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Scale-up Lipase Production and Development of Methanol Tolerant Whole-Cell Biocatalyst from *Magnusiomyces spicifer* SPB2 in Stirred-Tank Bioreactor and Its Application for Biodiesel Production

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Abstract: This study aimed to economically develop the yeast whole-cell biocatalyst from the lipase-secreting *Magnusiomyces spicifer* SPB2 to serve green biodiesel production. The scaled-up productions of lipases were optimized using a 5-L stirred-tank bioreactor. The maximum extracellular lipase and cell-bound lipase (CBL) yields of 1189.65 U/L and 5603.74 U/L were achieved at 24 h and 60 h, respectively, in the modified IMY medium (pH 5.0) containing 2% of soybean oil as a carbon source and 0.2% Gum Arabic as an emulsifying agent. The optimized cultivation was initiated with an inoculum size of 1×10^7 cells/mL and conducted under an aeration rate of 0.75 vvm with an agitation speed of 400 rpm. The obtained whole-cell biocatalyst of *M. spicifer* SPB2 was applied to catalyze the transesterification reaction using palm oil and methanol as substrates. The greatest yield of 97.93% fatty acid methyl ester (FAME) was reached at 72 h using a palm oil/methanol ratio of 1:7, indicating high methanol stability of the biocatalyst. Moreover, substrate homogenization accelerated the reaction to achieve FAME production of 97.01% at 48 h and remained stable afterwards. Without homogenization, the highest FAME of 98.20% was obtained at 60 h. The whole-cell biocatalyst prepared from lipase-secreting *M. spicifer* SPB2 at an up-scaled level greatly enhanced efficiency and feasibility for commercial biodiesel production through a green conversion process.

Keywords: scale-up bioreactor; cell-bound lipase; fatty acid methyl ester; *Magnusiomyces spicifer* SPB2; transesterification reaction

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1. Introduction

Lipase (triacylglycerol acylhydrolases, E.C. 3.1.1.3) is an enzyme with the ability to catalyze various reactions including hydrolysis, acidolysis, aminolysis, esterification (reverse of hydrolysis reaction), and transesterification. The unique properties of the lipase enzyme have remained active in reactions that had organic solvents, yet cofactors are not required for the reaction [1–3]. Lipases are produced by various strains of bacteria, yeasts, and fungi, such as *Yarrowia lipolytica* [4], *Rhizopus arrhizus* [5], *Candida rugosa* [6], *Bacillus salmalaya* [7], *Pseudomonas* spp. [8], *B. subtilis* [9], *R. oryzae* [10], *Aspergillus niger* [11], *A. flavus* [11], *Cystobasidium oligophagum* [12], *Rhodotorula glutinis* [13], *Fusarium incarnatum* [14], and *F. solani* [15]. Among these organisms, yeasts are considered to be excellent lipase producers due to the outstanding properties of the enzymes. They secreted both extracellular and cell-bound lipases (CBL), which can tolerate various extreme physical conditions, including high solvent concentration, low pH, and high osmotic pressure [16].

Apart from its eco-friendly process, biodiesel production through a transesterification reaction catalyzed by lipases exhibited many advantages over acid/alkaline catalysts. These included mild reaction conditions, simple separation, and high quality of biodiesel [1–3]. However, production of biodiesel using lipases at an industrial level is unfeasible due

to high cost, low stability in methanol, and the low conversion and reaction rate of the enzymes. Therefore, cost effective and solvent tolerant lipases are ideal and essential for industrial applications. Many physical, chemical and genetic modifications have been attempted to improve activity, selectivity, stability and reusability of lipases in solvent-free system. These approaches generally required costly complicated steps [2,6,13,14,17].

Application of yeast cells as a lipase-displaying whole-cell biocatalyst is the most simple and economical approach to enhance solvent stability and repeatedly use of the enzymes. Since yeast cell walls have a rigid structure, they are stable in various organic solvents. Cell bound lipase (CBL) can be considered as a form of self-immobilization, which could maintain native conformation and activity of the enzyme. Moreover, CBL-producing yeast cells can be propagated through a conventional standard fermentation system. The obtained yeast cells can be directly used as biocatalysts without additional steps of enzyme purification and immobilization, hence the low production cost [17–21]. For this reason, development of methanol-tolerant lipase-producing yeast as a whole cell biocatalyst could effectively resolve the major technical hurdles in terms of enzyme stability and cost effectiveness, enhancing the feasibility of green biodiesel industrialization.

To date, much research relating to lipase production has focused on CBL-producing yeasts, including *Candida antarctica* lipase B-displaying *Pichia pastoris* [17], *Candida parapsilosis* [18], *Rhodotorula mucilaginosa* [19], *Y. lipolytica* [22], *Magnusiomyces capitatus* [23–25], and *Magnusiomyces spicifier* [21,25]. *M. spicifer* SPB2 was previously reported to secrete both CBL and extracellular lipase. The whole-cell biocatalyst prepared from *M. spicifer* SPB2 demonstrated extremely high methanol tolerant up to 1:8 molar ratio (palm oil to methanol) in solvent-free transesterification reaction, yielding maximum fatty acid methyl ester (FAME) of 93.86%. In the meantime, the biocatalyst was also stable in the presence of iso-butanol and thereby generated high levels of fatty acid butyl ester (FABE), up to 86.80% at the substrate molar ratio of 1:3 [21].

For its potential use at an industrial level, the lipase production as a methanol tolerant whole-cell biocatalyst suitable for biodiesel production through transesterification reaction must be explored at a larger scale. Thus, the scale-up of lipase production by *M. spicifer* in a stirred-tank bioreactor to yield methanol-tolerant whole-cell biocatalysts applied for effective production of fatty acid methyl ester (FAME) through transesterification reaction was the focus of this study. The impacts of various physical and chemical parameters, such as agitation speed, aeration rate, soybean oil concentration, Gum Arabic, and pH control, on lipase production from *M. spicifer* SPB2 were investigated and optimized in a 5-L stirred-tank bioreactor. Furthermore, the scaled-up FAME production via transesterification reaction catalyzing by the obtained whole-cell biocatalyst of *M. spicifer* SPB2 was also evaluated.

2. Results and Discussion

2.1. Effects of Inoculum Size on CBL Extracellular Lipase, and Biomass from M. spicifer SPB2 in Shake-Flask Cultivation

To investigate the effect of initial cells on lipase production, 1×10^5, 1×10^6, and 1×10^7 cell/mL of *M. spicifer* SPB2 were examined in shake-flask level. The lipase enzyme secreted by *M. spicifer* SPB2 was shown to be growth-associated. Considering the results, the increase of initial inoculum level positively supported CBL and biomass production. The highest CBL activity and biomass of 539.17 U/L and 11.77 g/L, respectively, were reached at 48 h (Figure 1a,c) and 144 h when the initial inoculum level of 1×10^7 cell/mL was introduced. On the contrary, the maximum production of extracellular lipase activity of 2004.43 U/L was obtained at 84 h when the inoculum level was 1×10^5 cell/mL. The lower lipase secretion of 1739.42 and 1859.88 U/L was observed when the greater inoculum size of 1×10^6, and 1×10^7 was applied, respectively (Figure 1b). The results were consistent with those of *Aspergillus nidulans* (Mbl-S-6) and *Aspergillus niger* NRRL-599 where extracellular lipase activity decreased with increasing inoculum size. This is because the increased cell mass could reduce nutrient availability [26,27].

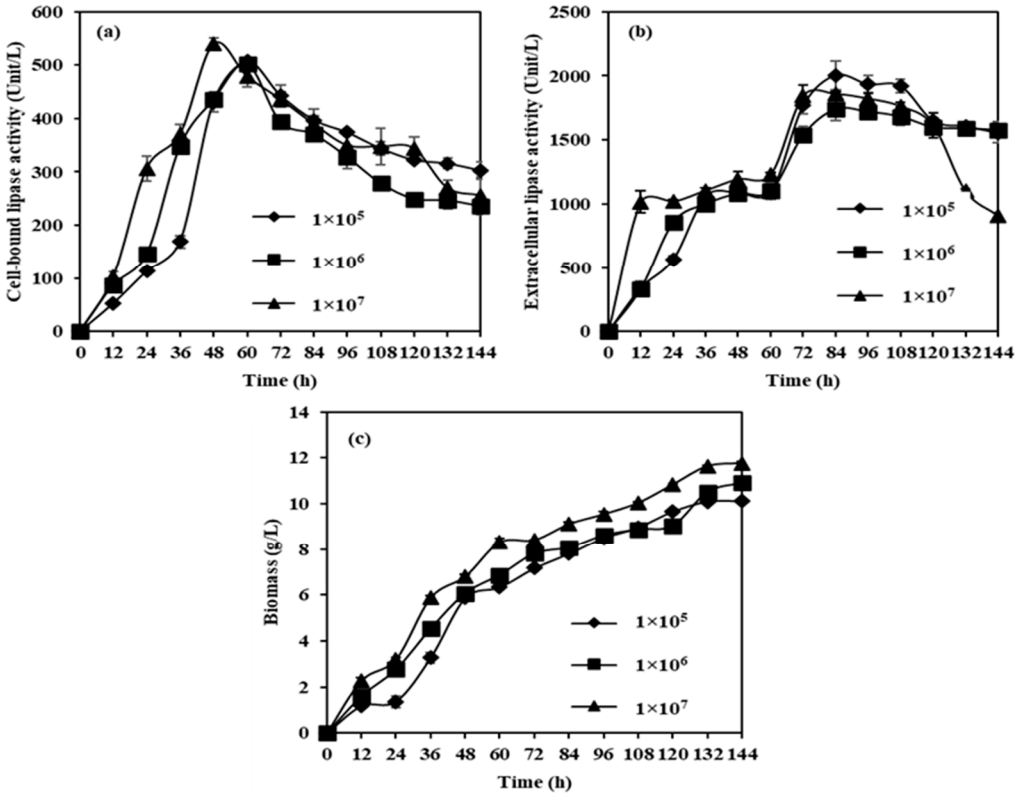

Figure 1. Effects of inoculum levels on production of cell-bound lipase (**a**), extracellular lipase (**b**), and biomass (**c**) of *M. spicifer* SPB2 cultivated in IMY medium containing 2% soybean oil and 0.2% Gum Arabic at 30 ± 2 °C under a shaking speed of 200 rpm.

This could be elaborated by suggesting that *M. spicifer* SPB2 cells possibly produced and accumulated intracellular CBL at the beginning of the cultivation. The CBL enzymes were released into the supernatants thereafter, leading to the increase of extracellular lipase and reduction of CBL [25]. In this study, the whole cell of *M. spicifer* SPB2 was later applied for use as a biocatalyst to accelerate transesterification reaction for the synthesis of fatty acid methyl ester (FAME). Therefore, inoculum size of 1×10^7 cell/mL, which yielded the highest CBL, was selected for the next step in the development of whole-cell biocatalyst.

2.2. Stirred-Tank Bioreactor Operation

2.2.1. Effects of Aeration Rates on CBL, Extracellular Lipase, and Biomass Production from *M. spicifer* SPB2

The effects of aeration rates on CBL, extracellular lipase, and biomass production from *M. spicifer* SPB2 were examined in a 5-L stirred-tank bioreactor using an inoculum level of 1×10^7 cell/mL. The productions of CBL and extracellular lipase from *M. spicifer* SPB2 were improved from 539.17 U/L to 683.72 U/L and from 1859.88 U/L to 3259.61 U/L, respectively, in comparison with those obtained from shake flask cultivation. As shown in Figure 2, the activities of both CBL and extracellular lipases were enhanced with the increase of the aeration rate, which was considered as an important factor for yeast multiplication. The maximum CBL and extracellular of *M. spicifer* SPB2 lipase production of 683.72 U/L and 3259.61 U/L, respectively were achieved at an aeration rate of 0.75 vessel volume per minute (vvm) (Figure 2a,b). At the higher aeration rate of 1.0 vvm, the lipase production of *M. spicifer* SPB2 slightly reduced. The results were consistent with that produced by *Candida cylindracea* previously reported by Salihu et al. [28], of which the highest lipase production of *C. cylindracea* reached 41.46 U/mL at the high aeration rate of 1.0 vvm applied in a stirred-tank bioreactor.

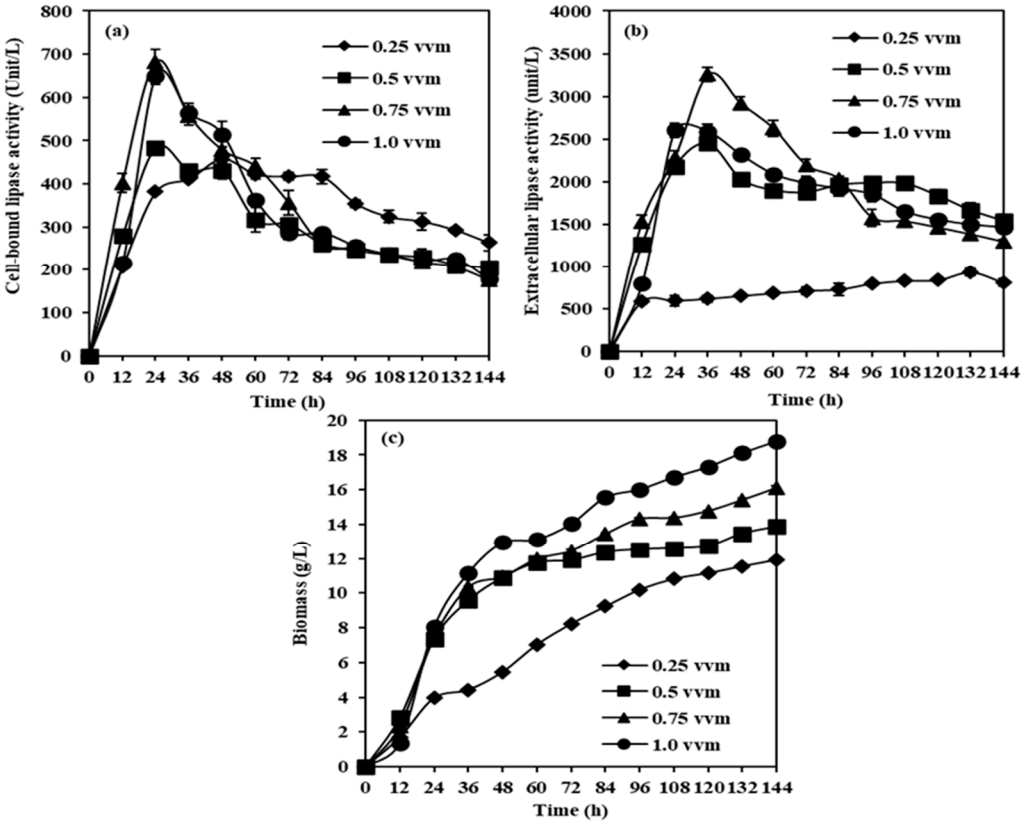

Figure 2. Effects of aeration rate on the production of CBL (**a**), extracellular lipase (**b**), and biomass (**c**) from *M. spicifer* SPB2 cultivated in IMY medium containing: 2% soybean oil and 0.2% Gum Arabic using inoculum level of 1×10^7 cell/mL at 30 ± 2 °C under agitation speed of 200 rpm.

Regarding *M. spicifer* SPB2, the increase of aeration rate to 1.0 vvm caused a significant elevation of its biomass production from 11.77 g/L in the shake flask method to 16.10 g/L in the 5 L bioreactor (Figure 2c). This is because the aeration rate applied for the 5-L stirred-tank bioreactor system corresponds to the greater level of dissolved oxygen incorporated into the culture medium, which is crucial for yeast proliferation. With a low aeration rate of 0.25 vvm, the lowest yeast biomass of 3.96 g/L was shown at 24 h. This reflected the limitation of oxygen, resulting in obstruction of metabolic respiration, hence the slow growth of yeast and the reduction of CBL and extracellular lipase production.

2.2.2. Effects of Agitation Speeds on CBL, Extracellular Lipase, and Biomass Production from *M. spicifer* SPB2

Generally, submerged fermentation has been performed using a stirred-tank bioreactor to increase the production of microbial enzymes in large quantities. Cultivation in a stirred-tank bioreactor comprises several parameters that can influence the lipase enzyme production. Since many yeasts are obligate aerobes requiring oxygen for growth, the aeration rate and agitation speed influence the oxygen transfer for yeast growth and metabolism. Shear force, which is the result of agitation speed, can also affect the cell morphology or damage the structure of the yeast cell [29]. In this study, the effects of agitation speed on CBL, extracellular lipase, and biomass production from *M. spicifer* SPB2 were examined under the optimal aeration rate of 0.75 vvm. The increase of agitation sharply elevated yeast growth and lipase production as shown in Figure 3. The maximum CBL and extracellular lipase activities of 1175.20 U/L and 5517.01 U/L were achieved at 24 h and 60 h, respectively, with an agitation speed of 400 rpm (Figure 3a,b). The biomass production reached a maximum of 17.90 g/L at 108 h with 300 rpm agitation speed (Figure 3c).

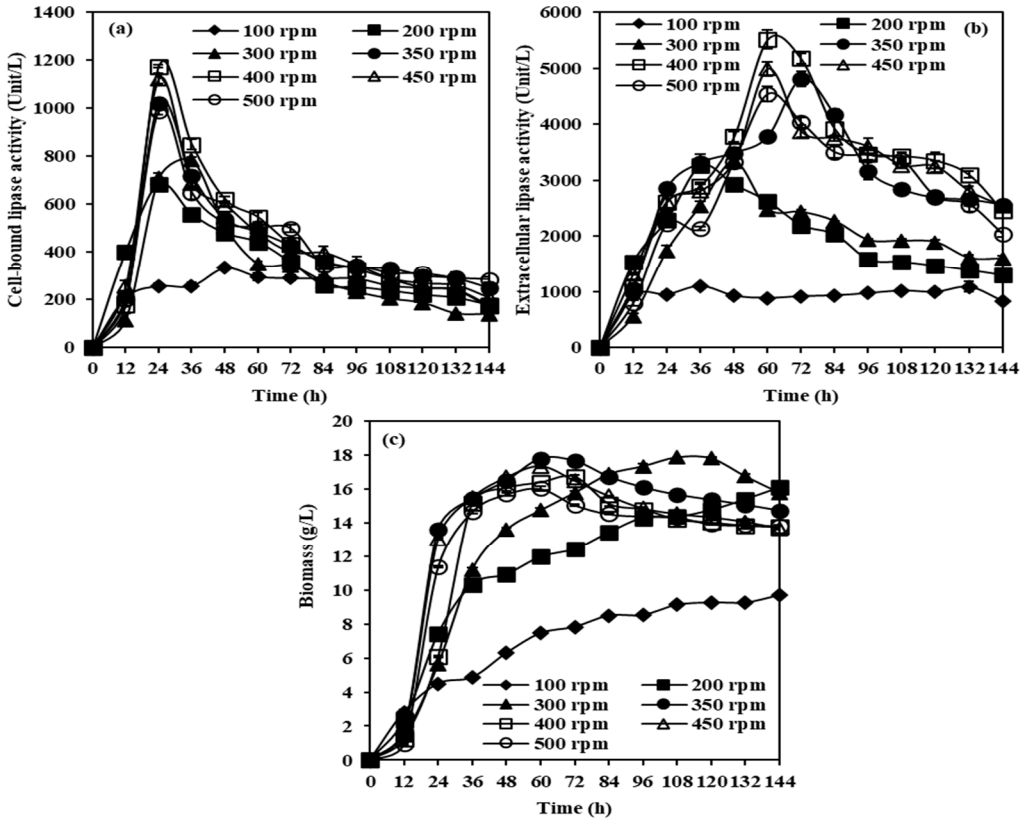

Figure 3. Effects of various agitation speeds on CBL (**a**), extracellular lipase (**b**), and biomass (**c**) production from *M. spicifer* SPB2 in IMY medium containing 2% soybean oil and 0.2% Gum Arabic, using an inoculum level of 1×10^7 cell/mL at 30 ± 2 °C under an aeration rate of 0.75 vvm.

In a corresponding manner, *Candida cylindracea* reached the highest lipase titer in a 30-L fermenter with an aeration rate of 1 vvm at an agitation speed of 400 rpm within a short fermentation process [28]. The lipase secretion of *Rhodotorula mucilaginosa* MTCC 8737 was maximized at an aeration rate and agitation speed of 2 vvm and 200 rpm, respectively. The better oxygen transfer through the optimal agitation and aeration contributed to the increasing productivity [30]. In addition, the greater agitation speed facilitated and enhanced the mixing efficiency of heterogeneous substrate between soybean oil and water, introducing higher oxygen mass transfer into the medium. On the contrary, significant reduction of growth and lipase production could occur at higher agitation speeds due to shearing effects on the microbial cells [28,30]. Therefore, the appropriate aeration rate and agitation speed are the necessary and essential fermentation parameters to be evaluated and controlled. For *M. spicifer* SPB2, the agitation speed of 400 rpm showed the increment of CBL, extracellular lipase, and biomass from 683.72 U/L, 3259.61 U/L, and 16.10 g/L to 1175.20 U/L, 5517.01 U/L and 16.73 g/L, respectively.

2.2.3. Effects of Soybean Oil Concentrations on CBL, Extracellular Lipase, and Biomass Production from *M. spicifer* SPB2

Soybean oil is an essential substrate required for inducing lipase production. Biomass, CBL and extracellular lipase production by *M. spicifer* SPB2 varied depending on the soybean oil concentration. The highest CBL production (1180.50 U/L) was observed at 24 h (Figure 4a), while the highest extracellular lipase production (5567.60 U/L) was detected at 60 h, when 2% soybean oil was introduced (Figure 4b). At 3% soybean oil, the biomass production reached its highest value, 20.47 g/L at 108 h (Figure 4c). In line with Colin [31], the highest lipase activity was achieved when using 2% olive oil, while the maximum biomass was obtained when using 3.5% olive oil. In contrast, CBL and extracellular lipase production decreased at higher concentrations of soybean oil. Such patterns were consistent

with that observed by Colla et al. [11]. The increase of soybean oil concentration (1–3%) decreased the lipase activity of *Aspergillus flavus*.

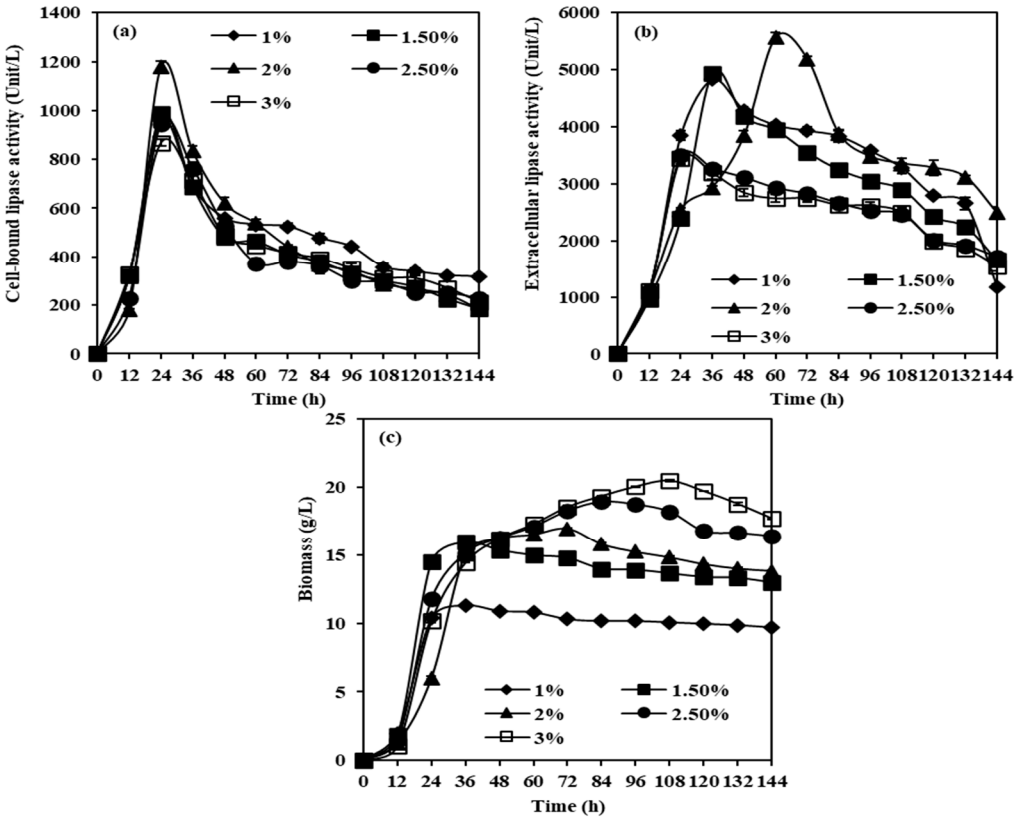

Figure 4. Effects of various concentrations of soybean oil on CBL (**a**), extracellular lipase (**b**), and biomass (**c**) production from *M. spicifer* SPB2 in the IMY medium containing 0.2% Gum Arabic, using an inoculum level of 1×10^7 cell/mL at 30 ± 2 °C under an aeration rate of 0.75 vvm and a stirring speed of 400 rpm.

The amount of oil substrate used as a carbon source can either enhance or inhibit lipase synthesis. Therefore, the appropriate amount of oil required in the medium for maximum lipase synthesis is strain-specific [31,32]. Apart from being an energy or carbon source to support cell growth, both lipid, oil and fatty acids substrates, such as soybean oil, olive oil and oleic acid, also acted as lipase inducers [31,33]. Soybean oil seems to be the most preferable inducer for lipase production by many yeasts and fungi. This could be due to its high content of unsaturated long-chain fatty acids. In particular, oleic and linoleic acids were the most effective inducers for lipase production [20,21,33–36]. Regarding this study, the appropriate concentration of 2% soybean oil yielded the maximum CBL and extracellular lipase production of *M. spicifer* SPB2 in a 5 L bioreactor. Such concentration provided the improvement of CBL, extracellular lipase, and biomass from 1175.20 U/L, 5517.01 U/L and 16.73 g/L to 1180.50 U/L, 5567.60 U/L and 16.97 g/L, respectively.

2.2.4. Effects of Gum Arabic on CBL, Extracellular Lipase, and Biomass Production from *M. spicifer* SPB2

Additions of many surfactants, particularly Triton X-100, Tween 80, polyethylene glycol 200, and Gum Arabic, were shown to generally promote extracellular lipase activity [36,37]. According to the previous study, Gum Arabic was the most essential surfactant for developing lipase whole-cell biocatalyst from *M. spicifer* SPB2 [21]. For a comparative study, the scaled-up cultivation of *M. spicifer* SPB2 was carried out in a 5 L bioreactor with and without Gum Arabic. The highest CBL of 1192.54 U/L (Figure 5a), extracellular lipase of 5464.01 U/L (Figure 5b), and biomass production of 16.80 g/L (Figure 5c) were obtained

in the presence of Gum Arabic. The CBL and extracellular lipase activities significantly increased up to 1.45-fold and 2.08-fold, respectively.

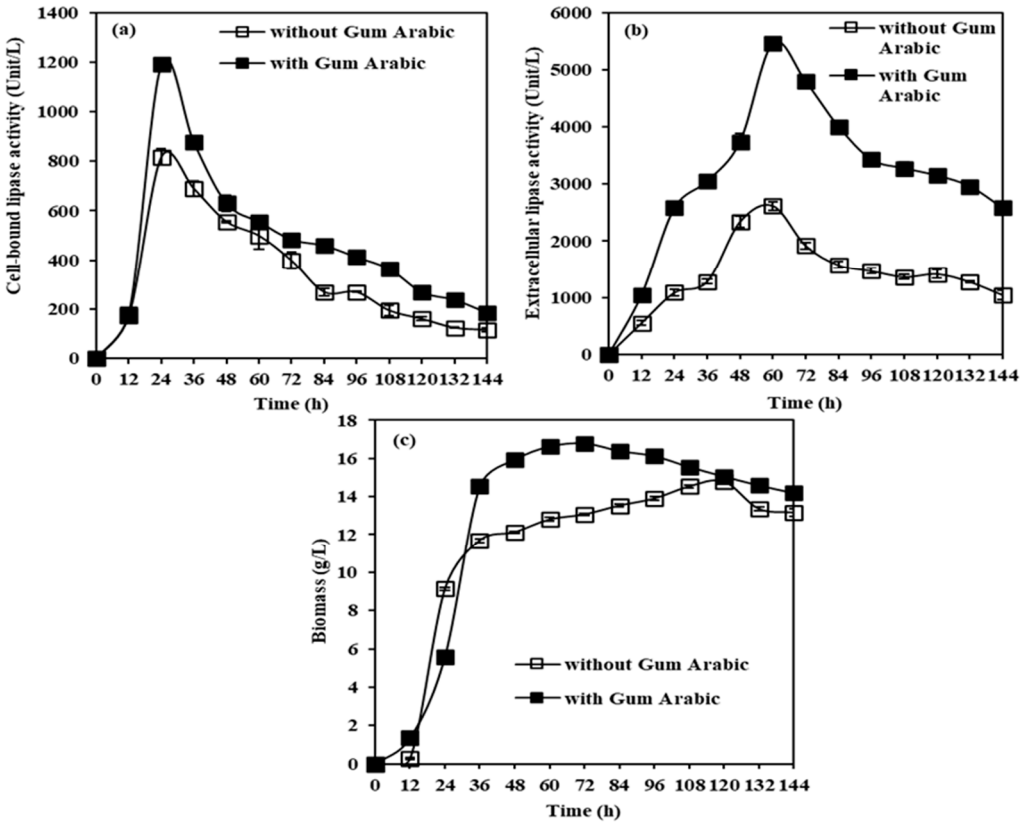

Figure 5. Effect of Gum Arabic (0.2%) on CBL (**a**), extracellular lipase (**b**), and biomass (**c**) production from *M. spicifer* SPB2 in the IMY medium containing 2% soybean oil using inoculum level of 1×10^7 cell/mL at 30 ± 2 °C, under an aeration rate of 0.75 vvm and stirring speed of 400 rpm.

Correspondingly, Gum Arabic played the greatest role in transesterification activity of whole-cell biocatalyst from *Rhodotorula mucilaginosa* P11I89, whereas its extracellular lipase was significantly induced by Tween-80 [20]. Gum Arabic could stabilize the interface of oil micelles in water phase due to its low HLB (hydrophile-lipophile balance) value. With appropriate agitation speed, interface area between oil and aqueous phases consequently enhanced the contact of yeast cells to oil substrate [20,38]. Gum Arabic also had the highest impact on lipase production of *Yarrowia lipolytica* W29 using waste cooking oil as a carbon source [39]. According to previous observations, the synthetic activity of CBL from *R. mucilaginosa* P11I89 was, remarkably, induced by adding Gum Arabic [20]. This supported the supposition that Gum Arabic was suitable to be used as an emulsifier that enhances CBL and extracellular lipase production for *M. spicifer* SPB2.

2.2.5. Effects of Initial pH for Cultivation on CBL and Extracellular Lipase Activity from *M. spicifer* SPB2

At an initial pH of 5.0, the secretion of CBL and extracellular lipases as well as biomass production of *M. spicifer* SPB2 were significantly greater than those obtained at pH of 7. The maximum hydrolytic CBL and extracellular lipase activities of 1206.51 U/L (Figure 6a) and 5538.69 U/L (Figure 6b), respectively were obtained at the fermentation periods of 24 h and 60 h, respectively. Moreover, the maximum biomass reached 17.00 g/L at 72 h (Figure 6c). This also confirmed that its lipase production was growth-associated. During fermentation, pH reduction was observed (Figure 6d) due to the generation of free fatty acids from soybean oil hydrolysis.

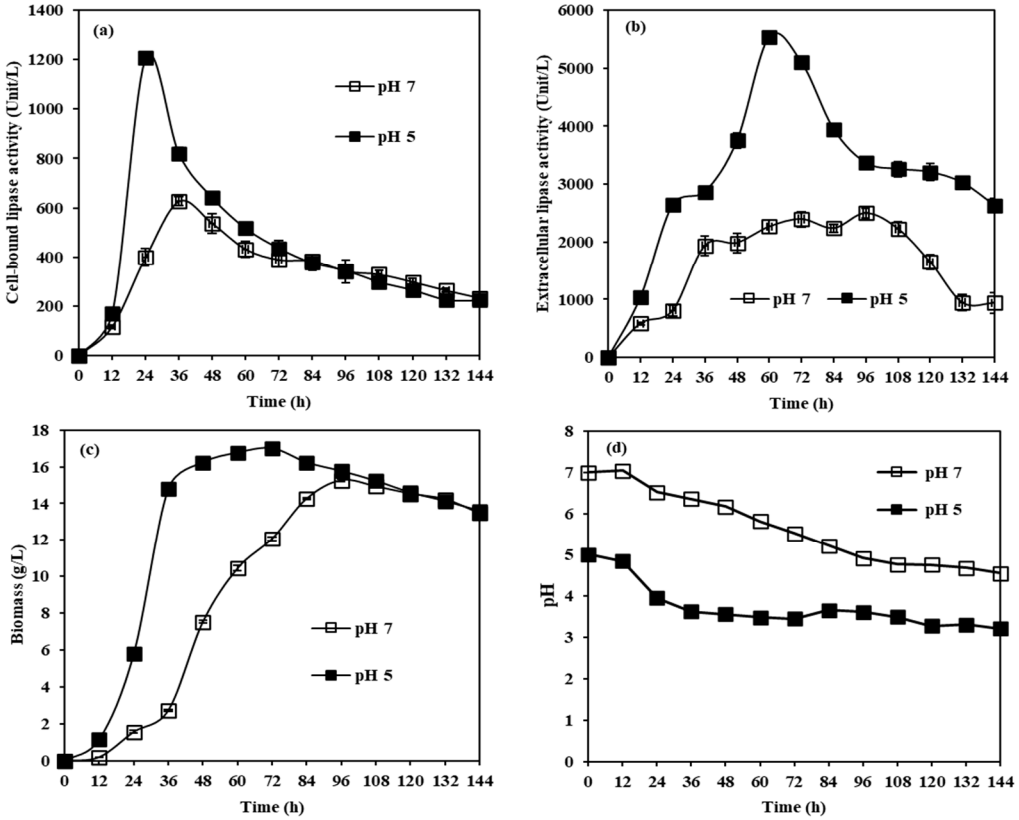

Figure 6. Effects of initial pH of culture medium on CBL (**a**), extracellular lipase (**b**), and biomass (**c**) production, and pH value of culture medium (**d**) from *M. spicifer* SPB2 in the IMY medium containing 2% soybean oil and 0.2% Gum Arabic, using an inoculum level of 1×10^7 cell/mL at 30 ± 2 °C under an aeration rate of 0.75 vvm and a stirring speed of 400 rpm.

Therefore, the acidic medium (pH 5.0) was more favorable for *M. spicifer* SPB2 growth and lipase production. This offers better advantage in controlling and preventing bacterial contamination in industrial large-scale fermentation, wherein a fully contamination-free process and complete sterilization are too laborious to economically accomplish. Several previous reports indicated that the pH of the medium could influence lipase production [20,21,26,32,40]. Lipase-producing microorganisms required different initial pH in the fermentation medium. *Aspergillus oryzae* and *Geotrichum candidum* favored initial pHs of 5.5 and 7.0, respectively [35,41]. Much related research suggested that pH alteration of microenvironment was essential for the movement of the lipase lid, thereby affecting catalytic behavior of the enzyme and its activity [42].

2.2.6. CBL, Extracellular Lipase, and Biomass Production from *M. spicifer* SPB2 under the Optimized Condition of Cultivation in a 5 L Stirred-Tank Bioreactor

The optimized IMY medium (pH 5) for cultivating *M. spicifer* SPB2 in the 5-L stirred-tank contained 2% soybean oil and 0.2% Gum Arabic as the essential carbon source and surfactant, respectively. The cultivation was performed using an initial inoculum of 1×10^7 cell/mL at 30 ± 2 °C under an aeration rate and an agitation speed of 0.75 vvm and 400 rpm, respectively. The maximum biomass of 16.83 g/L was accomplished at 72 h as shown in Figure 7. In the meantime, the greatest CBL activity of 1189.65 U/L was expressed at 24 h fermentation, which was the exponential stage of yeast growth. The extracellular lipase was secreted to the highest level of 5603.74 U/L at 60 h, at which yeast growth entered the stationary phase. The CBL production of *M. spicifer* SPB2 increased during the early stage of growth whereas extracellular lipase was secreted abundantly into the medium during the stationary stage. In comparison to the shake flask experiment (Figure 1a,b), the titers of CBL and extracellular lipase in the 5-L stirred-tank bioreactor increased from 539.17

to 1189.65 U/L and 1859.88 to 5603.74 U/L, respectively. Most remarkably, the production duration significantly decreased from 48 to 24 h for CBL and 84 to 60 h for extracellular lipase (Figure 7). The whole-cell lipase harvested at 24 h was then used as the biocatalyst to catalyze transesterification reaction in the next experiment.

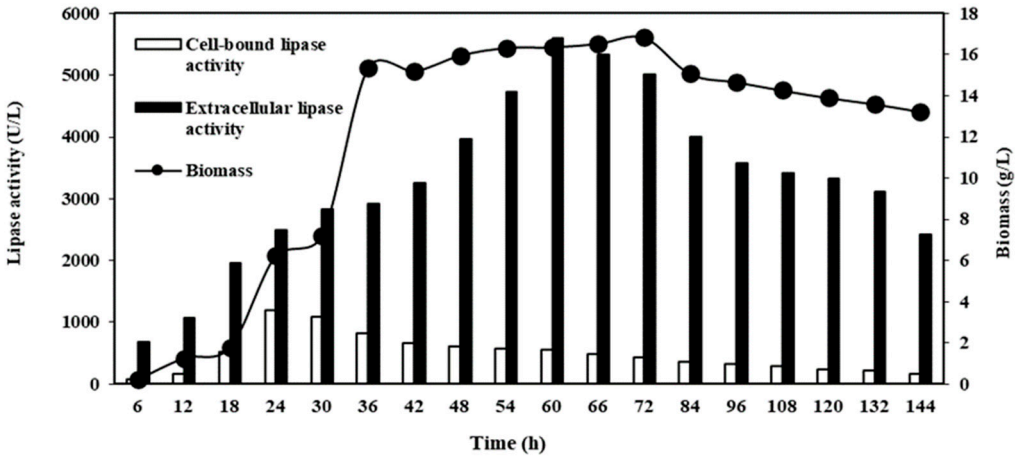

Figure 7. Time courses of CBL, and extracellular lipase, and biomass production from *M. spicifer* SPB2 in a 5-L stirred-tank bioreactor with the IMY medium containing 2% soybean oil and 0.2% Gum Arabic, using an inoculum level of 1×10^7 cell/mL at 30 ± 2 °C under an aeration rate of 0.75 vvm and a stirring speed of 400 rpm.

These beneficial impacts were contributed by aeration rate and agitation rate being applied to the up-scaled fermentation system. These factors consequently introduced oxygen continuously into the system. Moreover, the mixing efficiency to enhance interface contact of oil substrate and Gum Arabic was greatly improved in the stirred-tank bioreactor, resulting in the faster and higher production of CBL, extracellular lipase, and biomass than is obtained in the shake flask method. These results are consistent with a previous study, which an increased aeration rate, agitation speed, and Gum Arabic concentration influenced lipase production by *Y. lipolytica* strain W29 [39].

2.3. FAME Production Using Whole-Cell Lipase from M. spicifer SPB2 Cultured in a Bioreactor

The efficiency of FAME production using the whole-cell biocatalyst from *M. spicifer* SPB2 prepared from the up-scaled preparation in the stirred-tank bioreactor to catalyze palm oil by transesterification reaction was evaluated and examined. Interestingly, the obtained biocatalyst from *M. spicifer* SPB2 appeared to be highly methanol-tolerant as shown in Figure 8. Such solvent tolerant ability provides great technical advantage for the lipase catalyzing synthetic reaction. The excess methanol could reduce the viscosity of the reaction mixture and therefore enhance the mass transfer, resulting in an increased FAME yield. Regarding the whole-cell biocatalyst prepared from *M. spicifer* SPB2, the highest FAME yield reached 97.93% at 72 h when a 1:7 molar ratio of palm oil and methanol was used as a substrate. This is in good agreement with the methanol-tolerant biocatalyst prepared from whole-cell *Rhodotorula mucilaginosa* P11I89, which yielded about 93% of FAME at a 1:6 molar ratio of palm oil and methanol [20]. Inconsistently, the whole-cell biocatalyst from *M. spicifer* AW2 yielded 81.2% of FAME through transesterification reaction at 24 h and surprisingly dropped to <25% at 48 h, when 1:3 molar ratio of *Jatropha curcas* oil and methanol was used as a substrate [25].

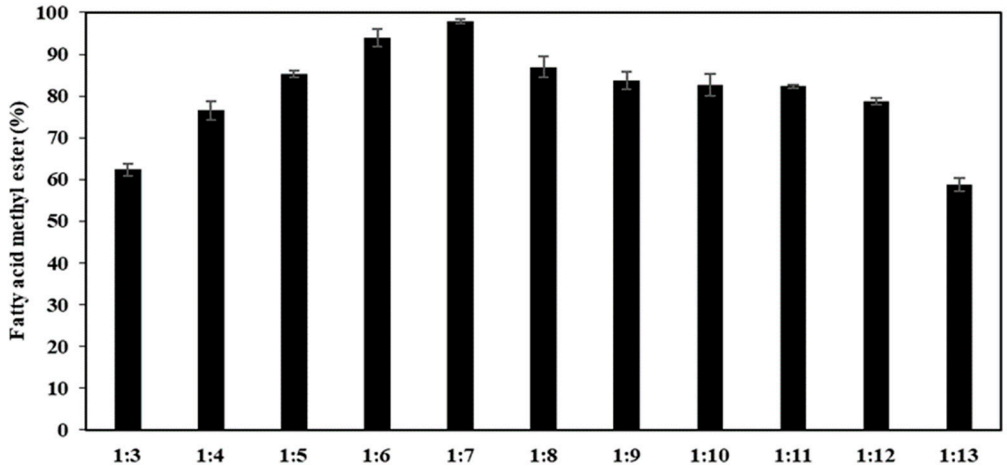

Figure 8. Effects of the ratio of the molar ratio on FAME production in the transesterification reaction using a 2 mL microcentrifuge tube catalyzed by *M. spicifer* SPB2 cells from a 5-L stirred-tank bioreactor. This was in the presence of palm oil and methanol, as substrates with 5×10^9 of yeast cells per gram substrate, incubated at 30 ± 2 °C in a vigorous shaker for 72 h.

The up-scaled transesterification reaction was carried out in a 20 mL vial. Palm oil was homogenized at a speed of 8000 rpm for 20 min before being used as a substrate in the transesterification reaction. Homogenization is a mechanical process to reduce the molecular size and viscosity of palm oil [43]. FAME production was facilitated by this palm oil pretreatment (Figure 9). At 24 h of the reaction, FAME yield of 75.17% was achieved, which was 1.34-fold higher than the yield of 56.20% generated using non-homogenized palm oil as a substrate. At 48 h, FAME yield of 97.01% was observed with homogenized substrate, while that using an unpretreated one yielded <85% and then slowly culminated to 98.2% at 60 h. The result indicated that the pretreatment of palm oil with homogenization increased the production of FAME in the early stages of the transesterification reaction when whole-cell lipase from *M. spicifer* SPB2 was used as the biocatalyst.

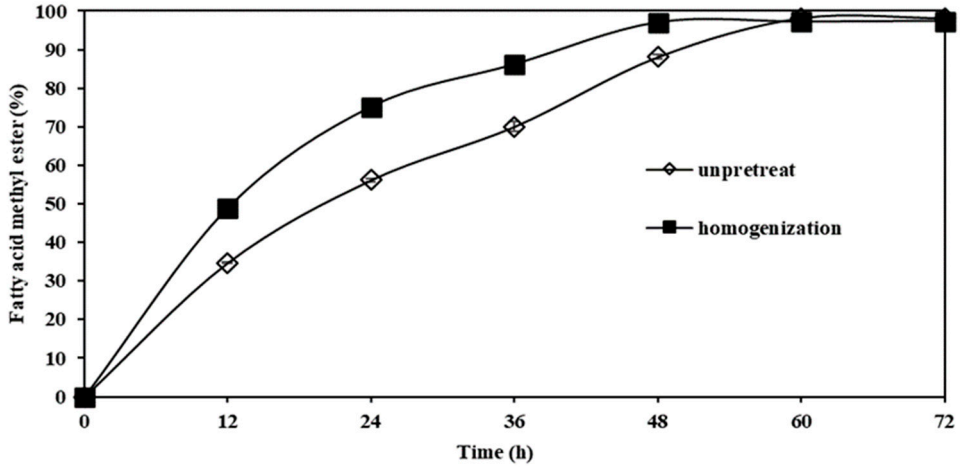

Figure 9. The scale-up of biodiesel production in a 20 mL vial and the effect of palm oil pretreatment by homogenization before using as a substrate in biodiesel production using *M. spicifer* SPB2 cells from a 5-L stirred-tank bioreactor 5×10^9 of yeast cells per gram substrate in the presence of palm oil and methanol with a molar ratio of 1:7, incubated at 30 ± 2 °C for 72 h.

This scale-up of FAME production produced FAME in quantities as high as those observed in the small-scale experiment, confirming the consistent performance of this developed whole-cell biocatalyst from *M. spicifer* SPB2. Moreover, the homogenization of palm oil could shorten the reaction period from 72 h to 48 h and 60 h compared with

the small-scale and the unpretreated palm oil counterparts, respectively. Consistently, the whole cell biocatalyst prepared from *R. mucilaginosa* P11I89 produced 83.3% FAME at 72 h via transesterification reaction using palm oil and methanol as substrates [19]. Further cultivation improvement of this particular biocatalyst could shorten production period to 48 h to achieve the same level of FAME yield, which eventually increased up to 92.98% at 72 h of reaction period [20].

3. Materials and Methods

3.1. Microorganism, Cultivation and Inoculum Preparation

Magnusiomyces spicifer SPB2 deposited at the TISTR Culture Collection Center (Bangkok, Thailand) was maintained in glycerol stocks and stored at −80 °C. The yeast inoculum was grown in 250 mL Erlenmeyer flasks containing 100 mL of YM medium consisting of 1% glucose, 0.5% peptone, 0.3% yeast extract, and 0.3% malt extract. The inoculated flask was incubated at 30 ± 2 °C for 72 h on a rotary shaker with a shaking speed of 200 rpm.

3.2. Effects of Inoculum Size on Lipase Production from M. spicifer SPB2

Lipase production was carried out with 1×10^5, 1×10^6, and 1×10^7 cell/mL of inoculum size prepared from *M. spicifer* SPB2, according to the method of Srimhan and Hongpattarakere [21]. Each inoculum level was introduced into a 250 mL Erlenmeyer flask filled with 100 mL of IMY medium containing 2.0% soybean oil, 0.4% peptone, 0.2% Gum Arabic, 0.47% KH_2PO_4, 0.03% $Na_2HPO_4 \cdot 12H_2O$, 0.1% $MgSO_4 \cdot 7H_2O$, 0.01% yeast extract, 0.001% $FeSO_4 \cdot 7H_2O$, 0.001% $CaCl_2 \cdot 2H_2O$, and 0.001% $MnSO_4 \cdot 4H_2O$ The flask was then incubated at 30 °C at a shaking speed of 200 rpm. The initial pH of the IMY medium was adjusted to pH 5.0. The fermentation sample (12 h sampling interval) was drawn and centrifuged at 10,000 rpm and 4 °C for 10 min. The cell supernatant and pellet were then subjected to the respective determination of the extracellular lipase and CBL activities using cupric acetate method [19]. The biomass production was estimated based on Srimhan and Hongpattarakere [21]. In brief, the obtained cell pellet from centrifugation of 1 mL culture broth was washed twice using sterile distilled water before drying at 105 °C until a constant weight was gained.

3.3. Stirred-Tank Bioreactor Operation

The experiments were carried out in a 5-L stirred-tank bioreactor (MDL-300, B. E. Marubishi, Chiba, Japan) with a working volume of 4.0-L culture medium sterilized at 121 °C for 15 min. The fermentation parameters were controlled by a digital control system. Four levels of airflow rates at 0.25, 0.5, 0.75, and 1.00 vvm were examined. The effects of agitation speeds were assessed at 100, 200, 300, 350, 400, 450, and 500 rpm. The impact of soybean oil concentration was observed at 1.0%, 2.0%, 3.0%, 4.0%, and 5.0% (*w*/*v*). Additionally, the presence of Gum Arabic in the medium was examined in comparison with the absence one. The effect of initial pH was investigated through two different processes setting initial pH at 5.0 and 7.0, respectively. The fermentation broth was then drawn every 12 h to determine for biomass (dry cell weight), CBL, and extracellular lipase activity via a two-phase emulsion method every 12 h. Finally, the reproducibility of the batch fermentation was repeated in triplicate of each analyzed sample.

3.4. Transesterification Reaction for Biodiesel Production

3.4.1. Effects of Palm Oil and Methanol Molar Ratios on FAME Yield Produced through Transesterification Reaction Catalyzed by Whole-Cell Biocatalyst from *M. spicifer* SPB2

The whole-cell biocatalyst optimally prepared from *M. spicifer* SPB2 cultivated in a 5 L stirred-tank bioreactor with the highest CBL activity was used to catalyze a palm oil transesterification reaction for FAME production. The effects of palm oil/methanol at various molar ratios of 1:3–1:13 were examined. The numbers of yeast cells required for the reaction were enumerated using a hemocytometer. To harvest the cells, the culture broth was centrifuged at 4 °C for 10 min at 10,000 rpm. The yeast pellet was then resuspended

in 0.1 M citric acid buffer pH 5.0 to achieve the final cell count of 5×10^9 cells per gram substrate with 5% aqueous phase in the reaction mixture. The transesterification reactions were carried out at 30 ± 2 °C for 72 h in a 2 mL microcentrifuge tube under vigorous shaking at the maximum speed of the Vortex Gennie Mixer-2 (Scientific Industries, New York, NY, USA).

3.4.2. Effect of Homogenization Pretreatment on FAME Yield Produced through Transesterification Reaction Catalyzed by Whole-Cell Biocatalyst from *M. spicifer* SPB2

The scale-up of FAME production was carried out using a 20 mL vial under vigorously stirring at the maximum speed of the Whiteman Magnetic Stirrer Model D12-160 (Whiteman, Bangkok, Thailand). The palm oil was pretreated by homogenization before being used as a substrate in FAME production. The homogenization was performed using the Ika-homogenizer (T25 basic Ika Labortechnik, Staufen, Germany) at the speed of 8000 rpm for 20 min. The transesterification reactions were carried out at 30 ± 2 °C for 72 h. Finally, the reaction mixture was drawn every 12 h for FAME analysis.

3.5. Determination of Lipase Activity

CBL and extracellular lipase activities were determined using the cupric acetate method [19]. The cupric acetate solution (5%, *w*/*v*) was prepared, and the pH was adjusted to 6.1 by adding pyridine. The two-phase system of substrate emulsion generated between cupric acetate solution (0.4 mL) and 10% palm oil in iso-octane (1 mL) was mixed with either the yeast cells suspended in 0.2 M phosphate buffer at pH 7.0 or the culture supernatants to determine the CBL or the extracellular lipase activity, respectively. The assay was conducted at 30 °C under a shaking speed of 300 rpm for 30 min. Then, 0.3 mL of 6 M HCl was added to stop the enzyme reaction. The upper layer of the mixture (0.1 mL) was drawn and diluted to 1 mL with iso-octane before mixing with 0.4 mL of cupric acetate solution. Free fatty acid dissolved in the iso-octane was determined by measuring the absorbance at 715 nm against the control, which contained no free fatty acid using GENESYS 10S Series UV-Visible Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). The hydrolytic lipase activity was determined by measuring the amount of fatty acid drawn from the standard curve of palmitic acid established prior the analysis 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 assay condition specified above.

3.6. Determination of Biomass Production

For the dry cell weight determination, 1 mL of the culture sample was transferred to a pre-weighed microcentrifuge tube and centrifuged at 4 °C for 10 min at 10,000 rpm. The yeast pellet was thoroughly washed twice with distilled water and allowed to dry at 105 °C until a constant weight was obtained.

3.7. Gas Chromatography Analysis for FAME

Gas chromatography with flame ionization detection (Hewlett Packard Plus 6850 series, Santa Clara, CA, USA) using the Agilent Varian Series GC capillary column of the Selected Biodiesel for FAME with 30 m × 0.32 mm, 0.25 µm film thickness (Agilent, Santa Clara, CA, USA,) equipped with flame ionization detector (FID) was used to determine the FAME product from the cell-bound lipase-catalyzed transesterification reaction of palm oil with methanol. The temperature of the column was initially maintained at 210 °C for 12 min, after which the temperature was raised to 250 °C at a rate of 20 °C/min and held for 8 min. Meanwhile, the temperature of the detector was maintained at 300 °C. Quantitative analysis of the weight percentage of the produced FAME was determined based on methyl heptadecanoate internal standard method [22].

3.8. Statistical Analysis

All the experiments were conducted in triplicate and statistical analyses were performed with SPSS software (version 15.0, SPSS, Inc., Chicago, IL, USA). The analysis of variance was carried out using the Duncan comparison procedure to determine the significant differences between the means.

4. Conclusions

In this study, the CBL, extracellular lipase, and biomass production from *M. spicifer* SPB2 were greatly improved in scaled-up fermentation conducted in a commercial standard type of the stirred-tank bioreactor. Such production was enhanced from 539.17 U/L, 1859.88 U/L, and 11.77 g/L to 1189.65 U/L, 5603.74 U/L, and 16.83 g/L, respectively in comparison to the shake flask fermentation. *Magnusiomyces spicifer* SPB2 prepared in the up-scaled production were demonstrated to be extremely tolerant to excessively high methanol content present in the palm oil transesterification reaction. This can resolve the technical hurdle in terms of enzyme stability in solvent. The yeast cell of *M. spicifer* SPB2 therefore displayed potential use as the whole-cell biocatalyst for lipase-catalyzed transesterification to generate a very high yield of FAME, up to 98% at the palm oil to methanol molar ratio of 1:7. Such successful development of whole-cell biocatalyst from *M. spicifer* SPB2 offered a simple and economical way to substitute the complicated conventional preparation and immobilization of lipases. The methanol tolerant biocatalyst of *M. spicifer* SPB2 can be easily prepared at an up-scaled level through yeast cultivation using a commercial standard fermenter. Thus, it greatly increases industrial feasibility and possibility to develop an eco-friendly process for biodiesel production.

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