the confidence level, v2 is the degree-of-freedom of

1 pooled error variance, Ve is the pooled error variance and r is

num- Table 1 Levels of variables: A to D for in situ transesterification and E to H for lowering the severity of in situ transesterification. Factors Parameters

1 Level 1 Level 2 Level 3

A Acetic acid (cm3) B Methanol (cm3) C N2 pressure (MPa) D Time (min) E SSR (cm3/g) F Time (min) G CO2 pressure (MPa) H Temperature (K) 0 7.5 15 22.5 1.0 2.5 30 60 5 7.5 30 60 1.5 3.0 473 498 ber of repeated trials. On the other hand neff is the number of effective measured results defined

1 as: neff $\frac{1}{4}$ total number of experiments sum of degrees of freedom used in

estimating the response p 1 δ 4p 2.6. Preparation of agar plates Y. lipolytica Po1g cells were obtained from YEASTERN Biotech Co., Ltd., (Taipei, Taiwan). The cells were perpetually maintained in yeast extract-peptone-dextrose (YPD) agar

11 (20 kg m 3 D -glucose, 20 kg m 3 peptone and 10 kg m 3 yeast extract, 10 kg m 3

agar) at 277 K. To test the possibility of utilizing JCL seed kernel hydrolysate, similar agar plates as mentioned above were prepared except that hydrolysate containing equivalent amount of glucose as the above media was used. The plates were then incubated at 301 K for 24 h. 2.7. Scanning electron microscope (SEM) imaging SEM images of the freeze-dried sample were scanned using a field emission electron microscope (JSM-6500F, JOEL, Ltd., Tokyo, Japan). 3. Results and discussion The crude hexane extractable lipid content of dry JCL seed kernel was $54.08 \pm 0.74\%$. A summary of the lipid components is shown in Table 2. Fig. 1 shows that SCW treatment

12 at a kernel to water mass ratio of 1:2

was enough for efficient lipid extraction. The water-soaked kernels (a kernel

12 to water mass ratio of 1: 0.5)

were also treated at 448 K for 15 min. Although a much lower lipid recovery (10%) was observed, this is still higher than the lipid extraction yield of the untreated whole kernels (4.87%). By referring to Table 2 and Fig. 1, no loss in the lipid was observed based on the dry weight of native kernels. SCW treated whole kernels contained 59% water. Lipid content in the dry SCW treated kernels increased to $62.84 \pm 0.1\%$ because 10–13% of the native kernel's mass was hydrolyzed. Visual inspection of the pretreated whole kernels did not reveal any major morphological change, except that the color of the kernel was changed from white to brown and kernels after SCW treatment were soft when wet and brittle when dry. Images of various cross sectional cuts (Fig. S1) of the kernel show that the pretreated 15 30 kernels are more porous leading to weaker structure. SEM micro- 4.0 graphs (Fig. S2) allow a better visualization of the morphological 90 changes of the kernels surface. Cracks were visible on the outer 10 surface of the SCW treated kernels, while micrographs of the cross 90 4.5 section reveal that the initially densely-packed structure of the 523 kernel became more porous with cavitations on the cross sectional surface. These morphological changes allowed easy extraction of Table 2 Mass fraction (%) of lipid components of ground kernels and SCW treated whole kernels based on hexane extractable lipid and initial dry kernel. Components Ground kernels SCW treated whole kernels Lipid Kernela Lipid Kernela FFA (%) Wax & Gum (%) Unsaponifiables (%) AG (%)

Hydrolysable (%) Theoretical FAME yield (%) 2.16 ± 0.21 13.73 ± 1.07 1.18 ± 0.06 82.93 87.38 ± 1.61 91.74 1.16 ± 0.12 7.43 ± 0.64 0.64 ± 0.03 44.84 47.25 ± 1.89 49.61 3.64 ± 0.87 9.58 ± 0.06 1.03 ± 0.28 85.75 87.99 ± 0.05 92.38 2.03 ± 0.52 5.36 ± 0.05 0.57 ± 0.12 47.37 49.28 ± 0.13 51.74 a Based on native kernel dry weight. Fig. 1. Effects of water loading on lipid yield of SCW treated kernels, treated at 448 K for 15 min and initially pressurized to 2.0 MPa using N₂. lipids from dried, SCW treated whole kernels without the need to grind the kernels. 3.1. Yield of FAME from SCW treated kernels FAME yields obtained by the in situ (trans)esterifications of SCW treated whole JCL seed kernels are summarized in Table 3. Based on the SN ratios obtained from the experiments, the main effects of each factor can be calculated and its average response graph is presented in Fig. 2. In Taguchi's experimental approach the main effect is calculated by the difference between the maximum and minimum of the mean SN ratio of each factor. In the first set of factors investigated the controlling factor was found to be the reaction time (D) followed by methanol loading (B), pressure (C) and acetic acid loading (A). The factor response graph (Fig. 2) shows that the optimal factor levels are A2, B2, C2 and D3. ANOVA was utilized to give a more objective judgment on the effects of factors investigated. Based on the experimental results, an F-ratio greater than 4.3 implies a significant effect contributed by the factor investigated. From Table 4, all factors investigated were significant and the sequence based on the degree of contribution agrees to that of Taguchi's analysis of the factors contributing to the main effects. Using Eqs. (3) and (4), the predicted yield and its confidence interval based on the optimal factor levels (A2, B2, C2, D3) was found to be $61.03 \pm 5.69\%$ and a conformational run carried out in duplicates resulted in an average value of $61.53 \pm 1.86\%$ with a S/N = 35.78 dB. This test shows that the optimized conditions can Fig.

42. Response graph of higher-the-better signal-to-noise ratios for

FAME yield from in situ (trans)esterification of SCW treated whole JCL seed kernels. Table 4 Summary of ANOVA analysis on FAME yield. Factor DOF SS (%) SSE F p (%) A 2 B 2 C 2 D 2 Error 9 Total 17 2.20 2.04% 2.59 2.44% 2.60 2.44% 3.32 3.17% 0.68 1.29% 11.38 – 7.13 17.95 8.51 21.44 8.52 21.47 11.04 27.80 – 11.33 – 100.00 DOF: degrees of freedom; SS: sum of squares; SSE: sum of square error; F: F-ratio; p: percent contribution. be well predicted by a simple addition model and the results falls within the 95% CI, showing the reliability and repeatability of this method. A yield of 61.53% based on dry kernel corresponds to 97.92% yield based on extractable lipids in kernel. Despite the fact that SCW treated kernels contain 59% water high FAME yield was achieved. The presence of water at 523 K promotes hydrolysis of lipids thus indirectly lowering the activation energy in FAME production since FFAs can be esterified at lower activation energies [7]. Another possible reason is the catalytic activity of SCW at the reaction condition employed as observed by other researchers [10,14,15]. High methanol loading used in this study favored the forward esterification reaction, contributing to high FAME yield observed. Moreover the presence of acetic acid may act as an acid catalyst making the process more water tolerant. The presence of acetic acid may favor the hydrolysis route of triglycerides and

4 Table 3 Results for L9 (34) orthogonal array experiments on

FAME yield from in situ (trans)esterification of SCW treated whole JCL seed kernels. Run Trial AA MeOH (cm³) P (MPa) t (min) Yield (%) Ave. (%) SD (%) S/N 1 2 3 4 5 6 7 8 9 1 0 2 0 1 0 2 0 1 0 2 0 1 7.5 2 7.5 1 7.5 2 7.5 1 15 2 15 1 15 2 15 1 15 2 15 1.0 15 1.0 22.5 2.5 22.5 2.5 30 4.0 30 4.0 15 2.5 15 2.5 22.5 4.0 22.5 4.0 30 1.0 30 1.0 15 4.0 15 4.0 22.5 1.0 22.5 1.0 30 2.5 30 2.5 30 28.6 30 32.1 60 57.2 60 50.2 90 54.0 90 54.9 90 54.1 90 50.8 30 45.9 30 49.7 60 46.2 60 45.6 60 37.6 60 35.4 90 40.6 90 46.7 30 42.1 30 39.7 30.30 2.48 53.69 5.00 54.46 0.63 52.42 2.36 47.80 2.62 45.87 0.43 36.53 1.55 43.63 4.36 40.90 1.66 29.59 34.54 34.72 34.38 33.57 33.23 31.24 32.73 32.22 Fig. 3. Effects of stirring on FAME yield employing the optimized condition (523 K, 3.0 MPa initial CO₂ and 7.5 cm³ g⁻¹ solvent to solid ratio). catalyze the later esterification step. Kusdian and Saka [26] found that conventional acid catalyzed esterification was only slightly affected by the presence of water at water content up to 50%. Methyl acetate could be produced which is a good extracting solvent and has considerable solubility in water thus may improve the overall solubility of the system. In this study, by using the subcritical solvent mixture of water, methanol and acetic acid high FAME yield can be obtained at 523 K and 13.0 MPa. The condition is less severe compared to that of the reported supercritical alcohol in situ processes, which operated at 548–573 K and 20.0 MPa [18,19]. Although higher methanol to solid ratio (7.5 vs. 5.9) and longer overall reaction time (135 min vs. 103 min) were required in this study due to the lower operating temperature (523 K vs. 573 K), this study demonstrated the direct use of wet, whole JCL seed kernels to produce biodiesel. Drying of seed and grind/flaking of seed kernel were not required thus the process can be simplified. 3.2. Lowering process severity The addition of co-solvent such as propane [27], heptane [28] and CO₂ [29] has been employed for reducing process severity in supercritical methanol process. One disadvantage of utilizing liquid co-solvents is the increase in the overall SSR, which leads to decrease in overall volumetric productivity. Hence the use of gaseous co-solvents like CO₂ is preferred. In addition to not decreasing the overall volumetric productivity, it can be easily removed after reaction. Taguchi method was again employed to find optimum FAME yield of the transesterification reactions of SCW treated seed kernels using CO₂ as the pressurizing gas and a solvent mixture (25% acetic acid and 75% methanol) based on the previous optimized results obtained from the first set of Taguchi DOE. The predicted FAME yield based on the optimal factor levels was found to be $65.20 \pm 4.63\%$ with a confidence interval of 95%. A conformational run carried

out in duplicate resulted in an average FAME yield of $62.15 \pm 1.09\%$ with a corresponding S/N ratio of 35.87 dB. Again the confirmatory test shows the reliability and repeatability of this method. Although statistical contribution of CO₂ on the yield of FAME was found to be the least, its presence allowed the lowering of the overall SSR from 10 to 7.5 cm³ g⁻¹. The reaction temperature was not lowered but the operating pressure was lowered to 11.0 MPa despite more CO₂ was loaded to the system compared to N₂.

3.3. Effects of stirring The effect of stirring on FAME yield was investigated and the results are shown in Fig. 3. Upon applying stirring, the reaction time required to reach 65% FAME yield (102% based on extractable lipids) was reduced from 90 min to 60 min. This corresponds to an overall reaction time of 105 min (including 45 min heating) which is comparable to those reported in literature utilizing supercritical methanol reactive extraction (103 min) as summarized in Table 5.

3.4. Recovery and possible utilization of pre-hydrolysate In SCW treatment of whole kernels, 12–16% of the initial dry solid was solubilized in water as water loading was increased from 2 to 6 cm³ per gram of dry kernel. Despite more solid was solubilized due to higher water loading the concentration in the filtrate decreased (57–28.5 mg cm⁻³) due to dilution. To better utilize the hydrolysate, TRS was analyzed. The trend in Fig. 4a is similar to what observed in the hydrolyzed and dissolved fraction of the whole kernel. Sugar comprises about 30–40% of the Table 5

Comparison of optimized in situ transesterification of JCL seed kernel. In situ (trans)esterification method Oil content SSRa Temp Pressure Time Yield Ref. (%) (cm³ g⁻¹) (K) (MPa) (min) (%) Acid catalyzed (H₂SO₄ 21.8%, w/w) 54.6 Microwave (110 W) assisted 2-step (Ethanolysis) (H₂SO₄ 7.5 wt.%) (KOH 5 mol/L) 42.5 Supercritical methanol (process intensification) 66.8 Supercritical methanol co-solvent (CO₂ 5.0 MPa) 64.6 Supercritical methanol, co-solvent (CO₂ 4.0 MPa) 66.8 Supercritical methanol, co-solvent (CO₂ 2.0 MPa) 66.8 Wet whole kernels (59% water content) sub-critical methanol-acetic acid (7.5:2.5), co-solvent (CO₂ 30 bar) 62.8 10.5 333 Step 1 – 10.5 Step 2 8.15 5.0 573 5.0 573 5.8 568 5.9 573 7.5 523 0.1 600 – 0.5 (0.8)b 9.5 30 (120)b 20.0 30 (120) 20.0 7.5 (98) 20.0 12.6 (103) 11.0 60 (105) 98.1c (53.53)d 90.0 (38.3) 99.67 (66.6) 102.3 (66.1) 87.1 (58.2) 92.0 (61.5) 101.7 (65.1) [30] [31] [33] [32] [19] [19] This Study a SSR = solvent to solid ratio. b Total reaction time including reactor heat up time. c Based on the hexane extractable lipid. d Based on the dry kernel. organics dissolved in the hydrolysate. With both consideration of productivity and sugar concentration in the hydrolysate, a kernel to water ratio of 1:2 g/g is preferred. Fig. 4b clearly shows that a SCW treatment of 30 min gave a nearly 50% increase over that of 15 min in both sugar yield and concentration. A treatment time over 30 min had little effect on sugar yield and concentration and lipids were completely recoverable (data not shown). These results agree with those reported on SCW treated microbial cells [2,3]. Although the maximum yield of reducing sugars ($22.09 \pm 1.48\%$) that can be obtained from acid hydrolysis of defatted kernels was not achieved, it should be noted that a high degree of hydrolysis would result in the collapse of solid matrix of seed kernel and release the lipids, which requires additional separation steps. Moreover a release of about 28% of the maximum available reducing sugars is considered as high noting that whole kernels were utilized, which greatly reduced the total surface area of contact. After SCW treatment, lipids can be easily extracted from the dried treated whole kernels without further grinding. Hydrolysates from baggase [20] and rice bran [11] have been utilized to grow oleaginous yeast *Y. lipolytica* Po1g for lipid production at a sugar concentration between 20 and 30 kg m⁻³. Peptone was the preferred nitrogen source for *Y. lipolytica* Po1g [21]. In the hydrolysate obtained in this study, inhibitors concentrations (furfural and 5-HMF) were found to be very low and the pH was about 5.06. Growth of yeast was confirmed in preliminary investigation, where *Y. lipolytica* was plated and maintained using the recovered hydrolysate (Fig. S3). In most processes, less production steps are preferred. However considering the possible use of hydrolysate as a sterilized media in Fig. 4a. Effects of water loading on yield of TRS in hydrolysate. SCW treatment at 448 K for 15 min. Initially pressurized to 2.0 MPa using N₂. Fig. 4b. Effects of time on yield of TRS in hydrolysate. SCW treatment at 448 K at a kernel to water ratio of 1:2 (g/g). Initially pressurized to 2.0 MPa using N₂. culturing yeast and other microbes to produce high value products, the SCW treatment step may reasonably be compensated. The 2-step process developed in this study allows the direct use of whole JCL kernels without the need of drying and size reduction.

4. Conclusion This work investigated the use of whole JCL kernels in a 2-step production of biodiesel. The first step involved the SCW treatment of seed kernel at 448 K for 15 min. In the second step, in situ (trans)esterification of wet kernel in subcritical solvent mixture was carried out at 523 K for 105 min. Taguchi method was successfully applied in optimizing the process conditions for in situ transesterification reactions. A high FAME yield (65.1%) was achieved which is comparable to those processed under supercritical methanol conditions reported in literatures. Preliminary investigation showed the possible use of hydrolysate from the SCW treatment step to grow yeast like *Y. lipolytica*. Acknowledgments This work was supported by the National Science Council of Taiwan (NSC 101-ET-0

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6Appendix A. Supplementary material Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.fuel.2013>.

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