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Palm Oil Decanter Cake Wastes as Alternative Nutrient Sources and Biomass Support Particles for Production of Fungal Whole-Cell Lipase and Application as Low-Cost Biocatalyst for Biodiesel Production

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Abstract: This is the first report on the possible use of decanter cake waste (DCW) from palm oil industry as alternative nutrient sources and biomass support particles for whole-cell lipase production under solid-state fermentation (SSF) by newly isolated fungal *Aspergillus* sp. MS15 and their application as a low-cost and environment-friendly biocatalyst for biodiesel production. The results found that DCW supplemented with 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 1% peptone and 2% urea and pH adjusted to 6.0 was optimal for whole-cell lipase production. The optimal moisture content and fermentation temperature was 60% and 37.5 °C, respectively. Environmentally friendly biodiesel production, through either esterification or transesterification using whole-cell lipase immobilized on DCW as a biocatalyst, was optimized. The optimal reaction temperature for both reactions was 37 °C. The whole-cell lipase effectively esterified oleic acid into >95% biodiesel yield through esterification under optimal water activity at 0.71 and an optimal methanol to oleic acid molar ratio of 2:1, and also effectively transesterified palm oil under optimal water activity at 0.81 and an optimal methanol to oil molar ratio of 3:1. The fuel properties of produced biodiesel are close to the international biodiesel standards. These results have shown the circular utilization of palm oil mill waste for the low-cost production of an effective biocatalyst, and may contribute greatly to the sustainability of renewable bioenergy production.

Keywords: *Aspergillus* sp.; biodiesel; biomass support particles; decanter cake waste; whole-cell lipase

1. Introduction

Biodiesel is produced from vegetable oils by the chemical reaction of transesterification, but not by esterification. However, it can be produced by the esterification of fatty acids in the presence of an alcohol (MetOH, EtOH) and catalyst (NaOH or KOH). It is becoming an interesting topic in every country's policy because of the limitation of energy reserves and the increasing amount of greenhouse gasses being released from fossil fuel, which causes global warming [1,2]. Enzymatic transesterification is a more attractive method than chemical methods due to its mild reaction condition, lower energy requirement and more environmentally friendly process [3]. Lipases are wildly used as biocatalysts which are capable of catalyzing diverse reactions including hydrolysis, esterification and transesterification [4]. Microbial lipases are gaining increasing attention in industrial applications due to their high product yields and high stability [5]. Several

commercial microbial lipases, including Lipase H (*Candida cylindracea*), Novozym 435 (*Candida antarctica*), Lipase OF (*Candida rugosa*), Lipase CVL (*Chromobacterium viscosum*), Lipase MML (*Mucor miehei*), Lipozyme TL (*Thermomyces lanuginosus*) and Lipase P (*Pseudomonas cepacia*), have been widely used for the production of biodiesel [6–8]. However, using these lipases at an industrial scale has not yet been practical due to their high costs.

The use of a whole-cell biocatalyst attached to biomass support particles for biodiesel production is one alternative way to reduce the cost of lipase because it can be directly used without a purification step. Fungi are a source of lipases as whole-cell biocatalysts which have been recently evaluated for biodiesel production. Over 90% of biodiesel yield has been achieved by using whole-cell *Aspergillus niger* lipase and *Aspergillus nomius* lipase [2,9]. Generally, fungi can grow on solid substrate and simultaneously produce lipase through the solid-state fermentation (SSF) process. It is therefore possible to use agricultural residues and agro-industrial wastes as low-cost nutrient sources for the fungi, which would make the process economically feasible [10–12]. Palm oil decanter cake waste (DCW) could be candidate solid substrate for SSF by the fungi and, due to its high residual oil content, it could be good source of lipase production. Few research groups have evaluated the use of DCW for enzyme production [10–12]. However, no attempt has been made to use DCW as a source of low-cost nutrients and biomass support particles for production of fungal whole-cell lipases or explore its further use as a biocatalyst for biodiesel production.

Enzymatic biodiesel synthesis can be performed either in organic solvents or in solvent-free (only the mixture of substrates) systems. In an organic solvent system, when the used total alcohol is added to the reaction, oils or related substrates can be converted into biodiesel depending on the catalytic activity and solvent tolerance of lipase. The biodiesel produced under a solvent-free system can be obtained during the stepwise-adding of alcohol to avoid the negative effect of alcohol on lipase activity [1–4]. Crucial parameters affecting the yield of enzymatic biodiesel synthesis are reaction temperature, water content, and molar substrate ratio [1,9]. They are related to catalytic efficiency and specific characteristics [4]. Although there are several reports on the effect of these three parameters on enzymatic biodiesel synthesis, none of them evaluate the optimal condition for maximizing biodiesel reaction catalyzed by immobilized whole-cell lipase on DCW. Therefore, these parameters need to be pursued and optimized for obtaining a higher production yield of biodiesel.

In this study, ten fungal isolates were screened for their abilities to produce lipase on DCW-based medium through SSF. The medium components and fermentation conditions for whole-cell lipase production by the selected fungi were then optimized. The applications of whole-cell lipase as a biocatalyst for biodiesel production through transesterification of oil and esterification of fatty acid were investigated. The important process parameters were optimized and the fuel properties of biodiesel obtained were evaluated and compared with international EN 14214 and ASTM D6751 standards.

2. Materials and Methods

2.1. Decanter Cake Waste from Palm Oil Mill

The DCW was obtained from local palm oil mills and then dried at 75 °C for 12 h, stored in plastic bags and kept at room temperature before use. The moisture and content of cellulose, lignin and lipid of dried DCW were determined by the standard method [13]. Carbon and nitrogen were measured according to Mazaheri et al. [14]. The cellulose, lignin, oil, carbon and nitrogen contents of DCW used in this study were: 33.16%, 15.80%, 9.45%, 41.58% and 2.45%, respectively. The pH and moisture content were 5.02 and 10.45%, respectively.

2.2. Fungal Strains

Ten isolates of fungi obtained from Faculty of Agro-Industry, Prince of Songkla University were used in this study. Five isolates including RT20, ST5, ST45, ST49 and ST52 were *Rhizopus* spp. while isolates RT24, RT29, MS15, MS26 and ST52 were *Aspergillus* spp. Fungal

strains were pre-cultured for 3 days at 30 °C on potato dextrose agar (PDA) as a pre-culture medium containing 200 mL of potato broth (boiled potato 1 kg and water 1 L for 15 min), dextrose 20 g/L and agar 20 g/L with the pH adjusted to 5.0. The spore suspension was prepared by suspending 3 day-old culture on the PDA slant with 0.1% (*w/v*) Tween 80. The stock suspension was diluted with sterilized water into the final concentration of 10^7 spores/mL.

2.3. Selection of Whole-Cell Lipase Producing Fungi

Ten fungal isolates were cultured through SSF using DCW-based medium as a renewable medium which contained 5 g DCW mixed with 6 mL of 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$ and 1% peptone with the pH adjusted to 5.0. Approximately 10^7 spores/mL of spore suspension of each isolate was added and the moisture content was adjusted at 60% and the cultures were performed at 37 °C for 72 h. After SSF, the whole-cell lipase attached to the DCW was washed twice with chilled 1:1 Tris-HCl buffer pH 7.0/acetone for 30 s. Washed whole-cell lipase attached to the DCW was dried in a fume hood at room temperature for 8 h and stored at −18 °C before use as a biocatalyst for hydrolysis, transesterification and esterification reactions. The whole-cell isolate that gave the highest hydrolytic, transesterification and esterification activities was selected for the next experiment.

2.4. Optimization of DCW-Based Nutrient Composition and Culture Conditions for SSF

The selected isolate (the initial spore concentration of 10^7 spores/mL) was cultured in five different media including DCW1: 5 g DCW added with 6 mL of 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$ and 1% peptone; DCW2: 5 g DCW added with 6 mL of 0.1% K_2HPO_4; DCW3: 5 g DCW added with 6 mL of 0.05% $MgSO_4 \cdot 7H_2O$; DCW4: 5 g DCW added with 6 mL of 1% peptone; and DCW5: 5 g DCW added with 6 mL distilled water. The effect of additional carbon sources (glucose, sucrose, lactose, molasses and palm oil) and inorganic nitrogen sources (NH_4NO_3, $(NH_4)_2SO_4$, $NH_4H_2PO_4$, $NaNO_3$ and urea) were also investigated. The cultures were incubated at 37 °C for 72 h.

2.5. Optimization of Whole-Cell Lipase Production

In this study, the selected isolate ($\approx 10^7$ spores/mL) was cultured under solid state fermentation for 3 days on the DCW-based medium. The effects of moisture content (50–70%), culture pH (4–7) and temperature (30–45 °C) on whole-cell lipase production were evaluated and optimized using response surface methodology (RSM). The response pattern and synergy among the factors were determined. According to Box-Behnken Design (BBD) with three factors at three levels, 17 runs with five replications at the central point were performed to estimate the purely experimental uncertainties. The response surface analysis of the data obtained from the 17 runs was carried out based on the multiple linear regressions including main, quadratic and interaction effects as shown in the following equation (Equation (1)):

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ij} x_i x_j + \sum \beta_{jj} x_j^2 \quad (1)$$

where Y is the responses, β is regression coefficient, and x are investigated factors including moisture content, culture pH and temperature. The fit of the model was evaluated by the coefficient of determination (R^2) and the analysis of variance (ANOVA). Response surface plots were performed to indicate the maximum levels of the response and the optimal levels of the factors by using the fitted equations obtained.

2.6. Application of Whole-Cell Lipase as a Biocatalyst for Biodiesel Production

2.6.1. Standard Procedures for Determination of Lipase Activity

Determination of Hydrolytic Activity

The hydrolytic activity of the whole-cell lipase was assayed using the modified cupric acetate method [15]. About 2 mg of whole-cell lipase attached to biomass support particles

(BSP) was mixed with 0.5 mL Tris-HCl buffer (pH 7.0) and 1.0 mL palm oil dissolved in iso-octane at 10% (*v/v*). The reaction mixture was incubated at 30 °C and 1200 rpm for 30 min in a thermomixer (TAITEC, Saitama, Japan). The reaction was stopped by adding 0.3 mL 6 M hydrochloric acid. The upper layer of the reaction mixture 0.5 mL was mixed with 0.5 mL of 5% (*w/v*) cupric acetate solution (pH 6.1 adjusted with pyridine). The mixture was mixed well for 15 s. The liberated fatty acids were determined using a spectrophotometer by measuring the absorbance at 715 nm against the control which contained a deactivated sample. The homogeneity was confirmed by the acceptable reproducibility. One unit of hydrolytic activity was defined as the amount of enzyme necessary to release 1 µmol fatty acids per minute at the specified condition. The hydrolytic activity of whole-cell lipase was expressed as unit per gram BSP.

Determination of Transesterification Activity

The whole-cell lipase with hydrolytic activity about 2 U/g-BSP was mixed with 0.4 g palm oil and 1.518 mL of methanol (=molar ratio of oil to methanol 1:3 and water content (a_w) of 0.73). The reaction was performed at 30 °C and 1200 rpm for 72 h [16]. The percent of fatty acid methyl ester (FAME) was analyzed by TLC-FID analyzer (IATROSCANTM MK-5, Tokyo, Japan).

Determination of Esterification Activity

The whole-cell lipase with hydrolytic activity of about 2 U/g-BSP was mixed with 0.4 g oleic acid and 506 µL of methanol (=molar ratio of fatty acid to methanol 1:1 and water content (a_w) of 0.71). The reaction was performed at 30 °C and 1200 rpm for 72 h [16]. The percent of FAME was analyzed by TLC-FID analyzer.

Analysis of FAME by TLC-FID

Five microliters of produced FAME were mixed with 200 µL of chloroform and 1 µL sample was spotted on Chromarod-SIII (Iatron Laboratories, Inc., Tokyo, Japan), developed using two solvent systems: *n*-hexane/diethyl ether/formic acid (50:20:0.3 *v/v/v*) for 15 min and subsequent benzene/*n*-hexane (1:1 *v/v*) for 30 min. The separated bands were identified by comparison with the standards. The Chromarods were then dried at 105 °C for 10 min and scanned with the TLC-FID analyzer under analytical conditions: hydrogen flow rate 150 mL/min, air flow rate 700 mL/min and scanning speed 30 s/scan. The peak area ratio was calculated with the ChromStar chromatography data system and used to calculate the percentage of fatty acid methyl esters (%FAME) [17]. All results throughout this manuscript were expressed as the percentage of peak areas of the reaction mixture's components. They were regarded as weight percentages and may vary slightly from the actual weight percent.

2.6.2. Optimization of Biodiesel Production by Whole-Cell Biocatalyst

The obtained whole-cell lipase was used as a biocatalyst to produce biodiesel, namely fatty acid methyl ester (FAME) through transesterification and esterification reactions. The reaction temperature (30–45 °C) and water activity (a_w = 0.7–0.9); water activity for both reactions, measured using the AQUALAB water activity meter (METER Group, Inc., Washington, DC, USA), were optimized. The molar ratio of methanol to oil was optimized for transesterification and the molar ratio of methanol to oleic acid was optimized for esterification.

2.6.3. Scale up of Whole-Cell Lipase and Biodiesel Production

The fungal *Aspergillus* sp. MS15 ($\approx 10^7$ spores/mL) was cultured under solid state fermentation under the optimal conditions from Section 2.5 for 3 days on DCW-based medium contained 50 g DCW and co-substrate with 60 mL of optimal components obtained from Section 2.4. After fermentation, the whole-cell lipase was used as a biocatalyst for methyl ester production through transesterification and esterification under the optimal conditions from Section 2.6.

2.7. Analytical Methods

The fuel properties of produced biodiesel including flash point, cloud point, pour point, viscosity at 40 °C and copper strip corrosion were determined according to the standard method as EN ISO 2719, ASTM D2500-17a, ASTM D97-17b, EN ISO 3104, and EN ISO 2160 published by the European Committee for Standardization the American Society for Testing and Materials (Table S1).

2.8. Statistical Analysis

All experiments were performed at least in triplicate. One-way ANOVA (analysis of variance) and Duncan's multiple range tests ($p < 0.05$) were used to evaluate statistical significance of the results.

3. Results and Discussion

3.1. Selection of Whole-Cell Lipase Producing Fungi

Ten fungal isolates were cultured on DCW as their nutrient source and BSP through SSF for 72 h. The hydrolytic, transesterification and esterification activities of whole-cell lipase attached to DCW were determined. Among the 10 isolates tested (Figure S1), the whole-cell *Aspergillus* sp. MS15 lipase gave the highest hydrolytic activity of 2.33 U/g-BSP followed by *Rhizopus* sp. RT20 lipase (2.10 U/g-BSP) and *Aspergillus* sp. MS26 lipase (2.09 U/g-BSP), respectively. The other seven fungal strains gave a low hydrolytic activity of 1.73–2.06 U/g-BSP. Our results are in good agreement with the previous publications reported by Rakchai et al. [1,2], Oliveira et al. [12] and Hama et al. [16]. They found that whole-cell lipase produced from different fungal strains, such as *Aspergillus nomius*, *Aspergillus niger, Aspergillus oryzae, Aspergillus awamori*, and *Rhizopus oryzae*, gave hydrolytic activity in a range of 0.10–20.70 U/g-dry cell weight. However, the medium components, culture conditions and substrate-related compound used in the activity assay influenced the hydrolytic activity [1,2]. Only *Aspergillus* sp. MS15 lipase showed both trans- and esterification activity by giving biodiesel yield of 13.33% FAME and 44.55% FAME, respectively. However, the whole-cell lipase from other strains could not catalyze the transesterification reaction. This might be due to differences in catalytic specificity of obtained lipases and lower accessibility of the whole-cell lipase yields [3,4]. Among ten isolates tested, *Aspergillus* sp. MS15 with high hydrolytic, trans- and esterification activities was chosen and the key parameters for production of whole-cell lipase on DCW were optimized.

3.2. Optimization of Medium Component and Culture Conditions for Whole-Cell Lipase Production

3.2.1. Optimization of Medium Components

Five DCW-based media were used for cultivation of the selected fungal *Aspergillus* sp. MS15. This experiment aimed to identify important additional nutrients. The hydrolytic activities of whole-cell lipase were comparable in the range of 2.33–2.60 U/g-BSP while DCW4 gave the highest esterification activity of 53.65% FAME and DCW1 gave the highest transesterification activity of 13.3% FAME (Figure 1). These results indicate that DCW1 and DCW4 that contain peptone as an additional nitrogen source could induce the production of whole-cell lipase with high activities of transesterification and esterification. It is possible that this organic nitrogen source may play an important role in regulating the synthesis of lipase type I and III which catalyze the transesterification and esterification reactions, respectively [18,19].

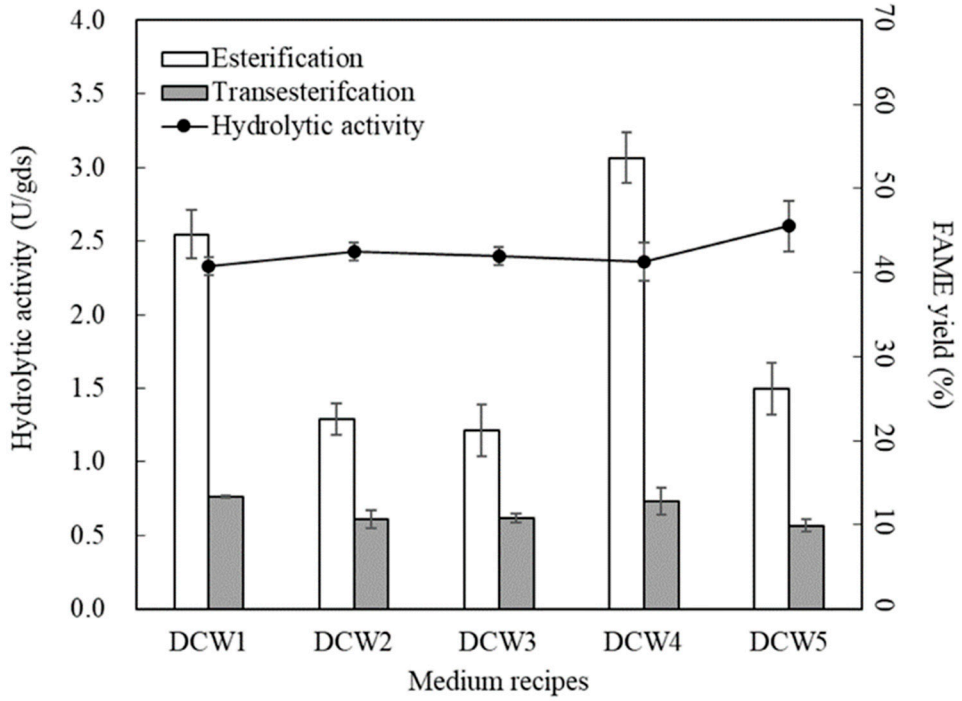

Figure 1. Hydrolytic, transesterification and esterification activities of whole-cell lipase produced by the selected fungal *Aspergillus* sp. MS15 cultured in decanter cake waste-based media.

3.2.2. Addition of Co-Carbon and Inorganic Nitrogen Sources

The effect of various co-carbon sources including sugars (glucose, sucrose and lactose), agro-industrial waste (molasses) and palm oil on whole-cell lipase production was investigated. Each carbon source was added at 2%. The addition of carbon source did not significantly increase the hydrolytic, transesterification and esterification activities of whole-cell lipase (Figure S2). This could be due the sufficient carbon source in raw decenter cake waste which contains cellulose and oil residue at 33.16% and 9.45%, respectively. The addition of inorganic nitrogen sources (NH_4NO_3, $(NH_4)_2SO_4$, $NH_4H_2PO_4$, $NaNO_3$) and urea on whole-cell lipase production were also studied. Each nitrogen source was added at 2%. Among the nitrogen sources tested, $NaNO_3$ gave the highest hydrolytic activity of whole-cell lipase up to 2.82 U/g-BSP (Figure S3). This would be because inorganic nitrogen sources can be assimilated quickly and this enhanced both cell growth and lipase production [19]. Helal et al. [20] evaluated the production of lipases using fish-frying oil added with various nitrogen sources such as $NaNO_3$, urea and KNO_3. They also found that $NaNO_3$ gave the highest lipase activity. Similarly, Oliveira et al. [21] also reported that $NaNO_3$ positively affected the hydrolytic activity of lipase produced by *Aspergillus ibericus*. However, the addition of urea promoted the transesterification and esterification activities of the whole-cell lipase. The biodiesel yields were as high as 85.41% FAME and 88.86% FAME, respectively (Figure S3). These values were much higher than those of the control. The differences in the catalytic activities could be due to the different structures of the lipase, especially at active site [22]. Therefore, only urea was chosen to be added with DCW1 for whole-cell lipase production.

3.2.3. Optimization of SSF through Response Surface Methodology

Moisture content, culture pH and temperature are considered as crucial factors for fungal growth during SSF and subsequent production of whole-cell lipase. These three factors were optimized through response surface methodology (RSM). Their individual and interaction effects on the production of the whole-cell lipase as well as their optimal levels were determined. The spores of *Aspergillus* sp. MS15 ($\approx 10^7$ spores/mL) were inoculated

on the optimal DCW-based medium from the previous section and SSF was performed under various culture conditions. The independent variables for the process were moisture content (%; X_1), initial pH (X_2) and fermentation temperature (°C; X_3). Their values were varied in the ranges shown in Table 1. The experimental results were concerned with the biodiesel yields from esterification reaction (%FAME; Y_1) and transesterification reaction (%FAME; Y_2) using three-factor Box-Behnken Design (BBD) experimental design. The conditions at the center point were: moisture content of 60%, initial culture pH 6 and fermentation temperature of 37.5 °C. The estimated response surface model in the form of a second order regression equation for FAME yields produced by esterification and transesterification reactions are shown in Equations (2) and (3), respectively.

$$Y_1 = -1352.63 + 12.86X_1 + 112.09X_2 + 44.32X_3 - 0.69X_1X_2 + 0.013X_1X_3 + 0.11X_2X_3 - 0.077X_1^2 - 6.26X_2^2 - 0.69X_3^2 \quad (2)$$

$$Y_2 = -3236.51 + 60.29X_1 + 355.43X_2 + 29.19X_3 - 1.27X_1X_2 - 0.027X_1X_3 + 0.079X_2X_3 - 0.43X_1^2 - 23.23X_2^2 - 0.44X_3^2 \quad (3)$$

Table 1. Box-Benhken design matrix of response surface methodology with experimental and predicted values of fatty acid methyl esters synthesis by transesterification and esterification reactions.

Run	X_1: Moisture Content (%)	X_2: pH	X_3: Temperature (°C)	Methyl Ester (%)			
				Esterification		Transesterification	
				Predicted	Actual	Predicted	Actual
1	50	5	37.5	69.11	67.12	15.31	18.42
2	70	5	37.5	83.36	83.84	30.96	20.13
3	50	7	37.5	82.68	82.21	47.26	58.06
4	70	7	37.5	69.39	71.39	11.99	8.85
5	50	6	30	88.70	89.24	63.09	46.01
6	70	6	30	87.17	85.23	57.39	54.24
7	50	6	45	0	0	0	0
8	70	6	45	0	0	0	0
9	60	5	30	90.30	91.77	77.78	91.71
10	60	7	30	88.39	88.34	83.09	89.33
11	60	5	45	0	0	6.27	0
12	60	7	45	1.46	0	13.95	0
13	60	6	37.5	90.07	90.32	93.04	92.47
14	60	6	37.5	90.07	90.69	93.04	92.79
15	60	6	37.5	90.07	91.03	93.04	93.95
16	60	6	37.5	90.07	91.46	93.04	92.59
17	60	6	37.5	90.07	86.89	93.04	93.33

Based on the experiment results (Table 1), the esterification activity ranged from 0 to 91.77% FAME. It was found that Run 9 (moderate moisture content of 60%, acidic pH of 5 and moderate fermentation temperature of 30 °C) gave the maximum esterification activity of 91.77% FAME with transesterification activity of 91.71% FAME. The two-dimensional contour plots from the calculated responses are shown in Figure 2. One variable was kept constant at its center point and the other two variables were varied within their ranges. These graphs were plotted in order to investigate the individual and interaction effects of the factors on esterification activity. The moderate moisture content of 60% and slightly acidic pH at 6.0 were most suitable for production of whole-cell lipase with high esterification activity. In SSF, the microbial growth and their metabolisms normally occur in the aqueous phase where nutrients can diffuse. In the case that moisture content is too low, the diffusion of nutrients will be hindered, while too high moisture content might cause a decrease in porosity of the solid substrate, hence limiting oxygen transfer and aerobic fermentation [23]. Gutarra et al. [24] reported that the hydrolytic activity of *Penicillium simplicissimum* lipase in SSF of babassu cake was high at pH 4–5. They also reported that at nearly a neutral pH of 6–7 the lipase was more stable and could perform better

transesterification and esterification reactions. Similar results have been also reported by Colla et al. [25]. With the same moisture content, the comparable esterification activity (>90%) could be obtained when using a fermentation temperature of 37.5 °C and initial culture pH of 6 (Run 13–16). The optimal temperature for production of whole-cell lipase with high esterification activity was in the range of 30–37.5 °C (Figure 2). Among the runs tested, Run 7, 8, 11 and 12 did not show esterification activity, possibly due to thermal inactivation at 45 °C. Most lipases produced by mesophilic fungi have low thermal stability, resulting in the loss of catalytic activity at higher temperatures above 40 °C [22,24]. Table S2 shows that the linear term of fermentation temperature and quadratic terms of each factor significantly influenced the esterification activity with *p*-value less than 0.05. In addition, the interaction between moisture content vs. initial culture pH significantly affected the esterification activity of whole-cell lipase (*p*-value < 0.05). Therefore, the degree of influences by three factors on esterification activity of whole-cell lipase is: fermentation temperature > moisture content > initial culture pH.

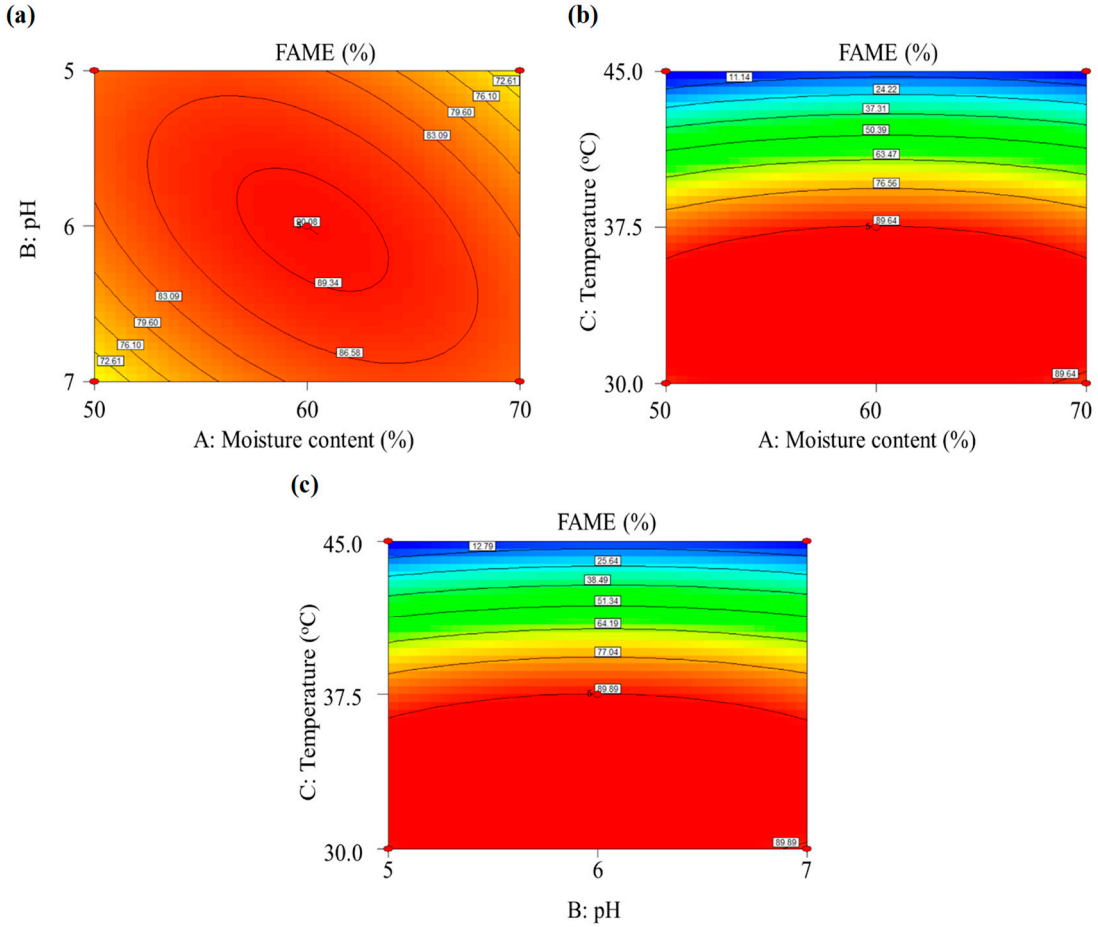

Figure 2. Two-dimensional contour plots showing the effect of (**A**): moisture content (%), (**B**): pH, (**C**): temperature (°C) on fatty acid methyl esters (FAME; %) synthesized by esterification. One variable kept constant at its center point and other two variables varied within the experimental range. As shown in (**a**–**c**).

The transesterification activity of whole-cell lipase ranged from 0 to 93.95% FAME (Table 1, Figure 3). The maximum response value for transesterification activity was estimated to be 93.95% FAME under the fermentation conditions of Run 15 (moderate moisture content of 60%, initial pH of 6 and fermentation temperature of 37.5 °C). With the same moisture content, initial pH of 6–7 and fermentation temperature of 30–37.5 °C gave transesterification activity as high as >89.33% FAME (Run 10, 13–17). Table S2 shows that the linear term of fermentation temperature significantly affected transesterification

activity, as was also observed for esterification activity. The quadratic term of each factor was also significant (p-value < 0.05), demonstrating that it required a suitable moisture content, initial culture pH and fermentation temperature. The degree of influences by three factors on the transesterification activity of whole-cell lipase is: fermentation temperature > moisture content > initial culture pH which was the same as that for esterification activity. It should be noted that the production of whole-cell lipase with transesterification activity was more sensitive to moisture content and initial culture pH (Figure 3) than the production of whole-cell lipase with esterification activity (Figure 2).

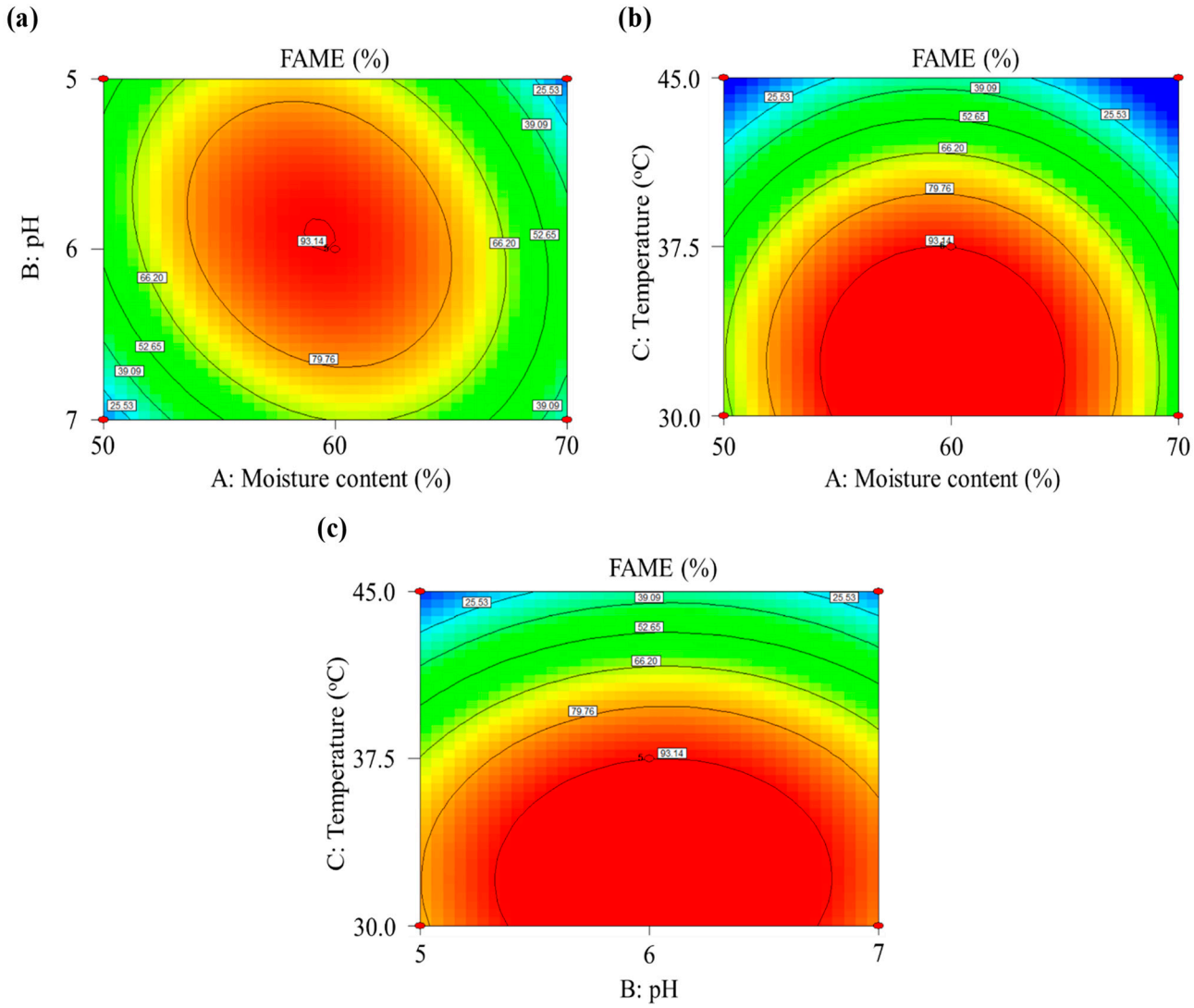

Figure 3. Two-dimensional contour plots showing the effect of (**A**): moisture content (%), (**B**): pH and (**C**): temperature (°C) on fatty acid methyl esters (FAME; %) synthesized by transesterification. One variable kept constant at its center point and other two variables varied within the experimental range. As shown in (**a**–**c**).

It could be concluded that the optimal conditions for both esterification activity and transesterification activity of whole-cell lipase produced by *Aspergillus* sp. MS15 were as follows: moisture content of 60%, initial culture pH of 6 and fermentation temperature of 37.5 °C. Under these optimized conditions, the experimental esterification and transesterification activity were 90.08% FAME and 93.03% FAME, respectively, which were very similar to the predicted values (90.07% FAME for esterification and 93.04% FAME for transesterification) without significant difference (p-value < 0.05), indicating the validity and adequacy of the predicted models. In addition, it was observed that the percentage

error between the predicted and actual value for the esterification and transesterification activity of whole-cell lipase were nearly 0%, confirming that the RSM is an efficient tool for predicting conditions for transesterification and esterification activity of whole-cell lipase.

3.3. Optimization of Biodiesel Production Using Whole-Cell Lipase

3.3.1. Effect of Reaction Temperature

The effects of temperature on the transesterification and esterification reactions using whole-cell lipase immobilized on DCW as a biocatalyst were investigated at various temperatures ranging from 30 °C to 45 °C. With increasing temperature from 30 °C to 37 °C, the biodiesel yield by transesterification slightly increased from 93.03% FAME to 95.74% FAME and that by esterification was slightly increased from 90.08% FAME to 90.72% FAME (Table 2). At higher temperatures the biodiesel yield decreased, possibly due to the thermal deactivation of the whole-cell lipase [1,2,26,27]. The reaction rates initially increased with increasing reaction temperature due to the increasing kinetic energy. Molecules of substrates require an input of activation energy to initiate the reaction. Typically, by increasing the temperature of the reaction, more molecules have the energy to reach a transition state. Therefore, as the temperature rises, molecules have more kinetic energy needed for the successful collision, so the rate increases. At the optimal temperature, the enzyme's catalytic activity is at its greatest. Above the optimal temperature, the enzyme structure breaks down or denatures due to breaking the intermolecular bonds (more kinetic energy), and enzyme activity decreases. Hence, increasing the temperature over the balance between increasing kinetic energy and thermal deactivation decreases the catalytic activity of the enzymes [26,27]. Thus, the reaction temperature of 37 °C seems to be the optimal temperature for biodiesel production catalyzed by whole-cell lipase through transesterification and esterification reactions.

Table 2. Optimization of the esterification and transesterification for synthesis of FAME.

Factors	Methyl Ester (%)
Optimization of esterification reaction	
Effect of temperature (°C)	
30	90.08 ± 1.83
37	90.72 ± 1.84
45	83.53 ± 2.05
Effect of water content (aw)	
0.71	90.72 ± 1.84
0.79	82.13 ± 5.19
0.83	83.82 ± 2.35
0.85	83.01 ± 1.87
0.87	76.38 ± 2.33
0.90	72.65 ± 3.60
Effect of molar ratio of methanol and oleic acid	
1:1	90.72 ± 1.84
2:1	96.25 ± 0.67
3:1	81.30 ± 0.37
Optimization of transesterification reaction	
Effect of temperature (°C)	
30	93.03 ± 0.61

Table 2. *Cont.*

Factors	Methyl Ester (%)
37	94.70 ± 0.60
45	72.16 ± 4.35
Effect of water content (aw)	
0.73	94.70 ± 0.60
0.81	95.74 ± 0.28
0.85	90.16 ± 2.09
0.87	87.11 ± 1.98
0.89	83.38 ± 4.67
0.91	31.12 ± 3.25
Effect of molar ratio of methanol to oil	
3:1	95.74 ± 0.28
3.5:1	87.31 ± 1.27
4:1	27.89 ± 6.06
5:1	7.60 ± 3.57
6:1	3.37 ± 0.97

3.3.2. Optimization of Water Content and Methanol Molar Ratio in Esterification Reaction

Water content was expressed as water activity in the reaction mixture which affects the enzyme activity and conversion yield for biodiesel production by enzymatic esterification reaction. Several studies have reported that a certain amount of water is required for optimal enzyme activity, but excess water adversely affects biodiesel yield [28–30]. The effect of a water activity (a_w) value of 0.71–0.90 on biodiesel yield in esterification was investigated, as shown in Table 3. A higher yield of biodiesel >80% FAME was obtained at a relatively low water activity of 0.71–0.85. The highest esterification activity of 90.72% FAME was obtained when using a water activity of 0.71. Similar results have been reported by Matsumoto et al. [31] who found that a water activity of 0.65–0.85 gave the maximum reaction rate of esterification of benzyl alcohol and octanoic acid. Therefore, the lowest water activity of 0.71 was selected for the esterification-based biodiesel process in this study.

Table 3. Fuel properties of lipids from transesterification of palm oil.

Properties	Diesel ASTM D975 *	ASTM D6751 **	EN 14214 ***	Produced Biodiesel
Methyl ester (%wt)	N.A.	N.A.	≥96.5	94–97
Viscosity at 40 °C (mm^2/s)	1.3 to 4.1	1.9 to 6.0	3.5 to 5.0	5.99
Flash point (°C)	>52	≥130	≥120	175
Copper strip corrosion	N.A.	≤No. 3	≤No. 1	No.1a
Cloud point (°C)	−15 to 5	N.A.	N.A.	11
Pour point (°C)	−35 to 15	N.A.	N.A.	8

N.A. is not available. * Diesel ASTM D975 is a standard specification for diesel fuel biodiesels published by the American Society for Testing and Materials. ** ASTM D6751 details standards and specifications for biodiesels published by the American Society for Testing and Materials. *** EN 14214 is a standard for biodiesel published by the European Committee for Standardization.

Table 2 also shows the effect of the molar ratio of methanol and oleic acid on esterification. It was found that an increase in the molar ratio of methanol and oleic acid from 1:1 to 2:1 increased biodiesel yield from 90.72% to 96.25%. The biodiesel yield reduced for a further increase in the molar ratio of methanol and oleic acid from 2:1 to 3:1, possibly due to substrate inhibition by a high amount of methanol. These results are in accordance with those previously reported by Nguyen et al. [30] who found that when a high methanol

ratio was used as acyl-acceptor in the esterification, the enzyme was deactivated and gave relatively low biodiesel yield. In this study, the maximum biodiesel yield was obtained at a methanol to oleic acid molar ratio of 2:1.

3.3.3. Optimization of Water Content and Methanol Molar Ratio in Transesterification Reaction

The effect of a water activity (a_w) value of 0.73–0.91 on biodiesel yield in transesterification is shown in Table 2. It can be seen that the water (a_w value of 0.81) was sufficient to give a high biodiesel yield (95.74%). Higher water activity decreased biodiesel yield. The suitable water activity for biodiesel production through transesterification of palm oil catalyzed by whole-cell lipase in this study is a water activity of 0.81. Noureddini et al. [32] found that the increased water content in transesterification of soybean oil using immobilized lipase from *Pseudomones cepacia* led to an increase in the amount of free fatty acid without decreasing biodiesel yield, while Rakchai et al. [1] reported that the excess water might shift the equilibrium toward hydrolysis rather than transesterification. In addition, the high amount of water might generate a thicker water layer around the enzyme surface and cause a diffusion problem of substrate and product toward enzyme activity sites [26].

As shown in Table 2, the highest biodiesel yield was achieved at a molar ratio of methanol to oil of 3:1. Increasing the molar ratio of methanol to oil decreased biodiesel yield. It would be possible that a higher methanol ratio than 3:1 might deactivate whole-cell lipase and reduce FAME yield. Rakchai et al. [1,2] reported the optimal molar ratio of methanol for transesterification of palm oil catalyzed by whole-cell *Aspergillus nomius* ST57 lipase was 3:1 to 4:1 giving a biodiesel yield >90%. Zhang et al. [33] reported that the excess methanol diluted oil concentration in the reaction system, decreasing the collision frequency between oil and catalyst. Therefore, a methanol to oil molar ratio of 3:1 is an appropriate for biodiesel production through transesterification of palm oil catalyzed by whole-cell *Aspergillus* sp. MS15 lipase in this study.

3.4. Selection of Whole-Cell Lipase Producing Fungi

To produce a large amount of whole-cell lipase, the fungal *Aspergillus* sp. MS15 were cultured in SSF on 50 g DCW-based culture medium containing suitable nutrient components as described above with the initial pH 6.0. The optimal fermentation conditions were a moisture content of 60% and fermentation temperature at 37.5 °C for 3 days. Then, the produced whole-cell lipase was used as biocatalyst for biodiesel production using the optimal reaction conditions for esterification (a_w value of 0.71 and molar ratio of methanol to oleic acid of 2:1) and those for transesterification (a_w value of 0.81 and molar ratio of methanol to oil of 3:1) with the optimal reaction temperature of 37 °C. It was found that the maximum biodiesel yields obtained through transesterification and esterification were 94–97%, which were insignificantly different to those of small scale production (95–97%). The fuel properties of produced biodiesel by whole-cell *Aspergillus* sp. MS15 lipase were: viscosity at 40 °C 5.99 mm^2/s, flash point 175 °C, copper strip corrosion No.1a, could point 11 °C and pour point 8 °C. These values were in accordance with the biodiesel EN 14214 and ASTM D6751 standards (Table 3). This study may contribute greatly to the industrialization of enzymatic biofuel production.

4. Conclusions

Palm oil decanter cake waste could be used as a low-cost nutrient source and biomass support material for whole-cell lipase production by selected fungal *Aspergillus* sp. MS15. The whole-cell lipase could effectively catalyze transesterification and esterification reactions which achieved a maximum biodiesel yield of 94–97% FAME, indicating its potential use as biocatalyst. The fuel properties of produced biodiesel meet with the international standards. This study has shown the promising biovalorization of waste which is effective not only for waste utilization but also contributes to environmentally friendly biofuel production.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/pr9081365/s1, Table S1: Standard methods used for determination of fuel properties of produced biodiesel, Table S2: ANOVA for response surface of fatty acid methyl esters synthesis, Figure S1: Hydrolytic activities of whole-cell lipase produced by different fungal strain cultured in decanter cake waste-based media. (Five isolates including RT20, ST5, ST45, ST49 and ST52 were *Rhizopus* spp. while isolate RT24, RT29, MS15, MS26 and ST52 were *Aspergillus* spp.), Figure S2: Hydrolytic, transesterification and esterification activities of whole-cell lipase produced by the selected fungal *Aspergillus* sp. MS15 cultured in decanter cake waste-based media (DCM1) containing different carbon sources, Figure S3: Hydrolytic, transesterification and esterification activities of whole-cell lipase produced by the selected fungal *Aspergillus* sp. MS15 cultured in decanter cake waste-based media (DCM1) containing different nitrogen sources, Figure S4: Three-dimensional contour plots showing the effect of A: moisture content (%), B: pH, C: temperature (°C) on fatty acid methyl esters (FAME; %) synthesized by esterification. One variable kept constant at its center point and other two variables varied within the experimental range, Figure S5: Three-dimensional contour plots showing the effect of A: moisture content (%), B: pH, C: temperature (°C) on fatty acid methyl esters (FAME; %) synthesized by transesterification. One variable kept constant at its center point and other two variables varied within the experimental range, Figure S6: TLC/FID chromatogram of standard mixture.

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