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Use of mono- and diacylglycerol lipase as immobilized fungal whole cells to convert residual partial glycerides enzymatically into fatty acid methyl esters

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ABSTRACT

The accumulation of partial glycerides such as monoglyceride (MG) and diglyceride (DG) is one of the rate-limiting steps in plant oil methanolysis catalyzed by *Rhizopus oryzae* producing triacylglycerol lipase. To convert partial glycerides efficiently into their corresponding methyl esters (MEs), we attempted to use a mono- and diacylglycerol lipase (mdlB) derived from *Aspergillus oryzae*. By considering cost efficiency, *R. oryzae* and recombinant mdlB-producing *A. oryzae* were immobilized independently within polyurethane foam biomass support particles and directly utilized as a whole-cell biocatalyst. The mdlB-producing *A. oryzae* effectively exhibited substrate specificity toward MG and DG and was then used for the methanolysis of intermediate products (approximately 82% ME), which were produced using *R. oryzae*. In the presence of 5% water, the use of mdlB-producing *A. oryzae* resulted in less than 0.1% of MG and DG, whereas a considerable amount of triglyceride was present in the final reaction mixture. On the basis of these results, we developed a packed-bed reactor (PBR) system, which consists of the first column with *R. oryzae* and the second column containing both *R. oryzae* and mdlB-producing *A. oryzae*. Ten repeated-batch methanolysis cycles in the PBR maintained a high ME content of over 90% with MG and DG at 0.08–0.69 and 0.22–1.45%, respectively, indicating that the PBR system can be used for long-term repeated-batch methanolysis with partial glycerides at low levels. The proposed method is therefore effective for improving enzymatic biodiesel production.

1. Introduction

Biodiesel, which is produced by plant oil methanolysis, has recently attracted considerable attention as a renewable, biodegradable, and nontoxic fuel. Although an alkali-catalysis method has been applied to biodiesel production, increasing environmental concerns have led to a growing interest in a lipase-catalysis method as it can avoid the conventional difficulties in the recovery of glycerol and potassium and/or sodium salt [1].

Recently, there has been considerable research into the use of microbial lipases as a whole-cell biocatalyst [2]. In particular, the direct use of lipase-producing fungal cells immobilized within porous biomass support particles (BSPs) [3] presents cost-effectiveness, because it requires no purification or further immobilization process [4]. *R. oryzae*, which originally produces a 1,3-position-specific triacylglycerol lipase, is one of the most studied fungi in lipase-catalyzed methanolysis [5–7]. Studies by our group revealed that BSP-immobilized *R. oryzae* can efficiently catalyze the methanolysis with the addition of a certain amount of water [5], and that a packed-bed reactor (PBR) using BSP-immobilized *R. oryzae* can be used for long-term repeated-batch methanolysis, with the methyl ester (ME) content around 80–90% during 10 batch cycles [8]. However, the repeated use of BSP-immobilized *R. oryzae* led to difficulties in maintaining a high ME content above 90%.

In a previous study of methanolysis using BSP-immobilized *R. oryzae*, we found that one of the major components remaining in the reaction mixture is partial glycerides such as monoglyceride (MG) and diglyceride (DG) and that increasing numbers of reaction cycles results in the marked accumulation of MG and DG [9]. It thus seems likely that the accumulation of MG and DG is responsible for the relatively low ME conversion. To prevent serious problems with biodiesel when used for diesel engines, impurities including MG and DG may be eliminated from the MEs through downstream processes [10]. From an economic point of view, however, it is desirable to maintain the residual MG and DG at extremely low levels.

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Therefore, in this study, we attempted to use a mono- and diacylglycerol lipase to convert MG and DG efficiently into their corresponding MEs.

Mono- and diacylglycerol lipase is an enzyme that hydrolyzes MG and DG, but does not hydrolyze triglyceride (TG). The mono- and diacylglycerol lipase-encoding genes have been cloned from the filamentous fungi, *Penicillium camembertii* (*mdlA*) [11] and *A. oryzae* (*mdlB*) [12]. These two genes indicate high amino acid sequence similarity and have the serine, aspartic acid, and histidine residues that form the catalytic triad in lipases. Although several researchers have so far reported the synthesis of partial glycerides from glycerol and fatty acids [13,14], little attention has been paid to the use of a mono- and diacylglycerol lipase for enzymatic biodiesel production.

To utilize a mono- and diacylglycerol lipase as immobilized fungal whole cells, the development of recombinant fungal cells is necessary. Our previous works have already established the technique of preparing immobilized *A. oryzae* producing recombinant lipases [15,16]. Thus, in this paper, we report on the characterization of mdlB-producing *A. oryzae* and the development of the process for biodiesel production catalyzed by both *R. oryzae* and mdlB-producing *A. oryzae*.

2. Materials and methods

2.1. Strains, culture media, and BSPs

Two fungal strains, *R. oryzae* IFO4697 and *A. oryzae* niaD300, were used. The former originally produces a 1,3-position-specific lipase, whereas the latter was genetically manipulated. The fungus *R. oryzae* was grown in a basal medium consisting of 70 g/l polypeptone, 1.0 g/l $NaNO_3$, 1.0 g/l KH_2PO_4, and 0.5 g/l $MgSO_4{\cdot}7H_2O$. Glucose or olive oil as sole carbon source was added to the medium at a concentration of 10 or 30 g/l, respectively. *A. oryzae* niaD300, which is a *niaD* mutant derived from the wild-type strain RIB40 [17] and has been deposited at the National Research Institute of Brewing (Hiroshima, Japan), was used as a host strain for transformation and grown in dextrin-peptone (DP) medium consisting of 20 g/l glucose, 20 g/l polypeptone, 5 g/l KH_2PO_4, and 0.5 g/l $MgSO_4{\cdot}7H_2O$. The pHs of the basal and DP media were initially adjusted to 5.6 and 6.8, respectively. Reticulated polyurethane foam (Bridgestone Co. Ltd., Osaka, Japan) with a particle voidage of more than 97% and a pore size of 50 pores per linear inch (ppi) was cut into 6 mm × 6 mm × 3 mm cuboids for use as BSPs.

2.2. Construction of lipase expression vector and transformation of A. oryzae

The basic procedures for the construction of the lipase expression vector and transformation of *A. oryzae* were previously described [15,16]. The *mdlB* gene (GenBank accession number D85895) was isolated from *A. oryzae* niaD300 using two primers, fw-SalI (5′-tgGTCGACatgcgcttcctctccggcttcgtttctgtt-3′) and rv-SphI (5′-gtGCATGCttagcgcaatggcaatccaggacccttgca-3′). PCR-amplified fragments were digested with SalI and SphI and inserted into the multicloning site of pNAN8142 [18]. The resulting plasmid was designated pNAN8142mdlB (Fig. 1).

For fungal transformation, *A. oryzae* protoplasts were prepared from mycelia grown at 30 °C for 48 h using Yatalase (Takara Bio Inc., Shiga, Japan). The constructed plasmid was digested with BamHI prior to transformation and then integrated into the *A. oryzae* chromosome according to the method of Gomi et al. [19]. Fungal transformants were selected using the *niaD* gene as a selectable marker.

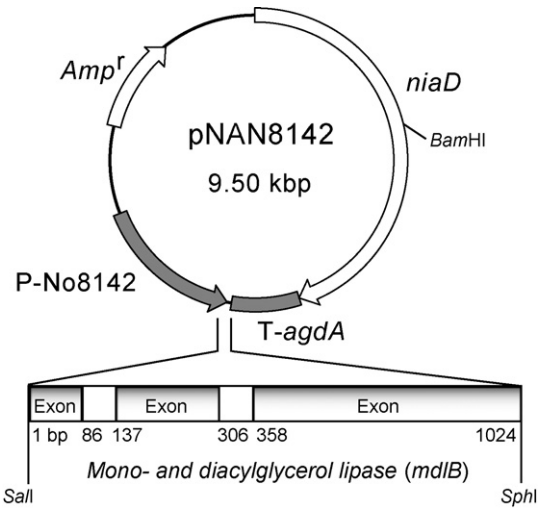

Fig. 1. Expression plasmid pNAN8142mdlB. The *mdlB* gene consisting of three exons and two introns was expressed under the control of P-No8142, which is the improved promoter region of No. 8AN genes [18]. The *niaD* gene was used as a selectable marker.

2.3. Preparation of immobilized cells

R. oryzae grown in the basal medium with glucose for 24 h (5% inoculation size) was transferred to a 20-l air-lift bioreactor (Kansai Chemical Engineering Co. Ltd., Amagasaki, Japan) [9], which contained 10 l of the basal medium with olive oil and 24,000 BSPs. The bioreactor was then aerated at 2.5 vvm to achieve liquid and particle mixing. The temperature was maintained at 30 °C by recycling water from a water bath. After cultivation for 40 h, the BSP-immobilized cells were separated from the culture broth by filtration, washed with tap water, and dried at room temperature for approximately 24 h. To stabilize the lipase activity, the dried cells were treated with a 0.1% (v/v) glutaraldehyde solution at 25 °C for 1 h, washed with tap water, dried at room temperature for more than 24 h, and then used as whole-cell biocatalyst for methanolysis.

For preparing BSP-immobilized *A. oryzae*, the spore solution was aseptically inoculated to a 5-l air-lift bioreactor (ABLE&Biott Co. Ltd., Tokyo, Japan), which contained 4 l of the DP medium with 4800 BSPs. The bioreactor was then aerated at 0.5 vvm to achieve liquid and particle mixing. The temperature was maintained at 30 °C. After cultivation for 72 h, the BSP-immobilized cells were separated from the culture broth by filtration, washed twice with tap water, lyophilized for approximately 24 h, and then used as whole-cell biocatalyst for methanolysis reaction.

2.4. Methanolysis reaction

2.4.1. Methanolysis in a screw-capped bottle

To confirm the substrate specificity of mdlB, methanolysis was carried out in a 30-ml screw-capped bottle using mono- di-, or triolein as a substrate. The reaction mixture contained 4.0 g of oil (mono-, di, or triolein) and methanol (one molar equivalent to each oil), 0.2 ml of distilled water, and 40 mdlB-producing *A. oryzae* immobilized within BSPs.

Intermediate products resulting from 72-h methanolysis using *R. oryzae* [5] were also used as a substrate for methanolysis using mdlB-producing *A. oryzae*. The reaction mixture contained 3.80 g of intermediate products (approximately 82% ME), 0.20 g of methanol, 0–0.4 ml of distilled water, and 40 mdlB-producing *A. oryzae* immobilized within BSPs. All the screw-capped bottles were incubated at 30 °C on a reciprocal shaker (150 oscillations/min, amplitude 50 mm).

2.4.2. Methanolysis in a packed-bed reactor (PBR)

A PBR system consisted of the first column with *R. oryzae* and second column containing both *R. oryzae* and mdlB-producing *A. oryzae*. The first column contained 1,500 BSP-immobilized *R. oryzae* and reaction mixture consisting of 96.5 g of soybean oil, 3.5 g of methanol (one molar equivalent to soybean oil), and 5 ml of distilled water. The reaction mixture was emulsified by ultrasonication for 5 min prior to the reaction. After starting the reaction, one molar equivalent of methanol was added stepwise at 12, 24, and 36 h. The effluent from the first column (47.5 g) was mixed with 2.5 g of methanol and 2.5 ml of distilled water, then added to the second column containing 375 each of BSP-immobilized *A. oryzae* and *R. oryzae*. Methanol (2.5 g) was further added at 12 h after starting the reaction. The PBR system was maintained at room temperature and the reaction mixture was continuously circulated at a flow rate of 25 l/h [8]. After one batch reaction cycle, the reaction mixture was replaced with fresh material before starting the next batch reaction. The shematic of the PBR system will hereinafter be described.

2.5. Gas chromatography analysis

Samples were obtained from the reaction mixture at specified intervals and centrifuged at 12,000 rpm for 5 min. The upper oil layer was analyzed by capillary gas chromatography as described previously [5]. Peaks were identified by comparison of their retention times with the references. The ME content in the reaction mixture was determined using a GC-18A gas chromatograph (Shimadzu Co., Kyoto, Japan) equipped with a DB-5 capillary column (0.25 mm × 15 m; J&W Scientific, Folsom, CA, USA). The temperatures of the injector and detector were set at 245 and 320 °C, respectively. The column temperature was initially maintained at 150 °C for 0.5 min, raised to 300 °C at 10 °C/min, and finally maintained at this temperature for 10 min.

The TG, DG, and MG contents in the reaction mixture were determined using a GC-2010 gas chromatograph (Shimadzu Co.) equipped with a ZB-5HT capillary column (0.25 mm × 15 m; Phenomenex, USA). The temperatures of the injector and detector were set at 320 and 380 °C, respectively. The column temperature was initially maintained at 130 °C for 2 min, raised to 350 °C at 10 °C/min, then to 370 °C at 7 °C/min, and finally maintained at this temperature for 10 min. All measurements were replicated at least three times.

3. Results and discussion

3.1. Substrate specificity of mdlB

To confirm the substrate specificity of mdlB toward partial glycerides, mdlB-producing *A. oryzae* was immobilized within BSPs and used as a catalyst for methanolysis. Fig. 2 shows the time course of ME content during the methanolysis of mono-, di-, or triolein. The mdlB-producing *A. oryzae* barely catalyzed the methanolysis of triolein, with the ME content of only 2.44% after 24 h, whereas its ability to produce MEs from mono- and diolein was higher than that from triolein, with the final ME contents of 16.0 and 23.8%, respectively. *A. oryzae* niaD300 as a control strain produced no ME from mono- and diolein under the conditions examined (data not shown). These results suggest that the mdlB-producing *A. oryzae* can effectively catalyze the methanolysis of MG and DG.

Several researchers have reported the two types of lipase that hydrolyze partial glycerides [13,20–22]. One is a lipase showing hydrolytic activity toward only MG [13,20]. The glyceride specificity of the lipase is strict, thus leading to the advantage of its application to MG synthesis. Another is a lipase showing the hydrolytic activity toward both MG and DG, but not TG (e.g., mdlA derived from *P. camembertii*) [21]. Toida et al. [22] found that the purified mdlB from *A. oryzae* can hydrolyze both MG and DG, but not TG, indicating that the hydrolytic characteristics of mdlB are similar to those of mdlA. In accordance with the results of Toida et al. [22], our results show that the mdlB-producing *A. oryzae* possesses glyceride specificity toward both MG and DG. Because the lipase-catalyzed methanolysis produces both MG and DG in the course of the reaction, such a catalytic property of the mdlB-producing *A. oryzae* is suitable for enzymatic biodiesel production.

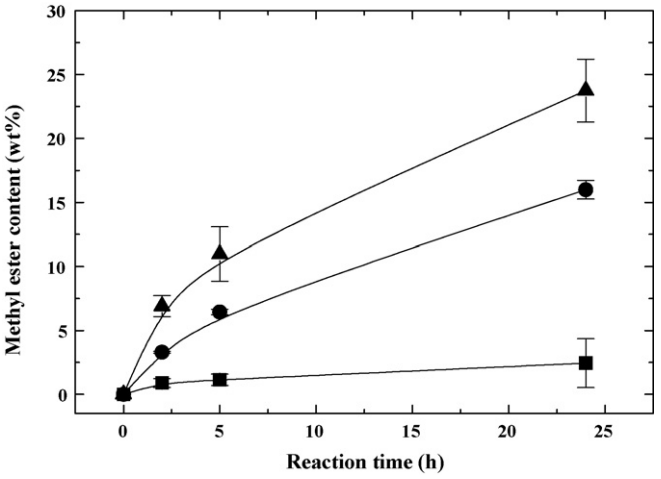

Fig. 2. Time course of ME content during methanolysis of mono-, di-, or triolein using mdlB-producing *A. oryzae*. Methanolysis was carried out in a screw-capped bottle using mono- (●), di- (▲), or triolein (■) as a substrate. Based on three independent experiments, the means and standard deviations were shown.

3.2. Effect of water content on methanolysis using mdlB-producing A. oryzae

Because the progress of methanolysis using several lipases depends on water content [23], mdlB-producing *A. oryzae* was used for methanolysis at varying water contents in the reaction mixture. Fig. 3 shows the ME content in the reaction mixture resulting from

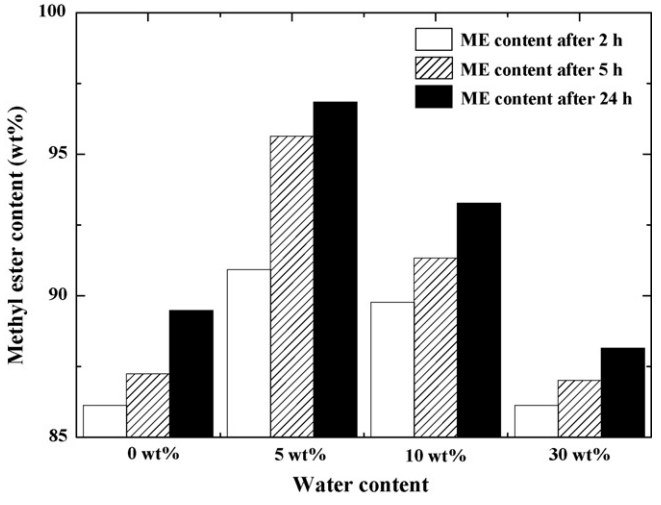

Fig. 3. Effect of water content on methanolysis of intermediate products using mdlB-producing *A. oryzae*. Methanolysis was carried out in a screw-capped bottle using the intermediate products as a substrate. The intermediate products were prepared by *R. oryzae*-catalyzed methanolysis and originally contained 82% ME. Methanolysis was initiated by adding the mdlB-producing *A. oryzae* and methanol to the intermediate products in the presence of 0, 5, 10, or 30% water. Based on three independent experiments, the means were shown.

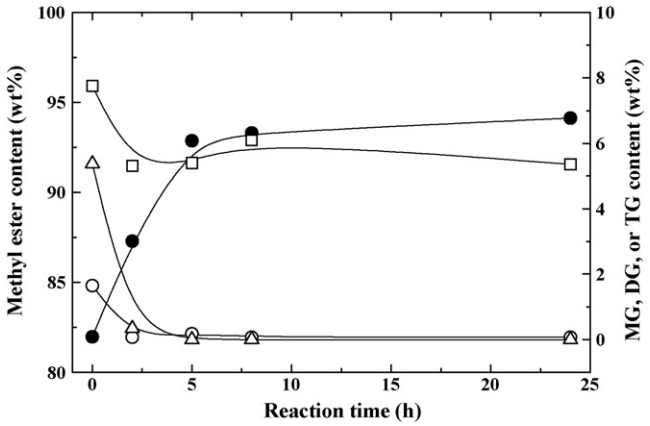

Fig. 4. Time course of ME, MG, DG, and TG contents during methanolysis of intermediate products using mdlB-producing *A. oryzae*. Symbols: (●) ME content; (○) MG content; (△) DG content; (□) TG content. Methanolysis was carried out in the presence of 5% water. Note that the MG and DG contents decreased rapidly. Based on three independent experiments, the means were shown.

methanolysis of the intermediate products, which were prepared by *R. oryzae*-catalyzed methanolysis and contained approximately 82% ME. The best result of methanolysis was obtained in the presence of 5% water, with the final ME content of over 95%. In the case of 10% water, the ME content reached over 90% after a 5-h reaction. In the presence of 0 and 30% water, however, the ME content resulted in less than 90% even after a 24-h reaction. Lipase inactivation might be responsible for the low ME content in the absence of water, because the lipase activity significantly decreased after a 24-h reaction (data not shown). Another possible reason is that mdlB-producing *A. oryzae* produces the MEs mainly by condensation reaction using methanol and free fatty acids, which are hydrolysis products from MG and DG. Adding water thus may be effective in both preventing lipase inactivation and facilitating the hydrolysis of MG and DG, whereas an excess amount of water may act as a competitive inhibitor for esterification and consequently inhibits methanolysis [24]. On the basis of the results, subsequent experiments were carried out in the presence of 5% water.

3.3. Composition of reaction mixtures in methanolysis using mdlB-producing A. oryzae

To confirm the effectiveness of mdlB-producing *A. oryzae* for reducing the levels of partial glycerides, the reactant components were analyzed by gas chromatography. Fig. 4 shows the time course of ME, MG, DG, and TG contents in the reaction mixture resulting from methanolysis of the intermediate products. In accordance with the progress of the ME content, the MG and DG contents decreased rapidly, both of which gave less than 0.1% after a 5-h reaction. However, considerable amounts of TG (5.31–6.09%) remained in the reaction mixture. This is probably because the mdlB-producing *A. oryzae* has a low ability to catalyze the methanolysis of TG, as was observed in Fig. 2. When *R. oryzae* was used for the same reaction with the mdlB-producing *A. oryzae*, the TG content decreased to 2.1% after 24 h, whereas the MG and DG contents were 0.88 and 1.61%, respectively, even after 24 h (data not shown). These results suggest that the mdlB-producing *A. oryzae* can catalyze the methanolysis of MG and DG in the intermediate products more efficiently than *R. oryzae*, and that *R. oryzae* is required for converting TG into MEs.

The substrate preference of the mdlB-producing *A. oryzae* to MG and DG should be one of the major reasons for its higher ability to catalyze the methanolysis of MG and DG in the intermediate products. Okumura et al. [25], for the first time, attempted to use a fungal

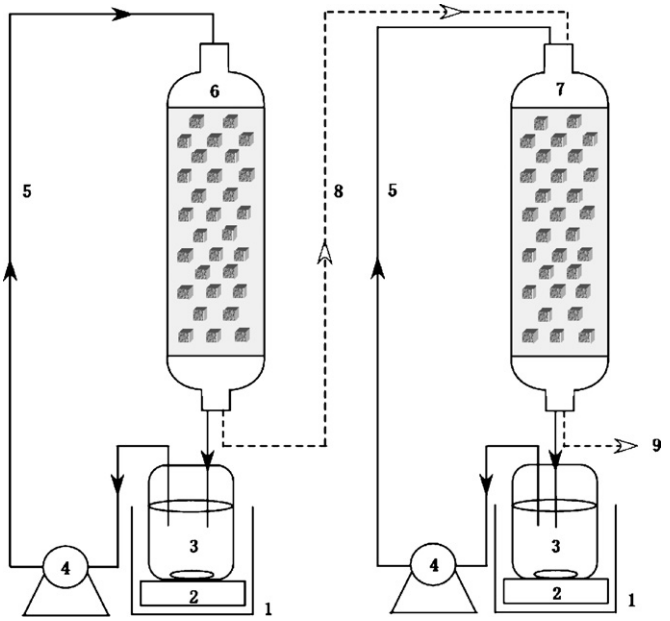

Fig. 5. Schematic of PBR system. (1) Bath sonicator; (2) magnetic stirrer; (3) reaction mixture reservoir; (4) peristaltic pump; (5) TYGON tube; (6) glass column (25 mm internal diameter and 400 mm height) packed with *R. oryzae*; (7) glass column packed with a mixture of *R. oryzae* and mdlB-producing *A. oryzae*; (8) intermediate products; (9) product. Methanolysis was carried out by circulating the reaction mixture in the first column (6). After 48 h, the reaction mixture was transferred into the second column (7) and then circulated for 48 h. One batch reaction cycle in the PBR system includes two steps of methanolysis in the first and second columns.

mono- and diacylglycerol lipase for plant oil hydrolysis, where the enhanced effect on hydrolysis was observed by using a mixture of triacylglycerol lipase and mono- and diacylglycerol lipase. Their results suggest that the mono- and diacylglycerol lipase can help in the hydrolysis of partial glycerides, to which the triacylglycerol lipase shows relatively low preference. It thus seems likely that the use of mono- and diacylglycerol lipase together with a triacylglycerol lipase is effective in improving enzymatic hydrolysis and methanolysis.

We also believe in the possibility that the nonpositional specificity of mdlB [22] contributes to the effective methanolysis activity of mdlB-producing *A. oryzae* toward MG and DG. Because *R. oryzae* lipase shows 1,3-positional specificity toward plant oils, it cannot

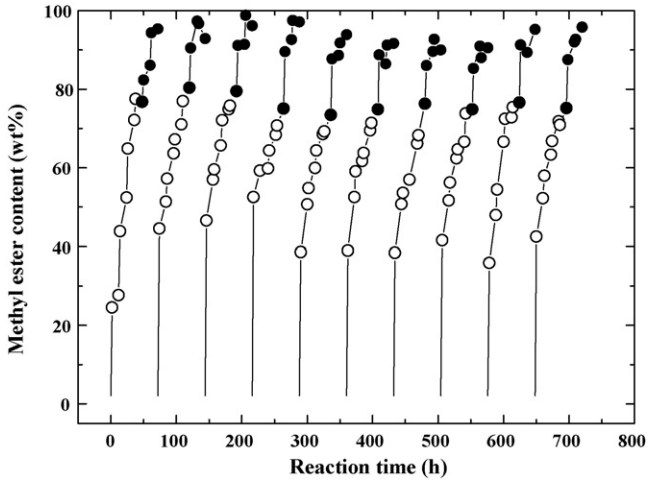

Fig. 6. Time course of ME content during 10 repeated-batch cycles of methanolysis in PBR system. Symbols: (○) first step of reaction using *R. oryzae*; (●) second step of reaction using a mixture of *R. oryzae* and mdlB-producing *A. oryzae*.

Table 1
MG, DG, and TG contents in reaction mixtures at the ends of first and second steps during repeated-batch methanolysis in PBR system.

Cycle number	MG content		DG content		TG content	
	First step[a]	Second step[b]	First step[a]	Second step[b]	First step[a]	Second step[b]
1	4.75	0.08	7.68	N.D.[c]	8.34	2.70
3	3.98	0.32	9.28	0.22	11.5	1.08
5	4.55	0.30	10.9	0.39	10.6	2.92
7	7.05	0.59	11.0	0.52	6.67	3.12
10	7.64	0.69	12.6	1.45	9.21	2.28

[a] MG, DG, or TG content in the reaction mixture at the end of the first step.
[b] MG, DG, or TG content in the reaction mixture at the end of the second step.
[c] Not detected.

directly convert the *sn*-2 acyl chains into MEs without acyl migration from the *sn*-2 to *sn*-1(3) position [26]. In addition, a study by Toida et al. [22] showed that the purified mdlB hydrolyzes both *sn*-1(3) and *sn*-2 acyl chains of MG and DG. Thus, it can be assumed that the nonpositional specificity of mdlB enables the mdlB-producing *A. oryzae* to convert 2-MG and 1(2)-DG into MEs more efficiently than *R. oryzae*.

3.4. Two-step batch methanolysis in PBR

On the basis of the results described above, we developed a PBR system consisting of the first column with *R. oryzae* and second column containing a mixture of *R. oryzae* and mdlB-producing *A. oryzae*. As illustrated in Fig. 5, one batch reaction cycle in the PBR system includes two methanolysis steps. Fig. 6 shows the time course of ME content during 10 repeated-batch cycles of methanolysis in the PBR system. At the first step of each cycle, the ME content reached 73.5–80.4% after 48 h (see open symbols in Fig. 6). When the effluent from the first column was transferred into the second column, the final ME content at the first batch cycle reached 95.4% (see closed symbols in Fig. 6). Moreover, the PBR system showed high lipase stability, reaching the final ME content of over 90% during 10 repeated-batch cycles. This ME production level is higher than that in our previous report using only *R. oryzae*, wherein the final ME content in a PBR reached 80–90% during 10 repeated-batch cycles [8].

To confirm the reduced levels of partial glycerides, we investigated the MG, DG, and TG contents in the reaction mixtures at the ends of the first and second steps (Table 1). In all the reaction cycles, the reaction mixture at the end of the first step contained considerable amounts of MG, DG, and TG, whereas these contents decreased significantly at the end of the second step. Note that, in all the reaction cycles, the MG content meets the standards such as EN14214 and JASO M 360 (<0.8%). The reduced levels of MG and DG should contribute to the substantial improvement in the ME conversion, because the PBR system using *R. oryzae* alone cannot maintain less than 2.0% of the MG and DG contents during repeated-batch methanolysis [8,9]. For further improvement in the DG and TG contents, it is necessary to optimize the mixture ratio of *R. oryzae* and mdlB-producing *A. oryzae*, improve lipase activity, and purify the reaction mixture through downstream processes. Future work by our group will also focus on the development of recombinant cells producing both triacylglycerol lipase and mdlB.

4. Conclusions

In this study, we attempted to use a mono- and diacylglycerol lipase to convert residual partial glycerides efficiently into their corresponding MEs. The newly developed mdlB-producing *A. oryzae* showed an effective methanolysis activity toward MG and DG. In the presence of 5% water, the methanolysis of intermediate products using mdlB-producing *A. oryzae* proceeded most efficiently, resulting in less than 0.1% of MG and DG. Ten repeated-batch methanolysis cycles in the PBR, containing both *R. oryzae* and mdlB-producing *A. oryzae*, maintained a high ME content of over 90% with MG and DG at 0.08–0.69 and 0.22–1.45%, respectively. These results indicate that the use of mdlB-producing *A. oryzae* allows maintaining MG and DG at low levels during long-term repeated-batch methanolysis. We thus conclude that the proposed method is effective for improving enzymatic biodiesel production.

Acknowledgements

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