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Development of a *Pichia pastoris* whole-cell biocatalyst with overexpression of mutant lipase I PCL^{G47I} from *Penicillium cyclopium* for biodiesel production

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Penicillium cyclopium lipase I (PCL) is a thermolabile triacylglycerol lipase with very low activity against monoacylglycerols, and there have been no reports on the transesterification of oil to produce biodiesel. A mutant PCL^{G47I} with an improved thermostability was previously obtained through replacing Gly47 with Ile in PCL. In this study, a novel *Pichia pastoris* whole-cell biocatalyst (WCB) with overexpression of PCL^{G47I} was constructed and characterized for biodiesel production from soybean oil. The optimum conditions for biodiesel preparation were 1 g soybean oil, 1 : 2 initial oil/methanol molar ratio with 3 times methanol addition of 1 : 0.75 oil/methanol molar ratio at 4 h intervals, 7% water content, 400 U lipase, temperature of 25 °C, and reaction time of 20 h. Under the optimum conditions, the FAME yield reached 60.7% and remained 47.3% after 4 batch cycles, and no glycerol was generated as a byproduct. These findings indicated that this WCB is a promising biocatalyst for biodiesel production in a relatively cost-effective manner. Additionally, the resulting enzymatic process may provide a potential method for biodiesel production at an industrial scale.

Introduction

Recently, biodiesel has received much attention as a promising alternative to replace the current fossil fuels as a mixture of fatty acid methyl esters (FAMEs). It has many advantages over traditional fossil fuels, including renewability, sustainability, biodegradation, nontoxicity, and environmental friendliness.[1] Many studies have shown that biodiesel could be produced in vegetable oils with an alkaline catalyst or the esterification of fatty acids with an acid catalyst by the transesterification of triacylglycerols.[2,3] However, chemical processes present various drawbacks, such as the formation of soap, difficulties in the recovery of glycerol and the removal of chemical catalysts, requirement of high energy, and production of wastewater.[1,4]

To address these problems, lipases-catalyzed biodiesel production has been studied as an substitute to overcome the limitations of chemical-catalyzed methods.[5] Lipases (triacylglycerol hydrolases, EC 3.1.1.3) exhibit the catalytic activity under mild conditions, resulting in lower energy consumption,[6,7] which will lower the cost of biodiesel production. Additionally, lipases have a wide diversity of substrates able to catalyze transesterification of triglycerides and esterification of free fatty acids (FFAs) simultaneously in vegetable oils,[8,9] leading to a more efficient strategy for biodiesel production. In addition, the separation and purification of biodiesel is a simple and environmentally friendly process.[10] However, lipases-catalyzed biodiesel production is limited in the scale-up process at present because of the high cost and low operational stability of free lipase.[9] An effective solution is to recycle lipases for batch reaction to reduce the cost. The use of immobilized lipase (*e.g.*, Novozym 435 and Lipozyme RM IM) is beneficial to improve the reusability and stability of lipases.[11] However, these lipases are immobilized by complicated processed including extraction, purification, and immobilization, which is an expensive method.[1] Moreover, the conformation of lipases are altered when they are bound to the carriers, which may change the specificity and reduce the activity of lipases.[7,12] These features are the main obstacles in the industrialization of lipase-catalyzed biodiesel production. Recent reports[13,14] indicated that the whole-cell biocatalyst (WCB) of lipases can eliminate the requirement for enzyme purification and immobilization, thus reducing the overall cost of lipases-catalyzed biodiesel production. In addition, WCB provides a natural environment for enzyme localization and conformation,[15]

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which can improve the stability and reusability of lipases without affecting their specificity and activity.[16–19]

Yeast surface display systems, including *Pichia pastoris*, *Yarrowia lipolytic*, and *Saccharomyces cerevisiae*, are widely used to prepare WCB for the expression of many heterologous proteins and display many advantages, such as simple genetic manipulation, standard fermentation for the production of target proteins, and use as a biocatalyst without purification and immobilization.[13,16] Nevertheless, studies of the lipases displayed on the yeast cell surfaces as WCBs for biodiesel production remain limited. *Rhizopus oryzae* lipase displayed on *S. cerevisiae* was employed for biodiesel production with a yield of 78.3% after 72 h.[20] *Rhizomucor miehei* lipase-displaying *P. pastoris* was used as the biocatalyst, reaching a biodiesel yield of 83.1% after 72 h.[21] Displayed *Candida antarctica* lipase B and *R. miehei* lipase, which were expressed on the cell surface of *P. pastoris*, afforded biodiesel yield over 90% in 12 h.[16,22] A biocatalyst through coexpressing of *C. antarctica* lipase B and *Thermomyces lanuginosus* lipase on the cell surface of *P. pastoris* was used in biodiesel production with biodiesel conversion of 95.45% in 12.6 h.[23]

During lipases-catalyzed biodiesel production, glycerol, the main byproduct of transesterification reactions, inhibits lipases activity by covering them, preventing the accessibility of lipases active sites to the substrates.[24] 1,3-Specific lipases can simultaneously synthesize biodiesel and monoacylglycerols (MAGs) to avoid the glycerol production. As is known, the obtained MAGs are considered valuable products widely used in food, pharmaceutical, and cosmetic industries.[25] Previous studies have reported that most lipases from *Penicillium* have a hydrolytic preference for the 1- and 3-positions of triacylglycerols (TAGs),[26] and *Penicillium cyclopium* lipase I (PCL) shows preferential specificity toward TAGs and low activity against diacylglycerols (DAGs) but almost no activity toward MAGs.[26,27] Compared with the commonly used and widely investigated lipases for biodiesel production, PCL has not received enough attention for its potential applications. PCL has an optimum activity at 25 °C and thereby being considered as a cold-active lipase. Cold-active lipases exhibit high activity at low temperatures compared to their mesophilic or thermophilic counterparts that drastically reduce their activities at low temperatures, which is beneficial to the energy saving for biodiesel production because the biodiesel synthesis by most other lipases was conducted at higher temperatures.[28] However, the poor thermostability prevented PCL from being applied in long-term processes of industrial applications. Compared with those mesophilic and thermophilic lipases, PCL displayed more difficulty in the enhancement of the thermostability because of its structural flexibility. In the previous study, PCL was engineered through the direct evolution with error-prone polymerase chain reaction (PCR), and a novel mutant PCL^{G47I} with significantly improved thermostability was obtained.[29] PCL^{G47I} could efficiently maintain its activity at 35 °C, 40 °C, and 45 °C with the time extension, leading to its potential long-term application at low temperatures in the process of biodiesel production.

Based on the above, the goals of this study was (i) to exploit the new preparation of PCL^{G47I} as the form of WCB using the *P. pastoris* displayed system, (ii) to optimize the conditions for biodiesel production, such as reaction temperature, oil/methanol molar ratio, enzyme loading amount, water content, methanol additional strategy, and reaction time, and (iii) to evaluate the reusability of WCB for efficient biodiesel production, thereby assessing the potential of using the PCL^{G47I} WCB as a biocatalyst for biodiesel production by the transesterification of oil with methanol. To our knowledge, this study is the first report on the use of recombinant *P. pastoris* yeast to functionally overexpress lipase from *P. cyclopium* as a WCB for biodiesel production.

Materials and methods

Chemicals and enzymes

Standard FAMEs were purchased from Sigma-Aldrich (St. Louis, MO, USA). Restriction endonucleases, T4 DNA ligase, Pyrobest DNA polymerase, and genetic manipulation kits were provided by Takara Bio (Dalian, China). Refined soybean oil was purchased from a commercial source. All other chemicals and reagents were obtained from standard sources and were of analytical grade. The recombinant PCL (rPCL) and PCL^{G47I} (rPCL^{G47I}) were produced by the expression of the corresponding genes in *Escherichia coli* and purified according to Liu *et al.*[29]

Plasmid, strains, and media

The *Pichia pastoris* surface display vector pKFS was provided by Professor Ying Lin (South China University of Technology). The flocculation functional domain (FFD) of lectin-like cell-wall protein (Flo1p) with its own secretion signal sequence was cloned into the N-terminal of vector pPIC9K without signal peptide to get the pKFS, which was used as the yeast cell surface display vector. The codon-optimized *pclm^{G47I}* was synthesized by replacing the rare codon in the *pcl^{G47I}* sequence according to the preferred codon usage of *Pichia pastoris* GS115 (BGI Co., Beijing, China). *E. coli* DH5α was used for *pclm^{G47I}* cloning and sequencing, and *P. pastoris* GS115 was used for the expression of the PCLM^{G47I}. *E. coli* strains were cultured in Luria–Bertani (LB) medium. The medium for yeast culture were prepared as described Liu *et al.*[30]

Construction of the expression plasmid on the yeast cell surface

The *pclm^{G47I}* fragment was digested with *Mlu*I and *Not*I and cloned into the *Mlu*I/*Not*I site of pKFS to produce the recombinant plasmid pKFS-*pclm^{G47I}*. Then, the resulting plasmid was transformed into *E. coli* DH5α competent cells. After restriction enzymes digestion analysis and DNA sequencing confirmation, the plasmid pKFS-*pclm^{G47I}* was linearized by *Sac*I and transformed into *P. pastoris* GS115 competent cells. The positive transformants harboring multicopy integration of *fdd-pclm^{G47I}* were screened on MD plates with G418 (0.5–2.5 mg mL^{-1}) for the production of the displayed PCLM^{G47I} (dPCLM^{G47I}).

Preparation of dPCLM^{G47I}-displaying whole-cell biocatalyst

The selected recombinant strain, GS115/pKFS-*pclm*^{G47I}, was inoculated into 5 mL of YPD medium and cultured at 30 °C for 24 h in 50 mL flask at 220 rpm shaking. Then, 1 mL of culture was transferred to a 250 mL flask containing 50 mL of BMGY medium and cultured at 30 °C at 220 rpm shaking until the optical density at 600 nm reached 2.0. The cells were harvested by centrifugation at 8000 × *g* for 20 min and re-suspended in a 250 mL flask with 50 mL BMMY medium. In addition, 0.5% methanol (final concentration, v/v) was added to the culture medium every 12 h to maintain the induction of the fusion protein FDD-PCLM^{G47I} expression with a total induction time of 120 h at 30 °C at 220 rpm shaking. In addition to the aforementioned operation, the fermentation conditions, including the methanol concentration, pH value, culture volume, temperature, and shaking speed, were optimized to improve the expression level of dPCLM^{G47I}. The induced cells were centrifugally harvested at 8000 × *g* for 10 min and washed twice with 50 mmol L^{-1} sodium phosphate buffer (pH 8.0) to remove media components, and then the harvested cells were lyophilized for 24 h. The obtained dPCLM^{G47I}-expressing cells were used as WCB for the following study.

PCL activity assay

The activity assay of lipase was carried out according to the method of Liu *et al.*[29] One unit of lipase activity was defined as the amount of enzyme required to hydrolyze olive oil to release 1 μmol of fatty acid per minute.

Transesterification of soybean oil with methanol

The enzymatic transesterification reactions were performed in a 20 mL screw-cap glass vial containing 1 g of soybean oil, dPCLM^{G47I} WCB, and distilled water with constant shaking at 200 rpm. The effects of different parameters, such as temperature, oil/methanol molar ratio, enzyme loading, water content, methanol addition, and reaction time, on biodiesel production were investigated using single-factor experimental design. After the reaction, the reaction mixture was collected and centrifuged at 5000 × *g* for 10 min, and the upper layer was analyzed *via* gas chromatography to determine the contents of FAMEs.

Gas chromatography analysis of biodiesel

The sample analysis was performed on Agilent 7890A gas chromatography (GC) system (Agilent Technologies, Santa Clara, California, USA) equipped with a flame-ionization detector, and a HP-5MS column (Agilent Technologies) [60 m (length) × 320 μm (inner diameter), 0.25 μm (film thickness)]. Injector and detector temperatures were set at 245 °C and 360 °C, respectively. Nitrogen was used as the carrier gas with the flow rate of 1 mL min^{-1}. For analyzing the oil samples, the column temperature was held at 90 °C for 0.5 min and then elevated to 170 °C at a rate of 10 °C min^{-1} and maintained at 170 °C for 8 min and then from 170 to 250 °C at a rate of 4 °C min^{-1}, and then the temperature was maintained at 250 °C for 4 min. For the analysis of glycerol samples, the column temperature was held at 120 °C for 1 min and then elevated to 180 °C at a rate of 10 °C min^{-1} and maintained at 180 °C for 1 min. The contents of methyl palmitate, methyl stearate, methyl oleate, methyl linoleate, and methyl linolenate were calculated based on the standard curves derived from standard solutions of each. FAMEs yield (%) was defined as the ratio between the amount of FAMEs produced, and the total amount of FAMEs when all soybean oil was converted.

Reusability of dPCLM^{G47I}

To evaluate the reusability of dPCLM^{G47I}, the biocatalyst was reused for the transesterification of soybean oil under the optimal conditions. After the reaction completion, the biocatalyst was collected from the reaction mixture by centrifugation at 5000 × *g* for 5 min. The recovered biocatalyst was washed with 50 mmol L^{-1} sodium phosphate buffer (pH 8.0) and remixed in fresh reactants to initiate the next new batch reaction under the same conditions. In each batch of reaction, the FAMEs yield from the reaction mixture was determined.

Results and discussion

Cloning of the *pclm*^{G47I} gene in *P. pastoris*

To maximize PCL^{G47I} expression, the codon optimized *pcl*^{G47I} gene (*pclm*^{G47I}) was synthesized based on the codon bias of *P. pastoris*. Notably, many heterologous proteins could be expressed at a higher level in *P. pastoris* through this approach.[19,30,31] After optimization, *pclm*^{G47I} gene had 155 nucleotide variations corresponding to 135 of 258 codons switch to *P. pastoris*'s favorite, which could enhance the PCLM^{G47I} expression level in *P. pastoris*. In addition, FFD, which is located near the N-terminus of Flo1p, can recognize and link to α-mannan carbohydrates of the yeast cell-wall by noncovalent bonds. It was reported that FFD was successfully used as anchor to immobilize the heterologous proteins on the yeast cell surface in many studies.[13,32,33] In this study, to display PCLM^{G47I} on the cell surface of *P. pastoris*, the PCLM^{G47I} was fused to the C-terminus of the FFD by cloning the *pclm*^{G47I} gene into the vector pKFS to engineer the plasmid pKFS-*pclm*^{G47I}. Then, the resulting plasmid was transformed into *P. pastoris* GS115, and a recombinant *P. pastoris* strain containing multiple integrated PCLM^{G47I} expression cassettes (*P. pastoris*/pKFS-*pclm*^{G47I}) was selected on MD medium plates with G418 at concentrations of 2.0 mg mL^{-1}.

Preparation of dPCLM^{G47I} WCB

As shown in Fig. 1, the hydrolysis activity of dPCLM^{G47I} on the *P. pastoris* GS115 cell surface expressed by *P. pastoris*/pKFS-*pclm*^{G47I} dramatically increased in a time course manner. After methanol induction for 108 h in a flask, the highest activity of dPCLM^{G47I} was 2377 U g^{-1} dry cell weight (DCW) under optimal conditions [0.5% (v/v) methanol in BMMY medium, pH 6.0, 32 °C, culture volume of 30 mL, and shaking at 260 rpm]. The culture supernatant displayed low hydrolysis activity (66 U mL^{-1}), indicating that most of the FFD-PCLM^{G47I} was successfully immobilized on the cell wall of *P. pastoris* GS115. After

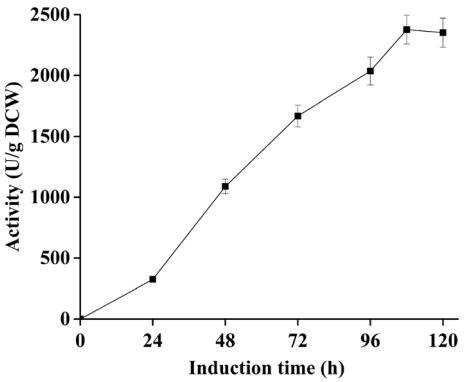

Fig. 1 Time course of the activity of dPCLM^{G47I} WCB during the methanol induction stage. The data presented are the average values of three independent experiments, and the error bars indicate the standard deviations.

freezing at −80 °C for 12 h and drying with a lyophilizer for 24 h, the dPCLM^{G47I} powder with activity of 2377 U g^{-1} DCW was obtained.

Currently, the main limitations for the scale-up industrial implementation of lipase catalyzed biodiesel production include (i) the high cost of most commercial lipases due to their low production efficiency and (ii) the relatively weak reusability of these lipases. So far, *E. coli* has been widely used for the expression of many recombinant proteins, however, the heterogeneous proteins are expressed at relatively low levels[34] and obtained with inconvenient preparation process because they are intracellularly expressed. In contrast, *P. pastoris*, as an established protein expression host, has been successfully applied for the overexpression of recombinant proteins by high cell densities in economical culture media.[35] At present, the *P. pastoris* cell surface display system has been used for the expression of heterogeneous proteins on the yeast cells as WCB with great success,[13] which can combine high-level gene expression, protein purification, and enzyme immobilization. In fact, various lipases have been effectively expressed on the surfaces of yeast cells for biodiesel production.[13,14] However, to our knowledge, the present study is the first report on the display of a lipase from *P. cyclopium* on the yeast cell surface using *P. pastoris*.

To date, most studies have focused on the lipases expression by yeast display systems for biodiesel production from soybean oil rather than from waste oils,[14] since waste oils have more

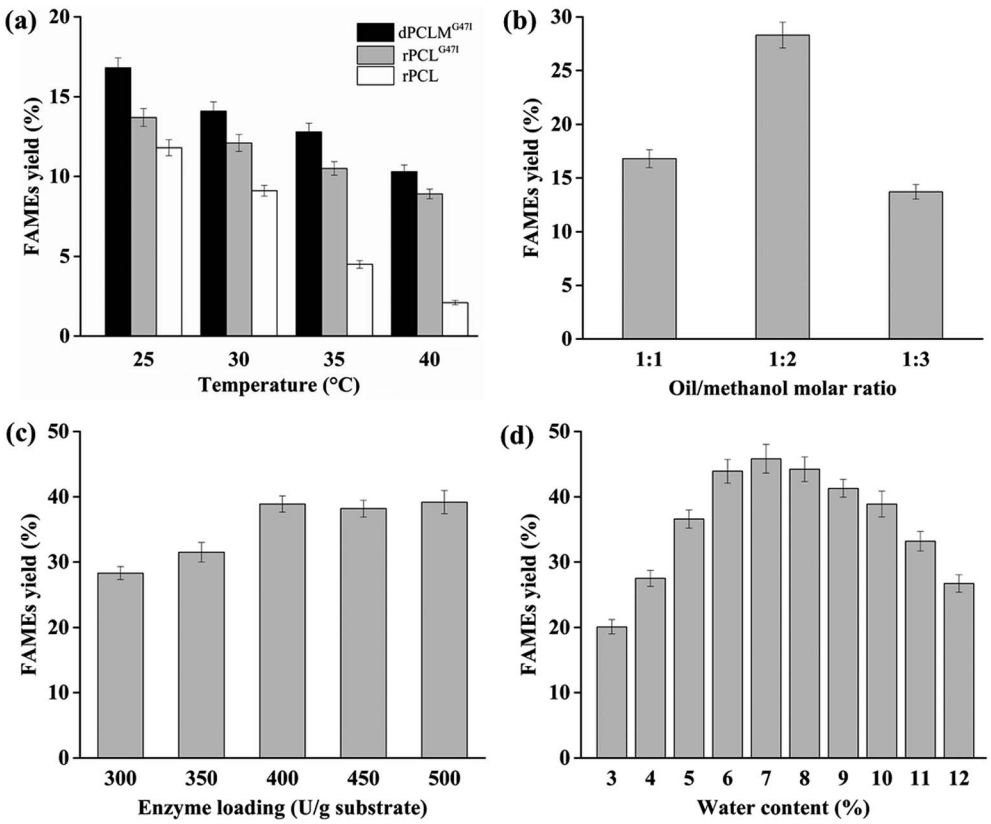

Fig. 2 Effects of reaction temperature, oil/methanol molar ratio, enzyme loading, and water content on FAMEs yield. (a) Effect of reaction temperature on FAMEs yield. Reaction conditions: 1 g soybean oil, 1 : 1 oil/methanol molar ratio, 300 U lipase, 10% (v/w) water content, temperatures of 25 °C, 30 °C, 35 °C, and 40 °C, and reaction time of 24 h. (b) Effect of oil/methanol molar ratio on FAMEs yield. Reaction conditions: 1 g soybean oil, 1 : 1, 1 : 2, and 1 : 3 oil/methanol molar ratio, 300 U lipase, 10% (v/w) water content, temperature of 25 °C, and reaction time of 24 h. (c) Effect of enzyme loading on FAMEs yield. Reaction conditions: 1 g soybean oil, 1 : 2 oil/methanol molar ratio, 300, 350, 400, 450, and 500 U lipase, 10% (v/w) water content, temperature of 25 °C, and reaction time of 24 h. (d) Effect of water content on FAMEs yield. Reaction conditions: 1 g soybean oil, 1 : 2 oil/methanol molar ratio, 400 U lipase, 3–12% (v/w) water content, temperature of 25 °C, and reaction time of 24 h. The data presented are the average values of three independent experiments, and the error bars indicate the standard deviations.

undesired properties than refined oils, and refined soybean oil is a high-quality substrate for biodiesel production, which was beneficial to the evaluation of a novel lipase for biodiesel production. Hence, to determine whether biodiesel production through transesterification is feasibly catalyzed by dPCLM^{G47I} WCB, the soybean oil was used as the feedstock substrate. In this research, we designed a series of experimental to study the optimal reaction conditions for biodiesel production with dPCLM^{G47I} WCB.

Effect of reaction temperature

Generally, the higher temperature can enhance the lipases activity and help substrates absorb required energy to break through the energy barrier, resulting in the improvement of the reaction rate.[36] However, the higher temperature has potential risk to denature the lipases, thereby decreasing the reaction rate. In addition, the lipase-catalyzed transesterification for biodiesel production was often performed for a long reaction time, which makes it necessary to maintain the lipase activity for a prolonged time. Hence, the optimum temperature and thermostability of the lipases are important for high biodiesel yield.

The effects of temperatures from 25 °C to 40 °C on the biodiesel production using rPCL, rPCL^{G47I}, and dPCLM^{G47I} were investigated when the other variables were set as follows: water content 10% (v/w), oil/methanol molar ratio 1 : 1, lipase loading 300 U g^{-1} substrate, and a reaction time of 24 h. The results are shown in Fig. 2(a). When the reaction temperature raised from 25 °C to 40 °C, the FAMEs yields obtained by rPCL, rPCL^{G47I}, and dPCLM^{G47I}-catalyzed transesterification gradually decreased, indicating that the optimum temperature for the stability of rPCL, rPCL^{G47I}, and dPCLM^{G47I} was 25 °C. Additionally, the dPCLM^{G47I} WCB exhibited higher yield of biodiesel than rPCL and rPCL^{G47I} at temperatures between 25 °C and 40 °C, suggesting that the thermostability of dPCLM^{G47I} was significantly improved in the form of the WCB engineered with the *P. pastoris* cell surface display system. Our findings were consistent with the findings of other studies.[33,37,38] The possible reason for this finding may be that the WCB provides a natural environment for enzyme localization and conformation, protecting cellular proteins against heat denaturation and helping them maintain their activity at higher temperature.[15] In our study, the maximum FAMEs yield (16.8%) was observed by dPCLM^{G47I} WCB catalysis at 25 °C. In many studies, the optimal temperature of the most lipases for biodiesel synthesis was between 30 and 55 °C.[4,36,39,40] The PCL is most active at 25 °C and stable at temperatures below 25 °C, suggesting that this enzyme cannot maintain its catalytic activity for a long time when the temperature is above 25 °C. In a previous study, the thermostability of PCL^{G47I} at temperatures above 25 °C was significantly improved by the substitution of Gly with Ile at position 47 in PCL.[29] The presented results showed that by immobilization of PCLM^{G47I} onto *P. pastoris* cells, dPCLM^{G47I} exhibited more stability than PCL^{G47I}, which made it more suitable for biodiesel production at 25 °C for a prolonged reaction time. It has been reported that lower temperatures could reduce the reaction rate, causing the prolongation of the reaction time for a high biodiesel yield.[39] These results showed that biodiesel production at a lower temperature and less reaction time had many advantages, such as energy saving, milder reaction conditions, and higher efficiency, and thus reducing the production cost of biodiesel. Therefore, the reaction temperature was fixed at 25 °C in the subsequent experiments.

Effect of oil to methanol molar ratio

High methanol concentration can shift the chemical equilibrium towards biodiesel synthesis, leading to an increased biodiesel yield.[41] However, on the other hand, an excessive amount of methanol can inhibit lipase activity, resulting in a decreased yield.[4]

Experiments were designed to examine the effect of the molar ratio of oil to methanol in the range of 1 : 1 to 1 : 3 on biodiesel production, while the other variables were set as follows: water content 10% (v/w), lipase loading 300 U g^{-1} substrate, temperature of 25 °C, and reaction time of 24 h. The results are shown in Fig. 2(b). Our data indicated that FAMEs yield increased when the molar ratio of oil to methanol varied from 1 : 1 to 1 : 2, and the maximum FAMEs yield (28.3%) was obtained at an oil to methanol molar ratio of 1 : 2. When the molar ratio of oil to methanol exceeded 1 : 2, the FAMEs yield decreased. The higher ratios (1 : 3) of oil to methanol could deactivate the dPCLM^{G47I} because of the significant excess of methanol in the reaction mixture, and the lower oil/methanol molar ratios (1 : 1) were not sufficient to perform a more complete equilibrium displacement towards the biodiesel. Consequently, the oil to methanol molar ratio was set at 1 : 2 in the subsequent experiments.

Effect of dPCLM^{G47I} loading

The amount of dPCLM^{G47I} is a key factor that affects the cost and yield of biodiesel production. To determine the effect of the amount of dPCLM^{G47I} on the biodiesel reaction, the loading of dPCLM^{G47I} varied from 300 to 500 U g^{-1} substrate were tested, while the other factors were fixed as follows: water content 10% (v/w), oil/methanol molar ratio 1 : 2, temperature of 25 °C, and reaction time of 24 h. The results are shown in Fig. 2(c). FAMEs yield increased from 28.3% to 38.9% when dPCLM^{G47I} loading amount ranged from 300 to 400 U g^{-1}. Subsequently, the FAMEs yield remained constant with an increase in dPCLM^{G47I} loading. More substrate molecules could be accessed by the active center of the lipase when the amount of available lipase was enhanced,[42] which should improve the yield of biodiesel. However, the presence of excess lipase in the reaction did not improve biodiesel yield as expected. The possible reason may be that not all active sites of lipase could be exposed to the substrates.[4] Thus, the lipase should be used with a minimum amount that can be consumed by the reaction since overdose of lipase does not effectively enhance the biodiesel yield. Hence, 400 U g^{-1} of dPCLM^{G47I} was selected as the optimum enzyme loading applied in the subsequent experiments.

Effect of water content

Water plays an important role in biphasic systems for biodiesel production. The appropriate water content is essential for maintaining the three-dimensional structure of lipase for its activity and increasing the available interface between the aqueous and organic phases for lipase catalysis.[41,42] However, excessive water content can increase the flexibility of lipase and activate the hydrolytic activity of lipase in aqueous phase, leading to a decrease in transesterification yields.[41,42] The optimum water content can help the lipase maintain maximal transesterification activity while only minimal hydrolysis activity in the reaction.

The effect of different water contents from 3% to 12% (v/w, based on soybean oil) on biodiesel production was determined when other variables were set as follows: oil/methanol molar ratio 1 : 2, lipase loading 400 U g^{-1} substrate, temperature of 25 °C, and reaction time of 24 h. The results are shown in Fig. 2(d). The FAMEs yield increased when the water contents varied from 3 to 7%, and the maximum yield reached 45.8% with 7% water content. When the water content was higher than 7%, the production significantly decreased. These results suggested that the optimum water content could enhance the efficiency of lipase on the transesterification reaction, leading to a higher production biodiesel yield. Many studies have shown that the best water content varied depending on the feedstock, the lipase type, the immobilized support, and the organic solvent employed.[43,44] Studies indicated that the optimum water content required to maintain the highest biodiesel yield is 1.15–20% based on the substrate weight.[1,2,4,36,42,45] Subsequently, the water content of the reaction was set at 7% in the remaining experiments.

Effect of methanol additional strategy

The addition of appropriate amounts of methanol as a reaction substrate to the reaction mixture can shift the reaction to biodiesel synthesis and improve the biodiesel yield, whereas excess amounts of methanol will lead to the denaturation and inactivation of lipase activity. Hence, it is necessary to reach the balance point of methanol concentration between catalysis and toxicity for the high yield of biodiesel. Until recently, many studies have reported that a strategy of step-wise addition of methanol into the reaction system could be used to successfully promote biodiesel production with high efficiency.[4,41,42,46] However, the detailed methods of every strategy were different, depending on the feedstocks variety, lipases property, and alcohols type.

Based on the optimal initial oil/methanol molar ratio of 1 : 2, we designed different strategies to introduce additional methanol to the reaction mixture (extra methanol with oil/methanol molar ratio of 1 : 0.5, 1 : 0.75, and 1 : 1, respectively, was added at different reaction time points of 4, 8, and 12 h). Meanwhile, the other variables were set as follows: water content 7% (v/w), initial oil/methanol molar ratio 1 : 2, lipase loading 400 U g^{-1} substrate, temperature of 25 °C, and reaction time of 24 h. The results are shown in Fig. 3. The maximum and second FAMEs yield of 60.9% and 52.2% were obtained using an oil to methanol molar ratio of 1 : 0.75 and 1 : 0.5, respectively, which were all higher than the yield from no additional methanol. These findings indicated that the accumulated amount of new additional methanol and remaining methanol in reaction mixture at 4, 8, and 12 h could promote the transesterification reaction without inactivating dPCLM^{G47I}, causing an improved FAMEs yield. Compared with the 1 : 0.5 molar ratio, the 1 : 0.75 molar ratio showed a higher yield because of the extra methanol content. However, FAMEs yield (40.2%) decreased when the extra methanol of oil to methanol molar ratio of 1 : 1 was added to the reaction mixture compared with that obtained without the addition of methanol. This result may be caused by the accumulation of methanol beyond the tolerance of dPCLM^{G47I}, leading to lipase denaturation. Therefore, the best methanol-adding strategy was methanol addition with oil/methanol molar ratio of 1 : 0.75 at 4 h intervals with initial molar ratios of oil : methanol of 1 : 2 in the remaining experiments.

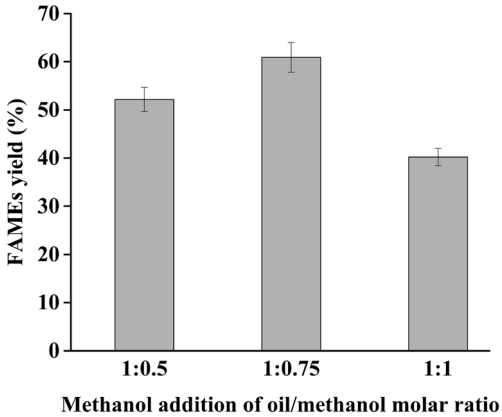

Fig. 3 Effect of methanol addition strategy on FAMEs yield. Reaction conditions: 1 g soybean oil, 1 : 2 initial oil/methanol molar ratio, 400 U lipase, 7% (v/w) water content, temperature of 25 °C, addition of methanol with oil/methanol molar ratio of 1 : 0.5, 1 : 0.75, and 1 : 1 at various reaction times of 4, 8, and 12 h, and reaction time of 24 h. The data presented are the average values of three independent experiments, and the error bars indicate the standard deviations.

Effect of reaction time

Extended reaction time typically results in higher biodiesel yield, but this may consume excessive energy, leading to higher cost for biodiesel production. The effect of reaction times from 0–24 h on the biodiesel production was investigated when the other variables were set as follows: water content 7% (v/w), lipase loading 400 U g^{-1} substrate, the initial oil/methanol molar ratio of 1 : 2, the addition of methanol with oil/methanol molar ratio of 1 : 0.75 at various reaction times of 4, 8, and 12 h, and temperature of 25 °C. The results are shown in Fig. 4. The FAMEs yield increased in a time course manner from 0 to 20 h. Generally, 2 moles of FAMEs and 1 mole of MAGs are produced by 2 mol of methanol and 1 mole of TAGs at a stoichiometric ratio when *sn*-1,3 regioselective lipases are used as catalysts, which can give the theoretical biodiesel yield of 66.7%.[9] In this study, the maximum yield reached 60.7% after 20 h, which was close to the theoretical maximum for *sn*-1,3

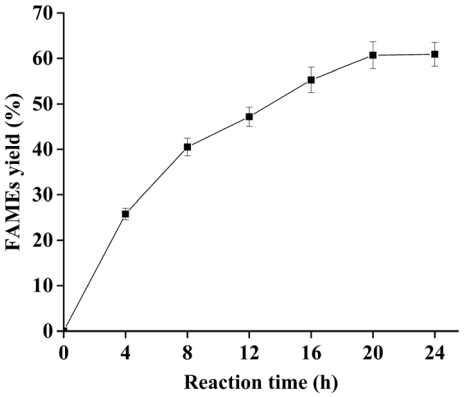

Fig. 4 Time course of the biodiesel production with dPCLM^{G47I} WCB. Reaction conditions: 1 g soybean oil, 1 : 2 initial oil/methanol molar ratio, 400 U lipase, 7% (v/w) water content, temperature of 25 °C, addition of methanol with oil/methanol molar ratio of 1 : 0.75 at various reaction times of 4, 8, and 12 h, and reaction time of 0–24 h. The data presented are the average values of three independent experiments, and the error bars indicate the standard deviations.

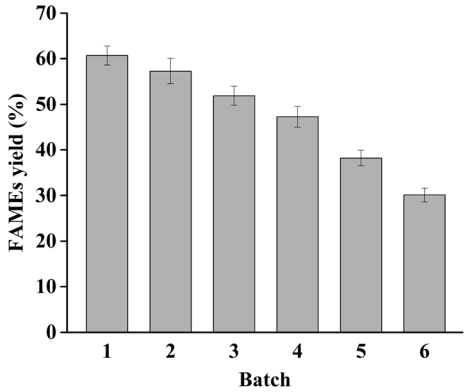

Fig. 5 Reusability of dPCLM^{G47I} WCB. Reaction conditions: 1 g soybean oil, 1 : 2 initial oil/methanol molar ratio, 400 U lipase, 7% (v/w) water content, temperature of 25 °C, addition of methanol with oil/methanol molar ratio of 1 : 0.75 at various reaction times of 4, 8, and 12 h, and reaction time of 20 h. The data presented are the average values of three independent experiments, and the error bars indicate the standard deviations.

regioselective lipases (66.7%). When the reaction time exceeded 20 h, FAMEs yield maintained a constant with the increasing reaction time because PCL shows very low activity against MAGs.[47] Most importantly, glycerol, the by-product that had negative effect on the biodiesel production, was not detected along the reactions. The previous studies reported that it was approximately 6–72 h for the suitable reaction time of biodiesel production,[13,14] and a shorter reaction time was preferred to save energy in industrial scale applications. Based on these results, a reaction time of 20 h was selected in the subsequent experiments.

Reusability of dPCLM^{G47I}

It is crucial to reuse the biocatalyst in most bioprocesses, and biocatalysts with good reusability can reduce the production costs effectively. To evaluate the reusability of dPCLM^{G47I}, the reaction was performed under the optimal reaction conditions. Generally, a considerable amount of by-product glycerol catalyzed by non-region specific lipase can attach onto the yeast cell surface, resulting in cell agglutination, forming an obstacle refraining the substrates from accessing the active sites of lipases.[14] In addition, the use of WCB absorbing the glycerol is inconvenient for subsequent reactions, and an additional step is required to remove the adsorbed glycerol, increasing the recycling process complexity and cost. The lipases having very low activity against MAGs can simultaneously produce biodiesel and MAGs, which is beneficial to biodiesel production by avoiding the generation of glycerol. Therefore, dPCLM^{G47I} can maintain its catalytic activity during the reaction process and can be simply recycled by centrifuging the reaction mixture after each batch for reuse.

The dPCLM^{G47I} was recovered from the reaction mixture by centrifugation after each batch reaction (20 h per batch), washed with 50 mmol L^{-1} sodium phosphate buffer (pH 8.0), and added to the fresh reactants for the next batch. As shown in Fig. 5, the FAMEs yield gradually decreased with increasing recycle number. The FAMEs yield in the first batch was 60.7%, and after 4 batches, the FAMEs yield was 47.3%, which was approximately 80% of the initial batch yield. From a practical perspective, dPCLM^{G47I} can be reused at least 4 times. This result indicated that WCB provides an excellent environment for displayed lipases to maintain their conformation. The high reusability of the biocatalysts may significantly reduce the costs of industrial biodiesel production. Recently, various heterologous lipases have been successfully displayed by yeast cell surface display systems as recyclable WCB for biodiesel production, and lipase WCBs could be reused from two to as high as twenty batches.[14] The reuse frequency difference between different studies might be caused by many factors such as the feedstock source, enzyme property, enzyme loading, oil/methanol molar ratio, and methanol adding strategy. Our data indicated that dPCLM^{G47I} had relatively good reusability and is a promising biocatalyst for commercial biodiesel production. Furthermore, dPCLM^{G47I}, could be combined with mono- and diacylglycerol lipases instead of one lipase, providing a route to increase the biodiesel yield and reduce the cost of biodiesel production.

Conclusions

This study is the first investigation of a novel *P. pastoris* WCB with the overexpression of PCLM^{G47I} for biodiesel production at a low temperature. The highest FAMEs yield (60.7%) was achieved under optimum conditions, and the biodiesel yield remained 47.3% after 4 batch cycles. The dPCLM^{G47I} WCB has many advantages, including convenient preparation, high catalytic activity and stability at low temperature, good tolerance to methanol, easy recovery, and relatively high reusability which would be further improved in the future. These features demonstrated its bright perspective as a biocatalyst for biodiesel production in a relatively cost-effective manner. Moreover, the resulting enzymatic process may provide a potential method for biodiesel production at an industrial scale.

Conflicts of interest

There are no conflicts to declare.

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