Synthesis of Fatty Acid Methyl Esters Using Mixed Enzyme in a Packed Bed Reactor

Jiin Ryu¹#, Nakyung Choi²#, Heejin Kim¹, Byung Hee Kim³, Hak-Ryul Kim⁴ and In-Hwan Kim¹, 2*  

¹ Department of Public Health Sciences, Graduate School, Korea University, 145 Anam-Ro, Sungbuk-Gu, Seoul, 02841, Republic of KOREA 
² Department of Integrated Biomedical and Life Science, Graduate School, KOREA University, 145 Anam-Ro, Sungbuk-Gu, Seoul, 02841, Republic of KOREA 
³ Department of Food and Nutrition, Sookmyung Women’s University, Seoul, 04310, Republic of KOREA 
⁴ School of Food Science and Biotechnology, Kyungpook National University, Daegu, 41566, Republic of KOREA 
* Jiin Ryu and Nakyung Choi contributed equally to this research.

Abstract: Fatty acid methyl esters were synthesized from palm fatty acid distillate (PFAD) and methanol in a packed bed reactor via lipase-catalyzed esterification. The PFAD consisted of 91 wt% of free fatty acids, 2 wt% monoacylglycerides, 3 wt% diacylglycerides, and 4 wt% triacylglycerides. t-Butanol was employed as a reaction medium and a mixed enzyme consisting of Lipozyme TL IM from Thermomyces lanuginosus and Novozym 435 from Candida antarctica was employed as the biocatalyst. The effect of mixed enzyme was investigated and the optimum blending ratio (w/w) of Novozym 435 to Lipozyme TL IM was 5:95. Using the mixed enzyme, the optimum molar ratio (PFAD to methanol) and temperature were determined to be 1:6 and 30°C, respectively. Under the optimized conditions, the maximum yield of ca. 96% was achieved.

Key words: fatty acid methyl ester, mixed enzyme, packed bed reactor, palm fatty acid distillate, esterification

1 Introduction

Biodiesel is currently used as a biofuel for diesel engines. It consists of mono alkyl esters of long chain fatty acids formed by the transesterification of various oils with alcohols[1]. Because of the concerns regarding pollution problems which were caused by the widespread use of fossil fuels, it has become increasingly required to develop renewable energy sources with low environmental impacts, such as biodiesel[2].

Many transesterification catalysts can be employed in biodiesel synthesis, including acids, bases, and free or immobilized enzymes[3]. Currently, the majority of industrial-scale biodiesel synthesis is accelerated by alkaline-catalysts. However, prior to the alkaline-catalyzed transesterification, the raw materials must be preprocessed to remove the water or free fatty acids so that saponification does not occur during transesterification. Moreover, a large amount of wastewater are also produced when alkaline catalysts are employed[4]. In contrast, biodiesel can be synthesized via enzyme-catalyzed reactions under mild conditions even when the oil feedstock contains high amount of free fatty acids[5]. Nevertheless, the industrial production of biodiesel via enzymatic transesterifications is limited because of its high cost. The cost of enzymatic method for large scale production is still more expensive than that of chemical method[6].

Recently, numerous efforts have been made to reduce the cost of biodiesel production because of falling crude diesel prices[7]. As such, low value feedstocks such as used frying oil, acid oil, and fatty acid distillate have been employed for the production of biodiesel. Palm fatty acid distillate (PFAD), which is a by-product obtained during the palm oil refining process, is one of the low-value feedstocks[8]. In fact, the price of PFAD is much less than that of other refined oils that represent the major feedstocks for the currently operational biodiesel plants[9]. In addition, low-cost lipases have been suggested for the production of less expensive biodiesel. Novozym 435, Lipozyme RM IM, and Lipozyme TL IM are representative commercial lipases that have been researched for the production of biodiesel. Although it is well-known that Novozym 435 is effective during various transesterifications, it would not be economically viable as a biocatalyst for the large-scale production of biodiesel because it is one of the most expensive immobilized lipases currently available. In contrast, Lipozyme TL IM from Thermomyces lanuginosus is one of the

*Correspondence to: In-Hwan Kim, School of Biosystem and Biomedical Science, Korea University, 145 Anam-Ro, Sungbuk-Gu, Seoul, 02841, Republic of KOREA 
E-mail: k610in@korea.ac.kr 
Accepted October 17, 2017 (received for review September 1, 2017)
least expensive immobilized lipases.

In the present study, the synthesis of fatty acid methyl esters (FAMES) from PFAD using the mixed enzyme in a packed bed reactor (PBR) was investigated. Novozym 435 from *Candida antarctica* and Lipozyme TL IM from *Thermomyces lanuginosus* were employed as the mixed enzyme. PFAD and methanol were used as the substrates and t-butanol was employed as the reaction medium. The effects of the mixed enzyme, the molar ratio of PFAD to methanol, and the reaction temperature were investigated.

### 2 Experimental

#### 2.1 Materials

PFAD obtained from a palm oil refining process was donated by SK Chemicals (Gyeonggi-do, Republic of Korea). The PFAD consisted of 91 wt% free fatty acids, 2 wt% monoacylglycerides, 3 wt% diacylglycerides, and 4 wt% triacylglycerides. The fatty acid composition of PFAD was 50.8 mol% palmitic acid, 4.8 mol% stearic acid, 35.7 mol% oleic acid, and 8.7 mol% linoleic acid. The composition of FAMES obtained under optimum condition was the same as that of PFAD. Lipozyme TL IM from *Thermomyces lanuginosus* and Novozym 435 from *Candida antarctica* were purchased from Novozymes (Seoul, Republic of Korea). The activities of the enzymes were 180,875 U/mg for Lipozyme TL IM and 5,500 U/mg for Novozym 435, respectively, where 1 U corresponds to the amount of enzyme which releases 1 μmol of butyric acid per minute from tributyrin at pH 7.0 and 30°C. All of the other chemicals used in this study were purchased as the analytical grade unless otherwise noted.

#### 2.2 Lipase-catalyzed esterification

Lipase-catalyzed esterification of PFAD with methanol was performed in a small scale PBR. t-Butanol was used as the reaction medium and was added into the substrates with the same amount of the PFAD (w/w). Fig. 1 showed a diagram of the PBR used in this study. The PBR consisted of a 5.1 cm long stainless steel tube (0.48 cm i.d.) and a reactor (6.5 cm length × 4.65 mm i.d.). The enzyme (0.42 g) was packed manually into the reactor. The PBR was placed in a water bath equipped with a water circulator and a digital temperature controller (Lauda, Lauda-Königshofen, Germany) to maintain a constant temperature. A syringe pump (Model 200; KD Scientific, New Hope, Pa., USA) was used to continuously supply the substrate mixture from a 50 mL glass syringe to the reactor.

For lipase-catalyzed esterification, the mixed enzyme was prepared from Novozym 435 and Lipozyme TL IM. The blending ratio (w/w) of Novozym 435 to Lipozyme TL IM were 100:0 (100% Novozym 435), 25:75, 10:90, 5:95, 1:99, and 0:100 (100% Lipozyme TL IM). The substrate mixture was placed in the 50 mL glass syringe and subsequently injected into the base of the reactor, where it displaced the air in the bed as the liquid flowed upwards. Each experimental trial involved first flushing the reactor to establish thermal equilibrium and to remove residual air bubbles from the column. For the first 15 min of operation, the substrate mixture was supplied at a rate corresponding to a mean residence time of 3 min. Hence, five void volumes of the substrate mixture passed through the reactor during this purge/thermal equilibration stage of the trial. Throughout each trial, reaction mixture aliquots were collected after the reactor had first been pre-conditioned using three void volumes of the substrate mixture to be used, after which the flow rate was adjusted to the desired residence time. All trials were conducted in duplicate.

#### 2.3 Analysis of products

Samples were withdrawn at appropriate time intervals during the enzymatic reaction and sample aliquots (10 mg) was dissolved in 1 mL chloroform for a gas chromatographic analysis. A gas chromatography (Model 3800; Varian Inc., Palo Alto, CA, USA) equipped with a fused silica capillary column (DB-1ht, 15 m × 0.25 mm i.d. × 0.15 μm film thickness, J&W Scientific, Folsom, CA, USA) and a flame ionization detector (FID) were used for analysis. Initially, the column was held at 120°C for 3 min and programmed to rise to 370°C at a rate of 20°C/min. Then, the column was held at 370°C for 5 min. The carrier gas was helium at a flow rate of 1.5 mL/min and the split ratio was 50:1. The injector and detector temperatures were maintained at 370°C. The amounts of acylglycerols, FAMES and free fatty acids were measured by a gas chromatography. The amount of free fatty acids was measured by neutralization of the fatty acids in the sample with a 0.1 M solution of KOH (AOCS Cd 3d-63) because the free fatty acids and FAMES were not separated by a gas chromatography. The yield (%) of FAMES was calculated as follows:

![Fig. 1 A diagram of packed bed reactor (PBR) used for lipase-catalyzed esterification.](image-url)
Synthesis of Fatty Acid Methyl Esters Using Mixed Enzyme

Yield (%) = \( \frac{a}{b} \times 100 \)

where \( a \) is the weight of FAMEs in the reaction mixture and \( b \) is the total weights of FAMEs, free fatty acids and acylglycerols in the reaction mixture.

3 Results and Discussion

3.1 Effect of the mixed enzyme

Novozym 435, which is an active and versatile enzyme, is highly stable in the presence of both methanol and ethanol\(^{12-14}\). Novozym 435 has been investigated extensively for biodiesel applications, although it is too expensive for the practical industrialized production of biodiesel\(^{15}\). On the other hand, Lipozyme TL IM has been reported to be more prone to deactivation by short chain alcohols such as methanol and ethanol\(^{16}\). Nevertheless, Lipozyme TL IM has been widely employed in various lipase-catalyzed reactions because it is one of the least expensive lipases\(^{16-18}\). In order to overcome these problems, esterification was performed for the synthesis of FAMEs using a mixed enzyme as the biocatalyst. The effect of the mixed enzyme on the yield of FAMEs as a function of residence time was shown in Fig. 2. The blending ratios (w/w) of Novozym 435 to Lipozyme TL IM examined were 100:0 (100% Novozym 435), 25:75, 10:90, 5:95, 1:99, and 0:100 (100% Lipozyme TL IM). In these trials, the molar ratio of PFAD to methanol, and the temperature were 1:6 and 50°C, respectively.

The reaction rate increased significantly as the proportion of Novozym 435 in the mixed enzyme was increased from 0 (100% Lipozyme TL IM) to 25%. In particular, a marked increase in the reaction rate was observed when the proportion of Novozym 435 was increased from 0 to 5%. For the trials between 5% Novozym 435 and 100% Novozym 435, the yields of FAMEs approached a plateau after 60 min. However, at 2.5% Novozym 435 and 0% Novozym 435 (100% Lipozyme TL IM), the yields of FAMEs increased gradually until longer reaction times, and did not reach a plateau throughout the entire period. The maximum yields of ca. 96% at 25% Novozym 435 and 100% Novozym 435 were achieved at 30 min, whereas those at 5% Novozym 435 and 10% Novozym 435 were achieved at 120 min. There have been prior studies regarding the production of biodiesel using mixed enzymes in a batch reactor. For example, Li et al., reported that the combined use of Lipozyme TL IM and Novozym 435 at a ratio of 3:1 gave the highest degree of methanolysis and subsequently produced a biodiesel yield of ca. 95% in a batch system\(^{17}\). Lee et al. determined that the mixed enzyme of R. oryzae lipase and C. rugosa lipase gave a higher yield of biodiesel than a single lipase\(^{18}\). There have also been some limited research regarding the synthesis of biodiesel using a mixed enzyme in a continuous reactor, but the reported yield was significantly lower than our present results\(^{20}\).

Overall, in the present study, although the reaction rate of the 25% Novozym 435 trial was faster than obtained with 5% Novozym 435, similar maximum yields of ca. 96% were obtained in both cases after 120 min. Therefore, 5% Novozym 435 was selected as the optimum condition, based on considering the economics of the process.

3.2 Effect of molar ratio

The effect of the molar ratio (PFAD to methanol) on the yield of FAMEs as a function of residence time was shown in Fig. 3. The range of molar ratio examined was from 1:2 to 1:8. In these trials, the mixed enzyme and the temperature were 5% Novozym 435 and 50°C. During the first 20 min of the reaction, the highest reaction rate was observed in the trials of 1:2 and 1:4. In the trial of 1:2, the yield did not further increase when a residence time was increased from 20 to 120 min. Meanwhile, in the trial of 1:4, the yield increased slowly after 20 min and reached the maximum yield of ca. 93% at 120 min. In the trials of 1:6 and 1:8, the esterification reached the equilibrium within 60 min, and the maximum yields (ca. 96%) were similar in both. For enzymatic reaction, the use of methanol can cause problems because it tends to denature lipases to a greater extent than longer aliphatic alcohols\(^{21,22}\). To overcome this drawback, trials using a stepwise addition of methanol to the reaction mixtures have been performed in previous studies\(^{23,24}\). However, our results demonstrated high yields of FAMEs was obtained, even when high amount of methanol was...
used during the esterification. This result can be possibly attributed to the t-butanol used as the reaction medium. t-Butanol increases the solubility of methanol in oil and thus lowers the inhibitory effects on the lipase. Therefore, it could increase the catalytic efficiency and stability. Based on the above results, a molar ratio of 1:6 (PFAD to methanol) was selected as the optimum and used in subsequent trials.

3.3 Effect of temperature

Enzymatic esterification is generally performed at lower temperature compared to the temperatures applied when using a chemical catalyst. Reaction temperature is critical in an enzymatic reaction because it controls the activity of the enzymes. An increase in temperature usually results in an acceleration effect, although overly high temperatures can deactivate an enzyme.

The effect of temperature on the yield of FAMEs as a function of residence time was shown in Fig. 4. The ranges of temperature examined were between 20 and 60°C. In these trials, the mixed enzyme and the molar ratio of PFAD to methanol were 5% Novozym 435 and 1:6. The times required to reach the equilibrium at 30 and 40°C were shorter than those at the other temperatures examined in this study. In addition, the maximum yields of ca. 96% were achieved within 60 min at both these temperatures, while it took 120 min to obtain the maximum yields at the other temperatures. The activity and stability of the mixed enzyme appear to be highly determined by Lipozyme TL IM, because this enzyme accounted for the majority of the mixed enzyme. Sim et al. have reported that the production rate of fatty acid methyl ester decreased at temperatures above 40°C, when the esterification of crude palm oil with methanol was carried out using Lipozyme TL IM.

4 Conclusion

The mixed enzyme consisting of Novozym 435 and Lipozyme TL IM was shown to improve the catalytic performance during the synthesis of FAMEs from PFAD in a PBR. The optimum conditions when using the mixed enzyme were determined and the highest yield of ca. 96% was attained under the optimum conditions. These results demonstrate the potential application of enzymes to industrial-scale biodiesel production.

Acknowledgements

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF), funded by the Ministry of Education, Science, and Technology (2015R1D1A1A01057958).

References

1) Knothe, G. Dependence of biodiesel fuel properties on
the structure of fatty acid alkyl esters. *Fuel Process Technol.* **86**, 1059-1070 (2005).
2) Meher, L.; Sagar, D.V.; Naik, S. Technical aspects of biodiesel production by transesterification-a review. *Renew. Sust. Energ. Rev.* **10**, 248-268 (2006).
3) Haas, M.J.; McAlone, A.J.; Yee, W.C.; Foglia, T.A. A process model to estimate biodiesel production costs. *Bioresour. Technol.* **97**, 671-678 (2006).
4) Atadashi, I.; Aroua, M.; Aziz, A.A. Biodiesel separation and purification: a review. *Renew. Energ.* **36**, 437-443 (2011).
5) Juan, J.C.; Kartika, D.A.; Wu, T.Y.; Hin, T.-Y.Y. Biodiesel production from jatropha oil by catalytic and non-catalytic approaches: an overview. *Bioresour. Technol.* **102**, 452-460 (2011).
6) Du, W.; Xu, Y.; Liu, D.; Zeng, J. Comparative study on lipase-catalyzed transformation of soybean oil for biodiesel production with different acyl acceptors. *J. Mol. Catal. B: Enzym.* **30**, 125-129 (2004).
7) Fjerbaek, L.; Christensen, K.V.; Norddahl, B. A review of the current state of biodiesel production using enzymatic transesterification. *Biotechnol. Bioeng.* **102**, 1298-1315 (2009).
8) Nielsen, P.M.; Brask, J.; Fjerbaek, L. Enzymatic biodiesel production: technical and economical considerations. *Eur. J. Lipid Sci. Technol.* **110**, 692-700 (2008).
9) Subramanian, K.; Singal, S.; Saxena, M.; Singhal, S. Utilization of liquid biofuels in automotive diesel engines: an Indian perspective. *Biomass Bioenerg.* **29**, 65-73 (2005).
10) Chongkhong, S.; Tongurai, C.; Chetpattananondh, P.; Bunyakan, C. Biodiesel production by esterification of palm fatty acid distillate. *Biomass Bioenerg.* **31**, 563-568 (2007).
11) Cho, H.J.; Kim, S.H.; Hong, S.W.; Yeo, Y.-K. A single step non-catalytic esterification of palm fatty acid distillate (PFAD) for biodiesel production. *Fuel* **93**, 373-380 (2012).
12) Hernández-Martín, E.; Otero, C. Different enzyme requirements for the synthesis of biodiesel: Novozym® 435 and Lipzyme® TL IM. *Bioresour. Technol.* **99**, 277-286 (2008).
13) Madras, G.; Kolluru, C.; Kumar, R. Synthesis of biodiesel in supercritical fluids. *Fuel* **83**, 2029-2033 (2004).
14) Ranganathan, S.V.; Narasimhan, S.L.; Muthukumar, K. An overview of enzymatic production of biodiesel. *Bioresour. Technol.* **99**, 3975-3981 (2008).
15) Wang, L.; Du, W.; Liu, D.; Li, L.; Dai, N. Lipase-catalyzed biodiesel production from soybean oil deodorizer distillate with absorbent present in tert-butanol system. *J. Mol. Catal. B: Enzym.* **43**, 29-32 (2006).
16) Basri, M.; Kassim, M.A.; Mohamad, R.; Ariff, A.B. Optimization and kinetic study on the synthesis of palm oil ester using Lipzyme TL IM. *J. Mol. Catal. B: Enzym.* **85**, 214-219 (2013).
17) Li, L.; Du, W.; Liu, D.; Wang, L.; Li, Z. Lipase-catalyzed transesterification of rapeseed oils for biodiesel production with a novel organic solvent as the reaction medium. *J. Mol. Catal. B: Enzym.* **43**, 58-62 (2006).
18) Reyes-Duarte, D.; Lopez-Cortes, N.; Torres, P.; Comelles, F.; Parra, J.; Peruia, S.; Ugidos, A.; Ballesteros, A.; Plou, F. Synthesis and properties of ascorbyl esters catalyzed by Lipzyme TL IM using triglycerides as acyl donors. *J. Am. Oil Chem. Soc.* **88**, 57-64 (2011).
19) Lee, D.H.; Kim, J.M.; Shin, H.Y.; Kang, S.W.; Kim, S.W. Biodiesel production using a mixture of immobilized *Rhizopus oryzae* and *Candida rugosa* lipases. *Biotechnol. Bioprocess Eng.* **11**, 522-525 (2006).
20) Tongboriboon, K.; Cheirsilp, B.; Aran, H. Mixed lipases for efficient enzymatic synthesis of biodiesel from used palm oil and ethanol in a solvent-free system. *J. Mol. Catal. B: Enzym.* **67**, 52-59 (2010).
21) Salis, A.; Pinna, M.; Monduzzi, M.; Solinas, V. Biodiesel production from triolein and short chain alcohols through biocatalysis. *J. Biotechnol.* **119**, 291-299 (2005).
22) Tan, T.; Lu, J.; Nie, K.; Deng, L.; Wang, F. Biodiesel production with immobilized *Rhizopus oryzae* and *Candida antarctica* lipases. *Biotechnol. Adv.* **28**, 628-634 (2010).
23) Shimada, Y.; Watanabe, Y.; Samukawa, T.; Sugihara, A.; Noda, H.; Fukuda, H.; Tominaga, Y. Conversion of vegetable oil to biodiesel using immobilized *Candida antarctica* lipase. *J. Am. Oil Chem. Soc.* **76**, 789-793 (1999).
24) Shimada, Y.; Watanabe, Y.; Sugihara, A.; Tominaga, Y. Enzymatic alcoholysis for biodiesel fuel production and application of the reaction to oil processing. *J. Mol. Catal. B: Enzym.* **17**, 133-142 (2002).
25) Chen, J.-W.; Wu, W.-T. Regeneration of immobilized *Candida antarctica* lipase for transesterification. *J. Biocat. Bioeng.* **95**, 466-469 (2003).
26) Watanabe, Y.; Shimada, Y.; Sugihara, A.; Noda, H.; Fukuda, H.; Tominaga, Y. Continuous production of biodiesel fuel from vegetable oil using immobilized *Candida antarctica* lipase. *J. Am. Oil Chem. Soc.* **77**, 355-360 (2000).
27) Bajaj, A.; Lohan, P.; Jha, P.N.; Mehrotra, R. Biodiesel production through lipase catalyzed transesterification: an overview. *J. Mol. Catal. B: Enzym.* **62**, 9-14 (2010).
28) Klibanov, A.M. Improving enzymes by using them in organic solvents. *Nature* **409**, 241-246 (2001).
29) Yang, T.; Xu, X.; He, C.; Li, L. Lipase-catalyzed modification of lard to produce human milk fat substitutes. *Food Chem.* **80**, 473-481 (2003).
30) Sim, J.H.; Kamaruddin, A.H.; Bhatia, S. Biodiesel...
FAME productivity, catalytic efficiency and thermal stability of lipzyme TL IM for crude palm oil transesterification with methanol. *J. Am. Oil Chem. Soc.* **87**, 1027-1034 (2010).

31) Dizge, N.; Keskinler, B. Enzymatic production of biodiesel from canola oil using immobilized lipase. *Bio-

mass Bioenerg.* **32**, 1274-1278 (2008).

32) Khor, G.K.; Sim, J.H.; Kamaruddin, A.H.; Uzir, M.H. Thermodynamics and inhibition studies of lipzyme TL IM in biodiesel production via enzymatic transesterification. *Bioresour. Technol.* **101**, 6558-6561 (2010).