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Production of Methanol Tolerant Cell-bound Lipase of *Magnusiomyces spicifer* SPB2 and Application as Whole-cell Biocatalyst in Transesterification Reaction

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Abstract

Cell-bound lipase (CBL) is a potential biocatalyst for production of fatty acid alkyl ester (FAAE), which is feasible replacement for diesel fuel. This study aimed to improve both hydrolysis and transesterification activities of CBL from the yeast *Magnusiomyces spicifer* SPB2, which could be employed as whole-cell biocatalyst for transesterification reactions through optimizing the growth medium using Taguchi orthogonal array experimental method (L9). The optimized medium, resulting the highest CBL transesterification activity, consisted of soybean oil, peptone, Gum Arabic and an initial pH of 5. Interestingly, the fatty acid methyl ester (FAME) yield increased up to 33.28 times when compared with non-optimized medium. Moreover, cell morphology of *M. spicifer* SPB2 varied depending on the culture medium component. The single cell was more active to be used as the whole-cell biocatalyst than the pseudomycelial form for transesterification reaction. The highest FAME yield was 93.86 % when refined palm oil to methanol molar ratio of 1:8 was used as substrates. Whole-cell lipase from *M. spicifer* showed favorable activity to iso-butanol as an acyl accepter, resulting in fatty acid butyl ester (FABE) yield of 86.80 % at 1:3 molar ratio of palm oil to iso-butanol. The FAME and FABE yields dramatically decreased from 93.86 and 86.80 % to 57.02 and 4.48 %, respectively after the reuse of the enzyme to the second batch of reaction. These results indicated that the whole-cell biocatalyst developed from *M. spicifer* was an efficient and economical approach to improve enzyme activity and stability in the transesterification reaction.

Keywords: *Magnusiomyces spicifer* SPB2, Hydrolysis activity, Cell-bound lipase activity, Transesterification activity, Taguchi orthogonal array method (L9), Whole-cell lipase, Fatty acid alkyl ester

Introduction

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) belong to the serine hydrolase enzyme family that catalyzes hydrolysis of acyl ester bonds of lipids at the oil/water interface [1,2]. These molecules act at the interface between oil substrate and aqueous phases in which the enzyme is dissolved. Lipases are also able to catalyze interesterification, transesterification, and enantioselective reactions in non-aqueous media [2,3]. Lipases are important biocatalysts and very useful in biotechnological processes that are being widely used in several industries; such as food, dairy, detergent, pharmaceuticals, and biodiesel production.

The transesterification reaction of plant oils is important because it produces fatty acid alkyl ester (FAAE), which is a great substitute for diesel fuel. Additionally, the enzyme-catalyzed reaction occurs under mild conditions, which is better for the environment than a chemical process using either bases or acids as catalysts to produce biodiesel [4,5]. However, production of biodiesel using lipases at industrial level is unfeasible due to high cost, low stability in methanol, low conversion and reaction rate of the enzymes. This is because lipases require many complicated steps, such as purification and immobilization. Therefore, cost effective and solvent tolerant lipases are ideal and essential for industrial applications. Thus, whole-cell biocatalysts are promising if their complications can be avoided or minimized in preparation process. The whole cells of various microorganisms were purposed to be used as biocatalysts for transesterification reactions. These included *Pseudomonas* SB15 MH715026 [6], *Aspergillus oryzae* [7], *Rhizopus oryzae* [8], *Rhizopus stolonifer* 1aNRC11 mutant F [9]. Among the reported microorganisms, yeasts are considered to be potential whole-cell biocatalysts for FAAE production because they exhibit high expression of cell-bound lipase (CBL) activity. Many lipase-producing yeasts were previously immobilized prior to use as biocatalysts to improve enzyme stability and reusability. In the meantime, a few yeast strains

expressing CBL could be used as whole cell-biocatalysts in transesterification reactions. The whole cell biocatalyst from *Rhodotorula mucilaginosa* P11I89 could yield 83.3 % FAME at 72 h through transesterification reaction between palm oil and methanol [10].

Yeast whole-cell CBL has been used to accelerate transesterification reaction, but FAAE yield is rather low [10]. One way to improve whole-cell lipase activity is to optimize the culture medium for increasing synthetic activity of CBL. Many researches optimized the culture medium to improve hydrolytic activity of lipases. Meanwhile, synthetic activity is also essential for catalyzing the transesterification reaction. Therefore, it is necessary to optimize the growth medium to improve both hydrolysis and transesterification activity of CBL. The Taguchi orthogonal array method (L9) is one of the statistical approach to enhance the efficiency and reproducibility of the experiments. It is a well-designed experiment useful for examining and screening many impact variables of factors and levels simultaneously. This therefore required lower number of experiment set to be carried on hence offering time and cost effectiveness. Although many researches used this design method to increase the yield of lipase, but few studies used it to identify and optimize the influencing factors affecting the transesterification activity of the enzymes [11-13]. Yeasts must be cultivated in the appropriate medium for improving CBL yield for transesterification activity, which is the essential step in the preparation of whole-cell biocatalyst suitable for successful FAAE production.

The whole-cell of CBL producing *M. spicifer* SPB2 (GenBank accession number MT027498) was preliminarily used as biocatalyst to catalyze the transesterification reaction of palm oil. Unfortunately, the percentage of FAME was very low (2.82 % of FAME). Therefore, this study focused on using Taguchi orthogonal array experimental method (L9) to determine significant impacts of cultivation factors and their levels influencing CBL hydrolytic and transesterification activities of *M. spicifer* SPB2. The cultivation factors; including carbon sources, nitrogen sources, surfactants, and initial pH were optimized under submerged fermentation condition. Moreover, other influencing parameters (temperature, alcohol type, substrate molar ratio, and reusability of whole-cell CBL) involving in transesterification reaction were also investigated by using palm oil which is abundantly produced from an economic-important crop in the southern part of Thailand as a substrate.

Materials and methods

Microorganism, cultivation media and growth conditions

Magnusiomyces spicifer SPB2 was grown on yeast malt medium (YM). The submerged culture of YM medium consisted of 1 % glucose, 0.5 % peptone, 0.3 % yeast extract, and 0.3 % malt extract. To obtain the yeast inoculum, *M. spicifer* SPB2 from frozen glycerol stock was grown in Erlenmeyer flasks (250 mL) containing 100 mL of YM medium. It was incubated at 30 °C ± 2 °C for 72 h under a shaking speed of 200 rpm. The lipase production was performed in Erlenmeyer flasks (250 mL) containing 100 mL of isolation medium for yeast (IMY) [14] according to parameter variation designed by Taguchi orthogonal array method (L9).

Taguchi orthogonal design

The Taguchi orthogonal array (OA) L9 (3^4) with 8 degrees of freedom was used to investigate the effects of cultivation factors, including carbon sources (A), nitrogen sources (B), surfactants (C) and initial pH (D) on lipase production expressed in term of hydrolytic and transesterification activities. The levels of the studied factors and the layout of the Taguchi OA L9 were displayed in **Tables 1** and **2**. L and 9 represent the Latin square and the number of experiments, respectively). The levels of the factors included palm oil, coconut oil and soybean oil for carbon sources; NH_4NO_3, tryptone and peptone for nitrogen sources; Gum Arabic, Tween 80 and Triton X-100 for surfactants; and pH 4, 5 and 6 for initial pH variables of the culture medium. All experimental values are presented as the mean of triplicate data from each experimental run designed in the Taguchi orthogonal array method.

The fermentation was performed in an Erlenmeyer flask (250 mL) containing 100 mL IMY medium based on the orthogonal test in **Table 1**. The IMY medium contained 0.1 % $MgSO_4 \cdot 7H_2O$, 0.47 % KH_2PO_4, 0.03 % $Na_2HPO_4 \cdot 12H_2O$, and 0.01 % yeast extract, various types of oils, nitrogen sources and surfactants according to the orthogonal test. The initial pH of the medium was adjusted to the corresponding values (pH 4, 5 and 6) using either 2 M HCl or 2 M NaOH. The medium was sterilized at 121 °C for 15 min before inoculation. Each flask was inoculated to achieve a final concentration of 1×10^6 cells/mL. Such inoculum was previously cultivated in YM medium at 30 ± 2°C for 120 h under shaking speed of 200 rpm. One milliliter of the fermentation broth was centrifuged at 10,000 rpm at 4 °C for 10 min to collect yeast cells for determining biomass, hydrolysis activity of CBL. The supernatant was subjected to the essay for

extracellular lipase. The yeast cells of 1×10^{10} cells/g substrate were used as a whole-cell biocatalyst in transesterification reaction for determining the CBL transesterification activity. To draw the main effect of the factors independently, *k* and *R* values were calculated from the experimental data. The major influencing factors and levels were considered based on the k and *R* values [12].

Table 1 Influencing factors and their levels used in the cultivation media according to Taguchi orthogonal array method (L9) for improvement of CBL hydrolytic and transesterification activities of whole-cell biocatalyst from *M. spicifer* SPB2.

Factor	Level 1	Level 2	Level 3
A: Carbon source (2 %)	Palm oil	Coconut oil	Soybean oil
B: Nitrogen source (0.4 %)	NH_4NO_3	Tryptone	Peptone
C: Surfactant (0.2 %)	Gum Arabic	Tween 80	Triton X 100
D: Initial pH	4	5	6

Optimization of fatty acid alkyl ester (FAAE) production *via* transesterification reaction catalyzed by whole-cell CBL of *M. spicifer* SPB2

Whole-cell CBL biocatalyst from *M. spicifer* SPB2 prepared under the optimal medium and growth condition was used to catalyze transesterification reaction of palm oil. The effects of alcohol type, reaction temperature, substrate molar ratio and enzyme reusability on FAAE production through transesterification reaction were investigated. The reaction was performed in a 2-mL microcentrifuge tube with vigorous shaking. Methanol, ethanol, propanol, 1-butanol, 2-butanol, and iso-butanol were used as acyl acceptors at palm oil to alcohol molar ratio of 1:3. To investigate the effect of temperature, the reactions were incubated at 30 ± 2 ℃, 37 and 45 ℃. The substrate molar ratio of palm oil to alcohol was performed under the range between 1:3 to 1:13 in increment of 1.

The reusability of whole-cell CBL in the transesterification reaction was evaluated under the optimized condition. This referred to palm oil to methanol molar ratio of 1:8, or palm oil to iso-butanol molar ratio of 1:3 were used at a reaction temperature of 30 ± 2 ℃ for 72 h. After each reaction completed at 72 h, the whole-cells biocatalyst was separated from the reaction mixture by centrifugation at 10,000 rpm for 15 min. The recovered whole-cells biocatalyst was washed with 0.1 M citric acid buffer (pH 5.0) and was then reused in catalyzing a new batch of reaction mixture carried out under the optimized condition. Fatty acid alkyl esters were determined using thin-layer chromatography-flame ionization detector (TLC-FID) (Iatroscan MK-5; Iatron Laboratories Inc., Tokyo, Japan).

Analytical method

Assays of hydrolytic activity of CBL and extracellular lipases

The hydrolytic activity of CBL and extracellular lipases were determined using cupric acetate method [10]. The cupric acetate solution (5 %, w/v) was prepared and adjusted to pH of 6.1 by adding pyridine. The substrate emulsion was the mixture of cupric acetate solution and 10 % palm oil in iso-octane. The lipase production of yeast cells and culture supernatant were expressed as CBL and extracellular lipase activities, respectively. The enzyme mixture was prepared by vigorously mixing yeast cells (CBL activity) or culture supernatant (extracellular lipase activity) with 0.2 M phosphate buffer pH 7. One milliliter of 10 % palm oil in iso-octane was subsequently added to the enzyme mixture to initiate the reaction. The reaction mixture was incubated at 30 °C for 30 min under shaking speed of 300 rpm. The enzyme reaction was then terminated by adding 0.3 mL of 6M HCl to the mixture.

Free fatty acid content present in the upper layer of iso-octane was then determined according to Nuylert and Hongpattarakere [15]. The upper layer (0.1 mL) of the mixture was drawn and diluted with iso-octane to a volume of 1 mL before mixing with 0.4 mL of cupric acetate solution for 15 s. Free fatty acid dissolved in iso-octane was determined by measuring the absorbance at 715 nm against the control iso-octane containing no free fatty acid using a spectrophotometer (GENESYS 10S Series UV-Visible Spectrophotometers, Thermo Fisher Scientific Inc., USA). The hydrolytic activity was determined by measuring the amount of fatty acid drawn from the standard curve of palmitic acid, which was established prior to the experiment from 0 to 8 µmol/mL. One unit of enzyme activity was defined as the enzyme necessary to release 1 µmol of palmitic acid per minute under the specified conditions (0.2 M phosphate buffer pH 7, 30 °C for 30 min). The hydrolytic activity of whole-cell CBL was calculated according to the below Eq. (1) [15].

$$Total\ hydrolytic\ activity\ \left(\frac{U}{L}\right) = Hydrolytic\ activity\ \left(\frac{U}{g} dry\ cell\right) \times biomass\ \left(g\ dry \frac{cell}{L}\right) \quad (1)$$

Transesterification reaction catalyzed by whole-cell CBL of *M. spicifer* SPB2

Transesterification activity of whole-cell CBL was performed by adding substrate mixture (palm oil and methanol/alcohol at molar ratio 1:3 to yeast cells (1×10^{10} cells/g substrate) suspended in phosphate buffer (pH 7.0) according to Srimhan et al. [10]. The reaction mixture was carried out under vigorously shaking at 30 °C ± 2 °C for 72 h. Fatty acid methyl ester (FAME)/fatty acid alkyl ester (FAAE) and free fatty acid were analyzed by thin-layer chromatography connected to flame ionization detector (TLC-FID) (Iatroscan MK-5; Iatron Laboratories Inc., Tokyo, Japan).

Before use, the scanned quartz rods (Chromarod S-II) were pretreated by dipping in 3 % boric acid solution for 5 min, dried at 105 °C for 5 min, and rescanned with the TLC-FID analyzer. Then, 1 µL of sample aliquot was spotted onto the rod, which was later developed in a solvent mixture of hexane-diethyl ether-formic acid (50:20:0.3 v/v/v) to reach 8 cm high, and subsequently reached 10 cm in a second solvent mixture of benzene-hexane (1:1 v/v). The rods were dried and installed in the TLC-FID analyzer thereafter. The analytical conditions were H_2 flow rate of 160 mL/min, air flow rate of 2000 mL/min and a scanning speed of 30 s/scan. The peak area ratio was calculated by the chromatography data system using ChromStar 6.3 Software. Transesterification activity/biodiesel yield was presented as the percentage of FAME/FAAE calculated based on the obtained peak area [10,16].

Biomass determination

One milliliter of culture sample was centrifuged at 10,000 rpm for 10 min at 4 °C to obtain yeast pellet, which was then washed twice with distilled water. The washed yeast pellet was thereafter allowed to dry at 105 °C until a constant weight of biomass was obtained.

Microscopic observation

Yeast cells from culture broth were diluted with 0.85 % w/v NaCl to achieve a final concentration of 10^6 cells/mL. Then, 30 µL of the cell suspension was dropped on the glass slide and covered with a cover slip. The yeast morphology was observed through a light compound microscope (Nikon, USA) at 400× magnification.

Molecular identification of lipase-producing yeast

The phenol-chloroform method was used to isolate the genomic DNA of the yeast [17]. The purified genomic DNA was subjected to PCR for amplification of the D1/D2 region of the 26S rRNA gene using primers Fw-5′-GCATATCAATAAGCGGAGGAAAAG and Rv-3′-GGTCCGTGTTTCAAGACGG [18]. The initial denaturation of DNA was performed at 95 ℃ for 2 to 3 min and 25 cycles of 26S rRNA gene amplification was performed using the following thermocycling program: denaturation at 95 ℃ for 30 seconds, annealing at 50 to 60 ℃ for 30 s, followed by a reaction extension for 2 min at 72 ℃. The final extension was carried out at 72 ℃ for 10 min. The amplified PCR product of the lipase-producing yeast was sequenced and analyzed by the National Center for Biotechnology Information (NCBI), Bangkok, Thailand. The obtained sequence was searched using BLAST tool in the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST). The phylogenetic analyses were performed using the Mega 5 program. The sequence data was then submitted to the Genbank NCBI database under the accession number MT027498. The lipase-producing yeast was then deposited with the TISTR Culture Collection Center (TISTR 6024).

Results and discussion

Optimization of culture medium for improvement of hydrolytic activity of CBL from *M. spicifer* SPB2

The identification of *M. spicifer* SPB2 was confirmed using D1/D2 region of 26S rRNA gene (GenBank accession number MT027498). *M. spicifer* SPB2 was grown on palm oil-rhodamine B agar plate and lipase secretion was clearly observed by the presence of orange fluorescent halos around the colony. The significant effects of carbon sources (A), nitrogen sources (B), surfactants (C), and initial pH (D) with their levels and types on growth and lipase production of *M. spicifer* SPB2 were investigated using Taguchi orthogonal array method (L9). The impact of the 4 factors designed for optimization of hydrolytic CBL and extracellular lipase production ranging from 111.79 to 313.67 Unit/L and 729.98 to 2459.77 Unit/L, respectively (**Table 2**).

Regarding the Taguchi analysis of the orthogonal array results, *k* and *R* values were calculated and presented in **Table 3**. The impact factors supported hydrolytic activity of CBL was ranked from high to low in order of B (nitrogen sources) > C (surfactants) > D (initial pHs) > A (carbon sources) according to *R* values. The nitrogen source in the medium had the greatest influence (R = 124.64) on the production of hydrolytic CBL from *M. spicifer* SPB2. The hydrolytic activity of CBL from *M. spicifer* SPB2 was enhanced drastically in the presence of peptone. Based on k_1 (116.28) and k_3 values (240.92), the organic nitrogen showed to be greater influence on the hydrolytic of CBL than inorganic nitrogen (NH_4NO_3). Peptones are protein hydrolysates consisting of polypeptides, dipeptides and amino acids, which are important nutritional sources for cells growth, cell metabolism and enzyme synthesis [19]. Similar results were presented by Maldonado *et al*. [20] showing that peptone had a positive effect on extracellular lipase production of *Geotrichum candidum* and yielded approximately 16 Unit/mL after 48 h of fermentation.

Addition of surfactant in culture medium also demonstrated high impact on hydrolytic CBL activity of *M. spicifer* SPB2 (R = 109.05). Tween 80 was the best surfactant for stimulating the biosynthesis and secretion of lipase from *M. spicifer* SPB2. This was also observed in lipase production from *Candida viswanathii* [21]. These results were in accordance with Byreddy *et al*. [22] who found that 1 % Tween 80 improved the intracellular lipase production by *Schizochytrium* S31. Surfactant could facilitate enzyme accessibility to oil substrate. The highly increased production of lipase by a mesophilic OE3 bacterial strain in medium containing olive oil and Gum Arabic [23]. According to some earlier reports, the initial pH of the medium was also found to influence lipase production and secretion in various microorganisms [19,24]. Correspondingly, an initial pH of 5 was found to be the optimum for CBL activity of *M. spicifer* SPB2 in this study. However, pH showed no significant in lipase production for *Kocuria flava* [15,25]. Carbon sources displayed to be less influent than nitrogen sources and surfactants on hydrolytic CBL production of *M. spicifer* SPB2. Among plant oils used, palm oil showed the highest influence. Lipase is generally known to be oil/fatty acid-inducible enzyme. Therefore, types of plant oils containing different ratios of fatty acid composition affect lipase activity [15,21]. Therefore, the hydrolytic CBL activity of *M. spicifer* SPB2 was optimized, when 2 % palm oil, 0.4 % peptone, 0.2 % Tween 80 and initial pH at 5 were applied in growth medium preparation.

Optimization of culture medium for improvement of transesterification activity of whole-cell CBL from *M. spicifer* SPB2

The production of FAME when using whole-cell CBL of *M. spicifer* SPB2 as biocatalyst in the transesterification reaction was also examined by the Taguchi orthogonal array method (L9). The FAME yield from the transesterification reaction catalyzed by lipase was determined and presented in **Table 2**. The yields of FAMEs ranged from 0 to 56.40 % when whole-cell CBL were applied for biocatalyst in Runs# 1 to 9. The experimental data of FAME yield are showed in **Table 3**. The cultivation factors studied using the orthogonal array analysis to determine their effects on the transesterification reaction showed in the significant order of surfactants > nitrogen sources > initial pHs > carbon sources according to *R* values (**Table 3**). Gum Arabic acting as the best surfactant added to the culture medium was the greatest influence on the transesterification activity when the whole-cell lipase of *M. spicifer* SPB2 was used as biocatalyst. The result was consistent with what observed in transesterification activity catalyzed by whole-cell CBL from *Rhodotorula mucilaginosa* P11I89, which required Gum Arabic to achieve the highest yield of FAME [15]. The nitrogen source became less influential than the surfactant factor in transesterification activity of whole-cell CBL from *M. spicifer* SPB2. Use of peptone as the nitrogen source and initial pH of 5 in the culture medium facilitated its transesterification activity. These results corresponded to the hydrolytic CBL activity, indicating that peptone and the initial pH of 5 was the preferable factor for both hydrolysis and the synthetic activities of *M. spicifer* SPB2. Soybean oil, which is rich in oleic acid and linoleic acid, had the greatest effect on synthetic activity of lipase, followed by palm oil and coconut oil. Oleic or linoleic acid-enriched cells were previously shown higher initial methanolysis activity than saturated fatty acid-enriched cells [26]. However, NH_4NO_3, palm oil and initial pH of 4 highly improved transesterification activity *Rhodotorula mucilaginosa* P11I89 [15].

The results showed that the hydrolytic and transesterification activities of CBL were inconsistent (**Table 3**). The synthetic activity of the whole-cell CBL was not supported by the amount of lipase production measuring as the hydrolytic activity. The highest hydrolytic activity of CBL (313.67 Unit/L) yielded 24.16 % FAME in Run 4, whereas the highest FAME content of 56.40 % was obtained from the CBL with relatively lower hydrolytic activity of 223.57 Unit/L in Run 3. To achieve high FAME yield, the whole-cell CBL of *M. spicifer* SPB2 must be propagated in the cultivation medium containing 2 % soybean oil, 0.4 % peptone, and 0.2 % Gum Arabic at an initial pH of 5.

Table 2 L9 (3^4) orthogonal array and results obtained from Taguchi orthogonal array method (L9) for lipase production by *M. spicifer* SPB2.

Run	Factors				Biomass (g/L)	Extracellular lipase activity (U/L)	Cell-bound lipases	
	A	B	C	D			Y_1[b]; Hydrolysis activity (U/L)	Y_2[c]; Transesterification activity (% FAME)
1	2	1	3	2	2.27 ± 0.06	754.07 ± 52.29	111.79 ± 4.42	1.60 ± 0.17
2	1	1	1	1	3.30 ± 0.10	782.98 ± 36.38	112.75 ± 2.50	30.90 ± 1.42
3	3	3	1	2	9.50 ± 0.10	2459.77 ± 58.86	223.57 ± 5.47	56.40 ± 1.26
4	2	3	2	1	5.23 ± 0.06	1392.50 ± 52.29	313.67 ± 1.45	24.16 ± 2.46
5	1	3	3	3	18.43 ± 0.06	1009.44 ± 37.09	185.51 ± 1.67	0.00 ± 0.00
6	3	1	2	3	2.63 ± 0.06	1223.86 ± 35.65	124.31 ± 2.89	11.88 ± 0.77
7	2	2	1	3	12.50 ± 0.17	780.57 ± 14.46	166.23 ± 5.78	0.00 ± 0.00
8	3	2	3	1	1.47 ± 0.06	729.98 ± 40.24	122.39 ± 6.02	1.38 ± 0.22
9	1	2	2	2	14.70 ± 0.17	1763.52 ± 19.12	308.86 ± 7.96	3.43 ± 0.55

[a] Carbon source (A), Nitrogen source (B), Surfactant (C) and Initial pH (D)
[b] CBL and extracellular lipase activity were measured by cupric acetate method with palm oil hydrolysis at pH 7.0, 30 ℃
[c] FAME production by transesterification reaction catalyzed using 1×10^{10} cells of *M. spicifer* in the presence of palm oil and methanol at a molar ratio of 1:3 as substrates.

Table 3 Analysis of lipase production by *M. spicifer* obtained from Taguchi orthogonal array method (L9).

	Y_1; Lipase activity of CBL (U/L)				Y_2; Transesterification activity (% FAME)			
	A	B	C	D	A	B	C	D
k_1[a]	202.37	116.28	167.52	182.94	11.44	14.79	29.10	18.81
k_2	197.23	199.16	248.94	214.74	8.58	1.60	13.16	20.47
k_3	156.76	240.92	139.89	158.68	23.22	26.85	0.99	3.96
R[b]	45.61	124.64	109.05	56.06	14.64	25.25	28.11	16.51
Optimal level	A1	B3	C2	D2	A3	B3	C1	D2
	Palm oil	Peptone	Tween 80	pH 5	Soybean oil	Peptone	Gum Arabic	pH 5

[a] k_i = (Σ the value of one factor at level i) / 3
[b] R = max (k_i) – min (k_i) of one factor

Morphological observation of *M. spicifer* SPB2

Yeasts are eukaryotic organisms that differ in size and shape depending on growth nutrients and environments [27]. The cell size or morphology of *Endomyces magnusii* [28], *Candida utilis* ATCC 9950, and *Saccharomyces cerevisiae* [29] varied when yeast cells were cultivated in the presence of different types and concentrations of substrates. In this study, the correlation between the yeast morphology of *M. spicifer* SPB2 and its CBL transesterification activity was observed. Microscopic observation revealed that the cell morphology of *M. spicifer* SPB2 grown in the media designed according to the Taguchi orthogonal array method (L9) showed highly variable among 9 runs of the experiments as shown in **Figure 1**.

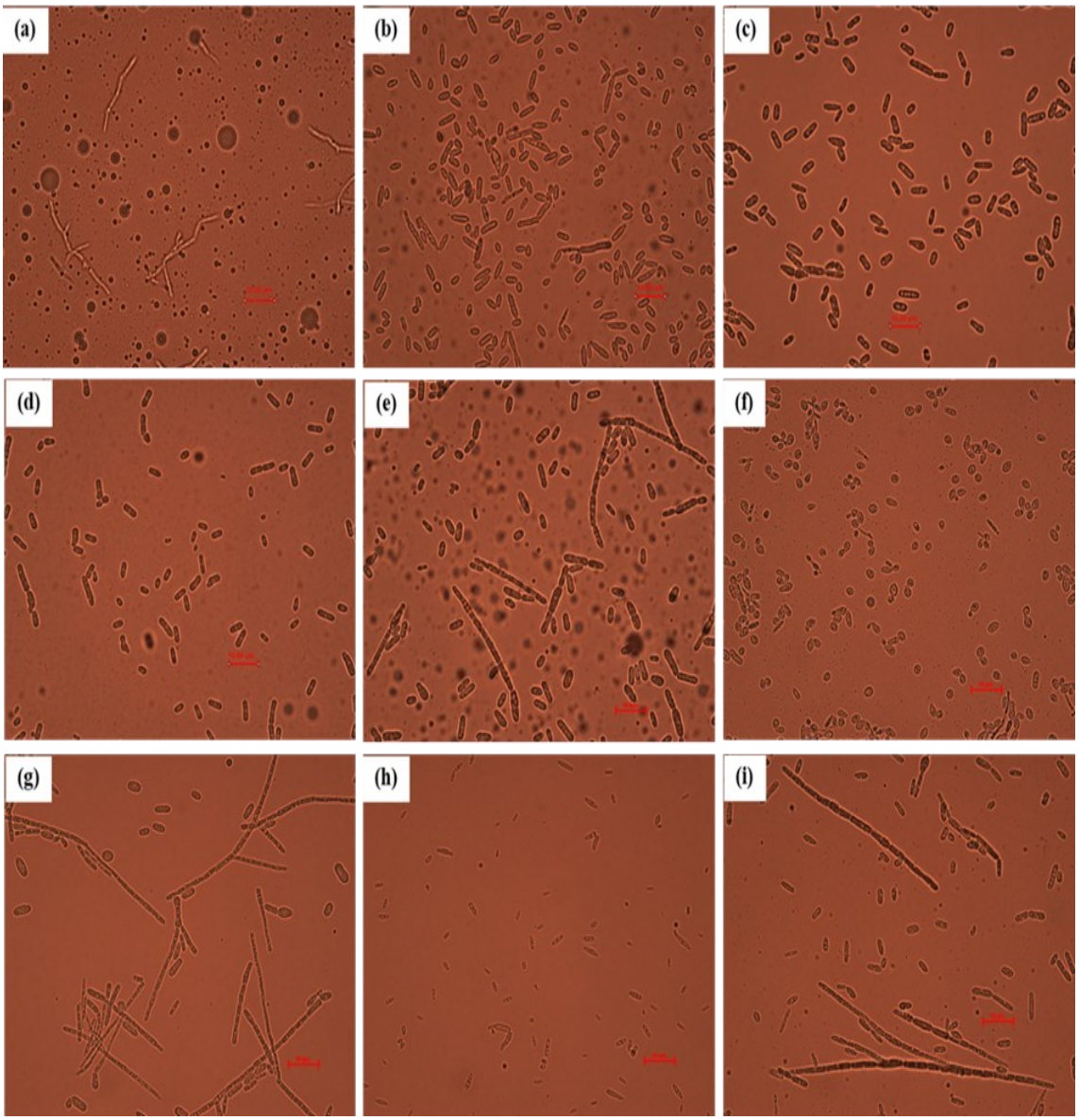

Figure 1 Morphological microscopic examination (Scale bar, 10 µm) observed under magnification of 400× of *M. spicifer* SPB2 grown in the cultivation media containing various carbon sources, nitrogen sources, surfactants and initial pH according to the designed experimental runs. (a) Run 1: Coconut oil, NH_4NO_3, Triton X-100, and pH 5. (b) Run 2: Palm oil, NH_4NO_3, Gum Arabic and pH 4, (c) Run 3: Soybean oil, peptone, Gum Arabic, and pH 5. (d) Run 4: Coconut oil, peptone, Tween 80 and pH 4. (e) Run 5: Palm oil, peptone, Triton X-100, and pH 6 (f) Run 6: Soybean oil, NH_4NO_3, Tween 80, and pH 6. (g) Run 7: Coconut oil, tryptone, Gum Arabic and pH 6 (h) Run 8: Soybean, tryptone, Triton X-100 and pH 4 (i) Run 9: palm oil, tryptone, Tween 80 and pH 5.

Based on yeast morphology observed at 120 h of growth, the morphological characteristics of *M. spicifer* SPB2 could be divided into 3 groups. First, pseudomycelial form of *M. spicifer* SPB2 was detected in Run 1 (**Figure 1(a)**). Second, a mixture of pseudomycelial form and single cells was noted in Runs 5, 7, and 9 (**Figures 1(e), 1(g)** and **1(i)**). Third, only single cells were observed in Runs# 2, 3, 4, 6, and 8 (**Figures 1(b), 1(c), 1(d), 1(f)** and **1(h)**). In the other runs, in which soybean oil was used as the carbon source regardless of the nitrogen source, surfactant, and initial pH of 5, 6 and 4 in the media (Runs# 3, 6, and 8), only single cells were observed. However, *M. spicifer* SPB2 formed a mixture of pseudomycelium and single cells, when palm oil and coconut oil were used as the carbon sources (**Figures 1(a), 1(b), 1(d), 1(e), 1(g)** and **1(i)**).

Regarding the synthetic activity presented in **Table 2**, it seems that single cells of *M. spicifer* SPB2 developed in the media containing soybean oil related to high transesterification activity leading to high production yields of FAME (Runs# 2, 3 and 4). Meanwhile, pseudomycelial forms detected in Runs# 1, 5, 7, 9 (**Figures 1(a), 1(e), 1(g)** and **1(i)**) exhibited extremely low level to none of synthetic activity (see Run# 1, 5, 7, 9 in **Table 2**). Such low activity was as well observed in runs# 6 and 8, in which the smaller cell size of *M. spicifer* SPB2 was developed (**Figures 1(f)** and **1(h)**). This could be caused by lack of essential nutrients for growth as low amount of biomass was determined (see Run# 6, 8 in **Table 2**). In Runs# 2, 3 and 4 in which single cells were developed, FAME yields reached 30.9, 56.4 and 24.16 %, respectively. This was irrelevant to the highest hydrolytic activity (313.67 U/L) of CBL (**Table 2**) observed in Run# 4, in which some elongated single cells were observed. The single cell form of *M. spicifer* SPB2 prepared to be applied for catalyzing palm oil transesterification reaction in Run#3 showed the greatest activity yielding 56.4 % FAME. Their cell surface appeared clearly bold (**Figure 1(c)**), whereas that of the ones from Run# 2 and 4 were more faded (**Figures 1(b)** and **1(d)**). Therefore, certain cell surface characteristics of the single yeast cells contributed to variable effects on the yields of FAME when using whole-cell lipase as the catalyst. These observations strongly highlighted the correlation between morphological and cell surface characteristics of *M. spicifer* SPB2 and transesterification activity.

The interface between the hydrophilic and hydrophobic substrate phases was crucial, when lipase was directly used as a biocatalyst to accelerate transesterification reaction. The morphological form and arrangement of yeast cells was greatly important for biodiesel production using whole-cell lipases. The mixing of heterogeneous substances between oil and methanol was better when using single-cell yeast as whole-cell biocatalyst. The single cells offered greater surface area of cell interface, at which CBL reacted with non-aqueous substrates hence enhancement of substrate availability. The mycelial and elongated forms reduced the cell interface leading to substrate accessibility and limitation in mass transfer [30,31].

Effects of acyl acceptor on synthesis of fatty acid alkyl esters (FAAE)

It is commonly known that excessive alcohol is a major cause of lipase inactivation in biodiesel production. The development of whole-cell biocatalyst was previously proven to overcome this obstacle. In this study, the optimized whole-cell biocatalyst of *M. spicifer* SPB2 catalyzed palm oil transesterification reaction using various alcohol types. FAAE yields of 62.17, 59.68, 73.11, 83.64 and 83.68 % when methanol, ethanol, propanol, 1-butanol, and 2-butanol were applied in the reaction, respectively (**Figure 2**). The FAAE increased with increasing length of the carbon chain of the alcohols [32]. The maximum FAAE yield of 86.80 % was obtained, when iso-butanol was used in the reaction. The results showed that the whole-cells lipase from *M. spicifer* preferred branched alcohol (2-butanol and iso-butanol) to the linear ones (methanol, ethanol, propanol and 1-butanol). The results were not in agreement with the previously reported, in which methanol was more favorable than the longer-chain alcohols for whole-cells lipases developed from *Rhodotorula mucilaginosa* [10], *Kocuria flava* ASU5 [25] and *Bacillus circulans* ASU11 [25]. Moreover, the biodiesel production using longer and branched chain length alcohol provided product advantages of low temperature performance, which is important when using diesel engines in winter or cold climates [33].

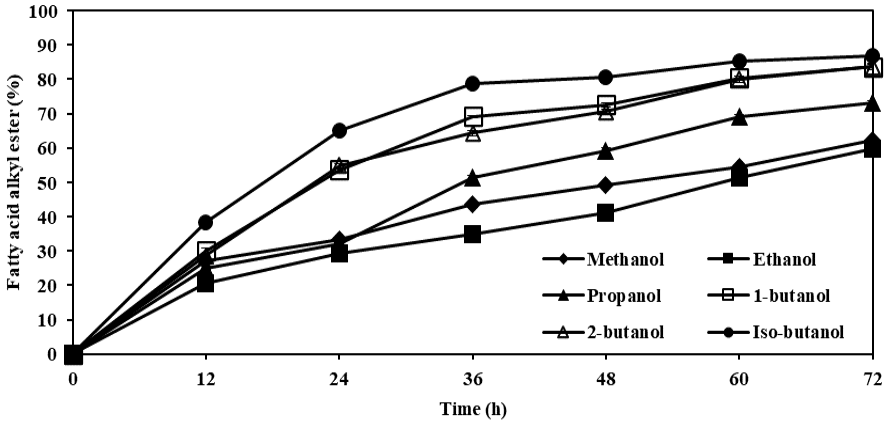

Figure 2 Effect of alcohol types on alkyl ester production *via* transesterification reaction catalyzed by the whole-cell biocatalyst from *M. spicifer* SPB2 with CBL activity of 6.90 unit/g substrate in the presence of palm oil and alcohol at a molar ratio of 1:3 as substrates.

Effects of temperatures on fatty acid butyl ester (FABE) production

The effect of temperature on FABE production *via* transesterification reaction catalyzed by the whole-cells lipase from *M. spicifer* was determined. As presented in **Figure 3**, The percentage of FABE rapidly elevated in 24 h and gradually increased to 86.28 % at the end of reaction period of 72 h when the reaction was performed at 30 ± 2 ℃. Unfortunately, none of FABE was generated at 72 h. 37 and 45 ℃. The optimum temperature of the whole-cell lipase from *M. spicifer* SPB2 is highly specific in catalyzing palm oil transesterification reaction. The inactivation of whole-cells lipase was observed because denaturation of the enzyme possibly occurred at higher temperatures [30,34]. The optimum temperature for FABE production using the whole-cells lipase from *M. spicifer* was 30 ± 2 ℃.

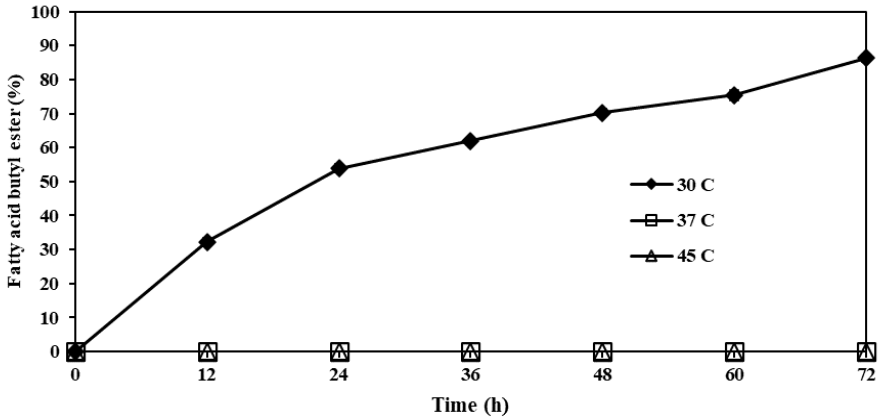

Figure 3 Effects of temperatures on the production of FABE *via* transesterification reaction catalyzed by the whole-cell biocatalyst from *M. spicifer* SPB2 with CBL activity of 7.28 unit/g substrate in the presence of palm oil and iso-butanol at a molar ratio of 1:3 as substrates.

Effects of substrate molar ratios on FABE and FAME production

The influence of palm oil and iso-butanol molar ratio on FABE production by the whole-cell lipase of *M. spicifer* SPB2 was investigated with various molar ratios of 1:3, 1:4, and 1:5. As shown in **Figure 4(a)**, the highest fatty acid butyl ester yield was 86.44 %, when palm oil and iso-butanol molar ratio of 1:3 was introduced as substrates. However, FABE yield dramatically decreased to 47.19 % at the molar ratio to 1:4. At the molar ratio of 1:5, no production of FABE was observed.

Methanol is widely used as an acyl acceptor for FAME production. The impact of palm oil and methanol molar ratio ranging from 1:3 to 1:13 was performed to produce FABE *via* transesterification reaction catalyzed by *M. spicifer* SPB2. As shown in **Figure 4(b)**, the FAME production greatly increased and eventually reached the maximum FAME yield of 93.04 % at the molar ratio of 1:8 at 72 h. FAME yield of 92 % were achieved at 60 h of reaction period. This is because the excess of methanol could promote

good mixing of the reaction mixture. The higher ratio of palm oil to methanol caused the oil substrate to form smaller droplets. This therefore increased overall interfacial area of the entire reaction mixture hence the promotion of collision frequency between the substrates and whole-cell lipases, resulting to a higher transesterification rate and FAME yield. The increments of the molar ratios from 1:9 to 1:13 resulted in significant decrease of FAME yield. This finding strongly suggested the irreversible inactivation of whole-cells lipase occurred by contact with immiscible methanol existing as droplets within the oil [35,36]. The developed whole cell biocatalyst of *M. spicifer* SPB2 exhibited highly methanol tolerant suitable for industrial application.

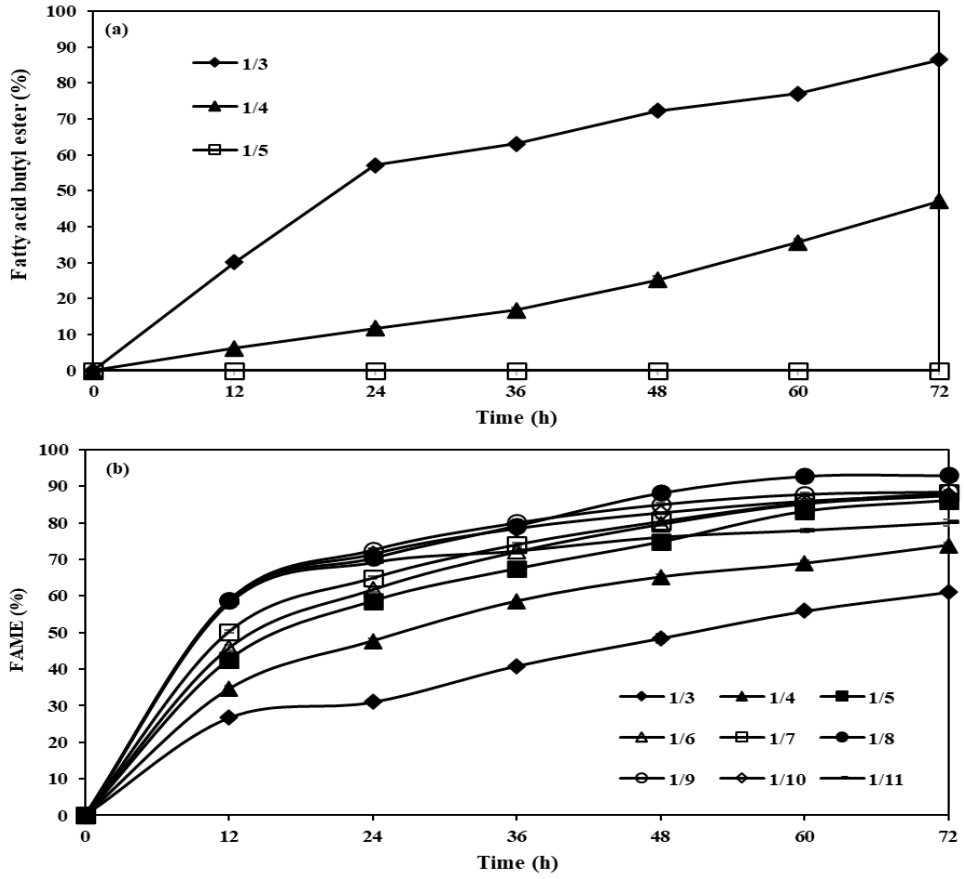

Figure 4 Effects of substrate molar ratios on the production of FABE (a) and FAME (b) *via* transesterification reaction catalyzed by the whole-cell biocatalyst from *M. spicifer* SPB2 with CBL activity of 7.31 unit/g substrate in the presence of palm oil and iso-butanol/methanol as substrates, respectively.

Reusability of the whole-cell biocatalyst from *M. spicifer* SPB2

The whole-cells lipase of *M. spicifer* was reused in FAAE production performed under the optimal conditions (5×10^9 cells per gram of palm oil, 5 % water content, pH 5, palm oil to iso-butanol molar ratio of 1:3 or palm oil to methanol molar ratio of 1:8 and incubated at 30 °C ± 2 °C). The whole-cell biocatalyst was removed from the reaction mixture once the reaction period ended at 72 h and then reused in the next following batch. As shown in **Figure 5(a)**, FABE yield in the second cycle dropped to 57.02 %. In the third and fourth cycles of the reuse, FABE yield decreased to 31.31 and 8.69 %, respectively. When methanol was used as the acyl acceptor (**Figure 5(b)**), FAME yield decreased from 93.86 to 4.32 % in the second batch. This is because a high concentration of methanol and glycerol as one of the by-products of the transesterification reaction may enhance cell surface polarity [36]. Consequently, the distribution of water could also be introduced into the fluid phase, leading to whole-cells lipase deactivation [37]. In the meantime, iso-butanol, a branched alcohol, could dissolve in the oil to a much greater degree than methanol. It then might have reduced the polarity, meanwhile the water still maintained the conformation of lipase enzyme. Therefore, the use of iso-butanol as the acyl acceptor was recommended for the repeated use of the whole-cell biocatalyst developed from *M. spicifer* SPB2

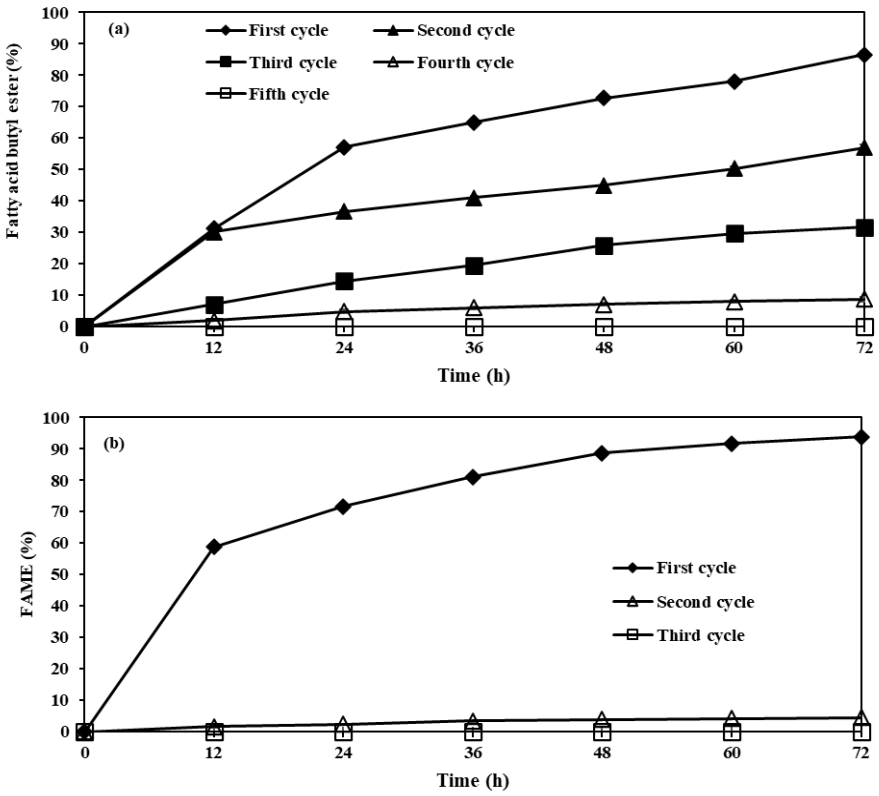

Figure 5 Reusability of the whole-cell biocatalyst from *M. spicifer* SPB2 on FABE production in the presence of palm oil and iso-butanol at a molar ratio of 1:3 (a) and FAME production in the presence of palm oil and methanol at a molar ratio of 1:8 (b) *via* transesterification reaction catalyzed by the whole-cell biocatalyst with CBL activity of 7.08 unit/g substrate.

Conclusions

The whole-cell biocatalyst of *Magnusiomyces spicifer* SPB2 was found to be extremely tolerant to methanol. It can be simply and economically developed during yeast cultivation in the optimized medium containing soybean oil, peptone, Gum Arabic, and an initial pH of 5. The experimental runs designed by the Taguchi orthogonal array method (L9) showed that the medium composition had influence on yeast morphology. Single cell characteristic seems to be the most effective whole-cell biocatalyst for increasing the transesterification activity. The whole-cell biocatalyst from *M. spicifer* SPB2 offered to be an efficient biocatalyst for FAME and FABE production from palm oil with a one-step addition of alcohol in a solvent-free system. However, further development is definitely needed to improve its poor stability in the enzyme recycle. This study demonstrated a simple, economical and convenient method for development methanol tolerant whole-cell biocatalyst with high potential and feasible for industrial application in biodiesel production.

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