

Comparison of methods for preventing methanol inhibition in enzymatic production of biodiesel

Dong Jin Jeon and Sung Ho Yeom[†]

Department of Biochemical Engineering, Gangneung-Wonju National University, Gangneung, Gangwon-do 210-702, Korea
(Received 8 November 2010 • accepted 27 December 2010)

Abstract—During the enzymatic production of biodiesel, methanol has a major inhibitory effect on enzyme activity whereas glycerol has a minor effect. Revitalization of the methanol-deactivated enzyme or pre-incubation of enzyme with various chemicals turned out to be unsuccessful. The stepwise feeding of methanol, a widely used conventional method for preventing methanol inhibition, was optimized in terms of the aliquot number and feeding interval to obtain a high conversion rate as well as a high degree of final biodiesel conversion. The use of six feedings of methanol with an equivalent molar ratio of 0.75 at 3-h intervals was found to be the optimal mode for preventing methanol inhibition; a biodiesel conversion rate of approximately 95% could be achieved within 20 h by using this method. Finally, to prevent contact between the undissolved methanol and the enzyme, methanol was pre-dissolved in water or biodiesel and fed to the mixture of soybean oil and the enzyme. This pre-dissolution method completely prevented enzyme inhibition, and a final biodiesel conversion rate of 82.3% was obtained.

Key words: Biodiesel, Methanol, Inhibition, Stepwise Addition, Pre-dissolution

INTRODUCTION

Global warming and a shortage of energy have recently been recognized as the most critical problems among current global issues [1]. Because fuel accounts for about 30% of the world's total energy usage, there is an urgent need for alternative fuels that are renewable and that emit less toxic exhaust than fossil fuel [2]. Among the alternative energy sources, biodiesel is a renewable, biodegradable, and non-toxic fuel that can be used in diesel cars without having to modify the existing engine design [3]. Biodiesel is a fatty acid methyl ester that is usually produced through transesterification with vegetable oils or animal fats. The processes that adopt chemical catalysts offer the advantage of a high reaction rate but require multi-step units; in addition, the catalysts must be neutralized to prevent corrosion of the engine, which discharges a large amount of wastewater [4,5]. Moreover, glycerol, which is now being used as a valuable intermediate in many industries, cannot be easily recovered with a high degree of purity [5,6].

In contrast, the enzymatic production of biodiesel has attracted a great deal of attention because of its environmentally friendly nature, the ease with which biodiesel and glycerol can be recovered, and the mild operating conditions involved, in terms of temperature and pH [7]. However, the high cost and easy deactivation of enzymes by short-chain acyl donors such as methanol are barriers to the enzymatic production of biodiesel on an industrial scale [8,9]. It is known that methanol inhibits enzyme activity when its molar ratio to soybean oil or to total fatty acids exceeds 1.5 and 0.5, respectively [10-12]. Several methods have been suggested to prevent methanol inhibition, including enzyme pretreatment [13], stepwise methanol feeding [10-12,14], the employment of other acyl donors such as

ethyl acetate [15] and introducing a solvent like t-butanol [8,16]. Watanabe et al. [12] attempted to prevent methanol inhibition by carrying out stepwise methanol feeding, a two-step batch reaction, and a three-step flow reaction. Although they successfully achieved more than 95% conversion with 4% (w/w) enzyme, it took 27 h for the conversion to be effected. Samukawa et al. [13] reported that they could increase the biodiesel conversion rate remarkably and minimize the deactivation of the enzyme through pre-incubation in methyl oleate. Royon et al. [16] reported that the addition of t-butanol eliminated the enzyme deactivation caused by undissolved methanol and increased both the reaction rate and the final biodiesel conversion rate. They were able to obtain a 97% conversion rate, but the reaction required 32.5% of t-butanol and was carried out at a relatively high temperature of 50 °C.

In this study, the factors that cause the deactivation of enzymes during biodiesel production were quantified. In addition, various methods, including the revitalization of deactivated enzymes, pre-incubation of enzymes, stepwise methanol feeding, and the pre-dissolution of methanol, were attempted for preventing methanol inhibition of enzyme activity. Finally, the most practical and feasible method for achieving this objective was suggested.

MATERIALS AND METHODS

1. Chemicals

Soybean oil was purchased from a domestic supplier (CJ, Korea); 99% of the oil was triglycerides composed of 51.8-56.0% linoleic acid, 22.0-27.1% oleic acid, 9.6-11.5% palmitic acid, 6.2-11.1% linolenic acid and smaller percentages of other acids. Methanol (Showa, Japan) was used as the acyl donor. The commercial lipase enzyme used in this study was Novozym 435 (Novo Nordisk, Denmark). Palmitic acid methyl ester, oleic acid methyl ester, linoleic acid methyl ester, and stearic acid methyl ester were purchased from Sigma-Aldrich

[†]To whom correspondence should be addressed.
E-mail: shyoom@gwnu.ac.kr

(USA) to identify and measure the components of biodiesel. The other chemicals were of analytical grade.

2. Analysis

To prepare a calibration curve for the components of biodiesel, each of the previously mentioned methyl esters was dissolved in chloroform (Wako, Japan) to become 100-1,000 mg/L. 1 μ l of the dissolved sample was injected into a GC (HP 5890, USA) equipped with an FID detector and HP-5 column (30 m \times 0.32 mm \times 0.25 μ m film thickness). The temperature of both the injector and detector was 250 °C and that of column was elevated from 150 to 250 °C, 5 °C/min after the oven temperature was initially maintained for 2 min. Helium was used as a carrier gas. Methyl heptadecanoate (Fluka, Japan) was used as an internal standard for GC analysis.

3. Enzyme Activity Measurement

Enzyme activity was measured according to the p-NPP method [17], in which p-NP (p-nitrophenol) and palmitic acid were produced from p-NPP (p-nitrophenylpalmitate) by lipase and p-NP was quantified for the determination of enzyme activity. One unit was defined as the amount of lipase needed to release 1 μ mol of p-NP in 1 min. The p-NPP was dissolved in acetonitrile to become 10 mM, and 1 mL of this solution was mixed with 4 mL of ethanol. After this, 95 mL of 50 mM Tris-HCl (pH 8.0) buffer was gently added to the solution, which was then stored in a refrigerator at 4 °C. 3 mL of the reagent solution was mixed with 0.1 mL or 0.1 g of the experimental sample and placed at 28 °C for 30 min in a shaking incubator. After centrifugation, the absorbance of the supernatant was measured with a UV spectrophotometer (Jasco V-550, Japan) at 405 nm.

4. Degree of Inhibition

1 g of enzyme, corresponding to 3% (w/w) of soybean oil, was added to a flask containing methanol, soybean oil, biodiesel, or glycerol and was then incubated at 30 °C for 6 h in a shaken incubator rotating at 200 rpm. The amount of methanol in the flask was determined according to the theoretical molar requirement for the ratio of methanol to soybean oil (3 : 1), and the amounts of biodiesel and glycerol were determined under the assumption of 100% conversion, that is, molar ratios of biodiesel and glycerol to soybean oil were 3 and 1, respectively. Following incubation, the enzyme was separated by using a filter paper (100 circles, 100 mm, Advantec) and its activity was measured. The degree of enzyme inhibition was calculated with the following equation:

$$DI(\%) = \frac{EA_i - EA_f}{EA_i} \times 100, \quad (1)$$

where, DI, EA_i , and EA_f represent the degree of inhibition, initial enzyme activity, and enzyme activity after incubation, respectively.

5. Revitalization of Deactivated Enzyme

Deactivated enzyme was prepared by mixing fresh enzyme with methanol at 30 °C for 1 h. Each 1 g of deactivated enzyme was incubated in a flask containing 50 mL of candidate revitalizing agents such as ethanol, n-propanol, and methyl oleate for 24 h at 30 °C. Enzyme was then separated from the solution by using the filter paper, in order to measure its activity. Biodiesel production that uses this potentially revitalized enzyme was also investigated in order to clearly show the effect of revitalization under the stepwise addition of methanol, which will be described later.

6. Pre-incubation

Each 20 g of enzyme was placed in a 100 mL flask containing

50 mL of methyl oleate, lauryl amine, or lanolin and incubated at 30 °C for 30 min. After being separated using filter paper, the enzyme was incubated again in 50 mL of soybean oil at 30 °C for 30 min to remove the remaining chemicals and was then separated again from the solution. After soybean oil was mixed with a 0.75, 1.0, 1.5, 1.3 or 4.5 equivalent molar ratio of methanol at 30 °C and 200 rpm for 1 h, biodiesel production was initiated by adding 3% (w/w) of pre-incubated enzyme.

7. Pre-dissolution of Methanol

Each 2.88 g of methanol was added to water or biodiesel that was previously prepared by enzymatic reaction, and the mixture was shaken at 30 °C and 200 rpm for 1 h. Biodiesel production was initiated by adding first 17.4 g of soybean oil and then enzyme to the pre-dissolved methanol. The effect of the pre-dissolution of methanol on the enzyme activity was investigated separately by measuring the enzyme activity after 0.1 g of fresh enzyme was added to the pre-dissolved methanol in water or biodiesel and incubated for 24 h.

8. Batch Biodiesel Production

Biodiesel was produced in a 50 mL flask in which 8.724 g of soybean oil and 0.24 g of methanol were initially placed and stirred for 10 min before the addition of enzyme. Although the methanol feeding mode was different in each case, the total amount of methanol fed to the solution was 1.44 g, which means that the total molar ratio of methanol to soybean oil was 4.5 [18]. The reaction was carried out at 30 °C and 200 rpm in a shaking incubator. All the experiments were performed in duplicate with a resulting deviation less than 10% and average values were presented.

RESULTS AND DISCUSSION

1. Factors that Cause Enzyme Deactivation

As stated, one of the major barriers to the enzymatic production of biodiesel is the ease with which the enzyme is deactivated. To determine the factors that cause this deactivation, the substances involved in biodiesel production-soybean oil, methanol, biodiesel, and glycerol-were tested. The degree of inhibition was quantified as described in Eq. (1) by measuring enzyme activity before and after incubation in each substance. The results show that methanol

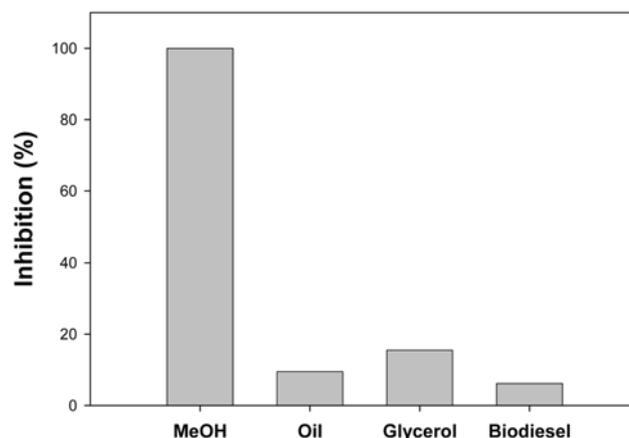


Fig. 1. Inhibition of enzyme activity by the compounds involved in the reaction of biodiesel production.

inhibited enzyme activity completely, while glycerol, soybean oil, and biodiesel did so by 15.5, 9.5, and 6.2%, respectively, as shown in Fig. 1. While other researchers have already reported the inhibition of the enzyme by methanol and glycerol [14,19], the inhibition by soybean oil and biodiesel was unexpected, although their adverse effects were relatively minor. As the results revealed, the inhibition of enzyme activity by methanol was fatal. This study has therefore focused on the prevention of methanol inhibition. Three other methods, as well as conventional stepwise methanol feeding to avoid methanol inhibition, will be investigated in the following sections.

2. Revitalization of Deactivated Enzyme

In the above section, we reported that methanol was found to be the major substance that causes inhibition of enzyme activity. The possibility of revitalizing deactivated enzymes is investigated in this section. Deactivated enzyme was first treated with candidate revitalizing agents, most of which were alcohols, and they were mainly selected from the relevant references regarding the maintenance or enhancement of enzyme activity [13,16,20-22]. These candidate revitalizing agents were ethanol, n-propanol, n-butanol, iso-butanol, butandiol, iso-pentanol, t-butanol, toluene, and methyl oleate. The biodiesel conversions using the chemical-treated enzyme, however, were less than 2%, as shown in Fig. 2. Temperature treatment at -20,

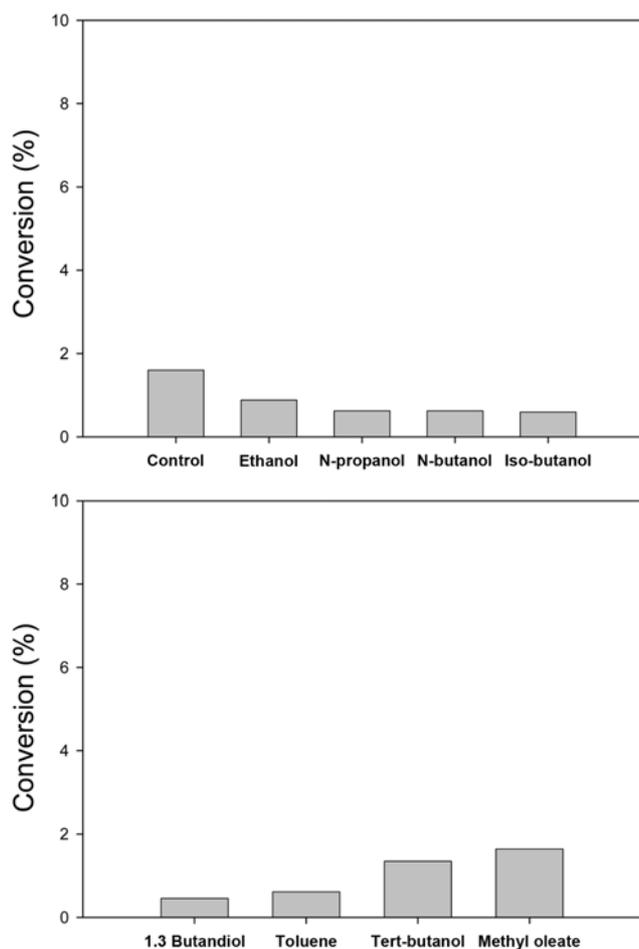


Fig. 2. Revitalization of methanol-deactivated enzyme by being treated with various agents.

Table 1. Effect of pre-incubation of the enzyme in various compounds

Molar ratio (MeOH to Oil)	Biodiesel conversion (%)			
	Non-treatment	Pre-incubation agents		
		Methyl oleate	Lauryl amine	Lanolin
0.75	20.7	23.6	15.4	9.2
1.0	20.3	22.1	15.9	12.6
1.5	18.0	19.9	18.4	11
3.0	2.8	9.1	7.7	4.3
4.5	1.6	8.1	5.3	3.9

4, 50, or 70 °C and pH treatment at 2, 4, 8, or 10 for 30 min were also carried out, though they proved unsuccessful (data not shown). To the best of our knowledge, the revitalization of deactivated enzymes does not seem possible once they have been deactivated by methanol. These results are in substantial agreement with other researchers' observations [10,12], which is that methanol deactivation is irreversible. We therefore concluded that it is more realistic to develop methods to prevent or mitigate methanol inhibition in advance than it is to attempt to revitalize deactivated enzyme.

3. Pre-incubation of Enzyme

In an effort to prevent methanol inhibition, the effect of the pre-incubation of enzyme in a number of promising chemicals was investigated; the results are summarized in Table 1. The chemicals were selected from the research of others [13]. In the case of methyl oleate, when the molar ratio of methanol to soybean oil was 3 or 4.5, the pre-incubated enzyme exhibited biodiesel conversion that was 5 times higher than non-treated enzyme, but the final conversion rate was less than 10%. In addition, the effects of pre-incubation were negligible when the molar ratio was 0.75, 1.0, or 1.5. In the case of lauryl amine or lanolin, pre-incubation produced a relatively minor increase in biodiesel conversion with a molar ratio of 3 or 4.5 compared to the control (Non-treatment), while these two chemicals showed an adverse effect when the molar ratio was 0.75, 1.0 or 1.5. On the whole, the pre-incubation of enzyme does not appear to be effective as a method to prevent methanol inhibition.

4. Stepwise Addition of Methanol

In the present study, the total molar ratio of methanol to soybean oil was 4.5 and the mass ratio of enzyme to soybean oil was 3% (w/w). Since methanol has an inhibitory effect on enzymes, it must be fed to the enzyme in a manner that avoids methanol inhibition. The stepwise addition of methanol has been widely employed to prevent methanol inhibition of enzyme activity and has turned out to be successful approach [12,14,23]. However, few attempts have been made to maximize the productivity of biodiesel, which could be a significant industrial application. In this study, the stepwise addition of methanol was optimized in terms of aliquot number and feeding interval.

4-1. Effect of Aliquot Number

To investigate the effect of the aliquot number of methanol on the initial conversion rate and final conversion, methanol of different aliquots was fed to a solution containing soybean oil and enzyme. The aliquots were 1, 3, 6, or 12 but the same total amount of methanol was fed, namely 4.5 molar ratios to soybean oil. The entire metha-

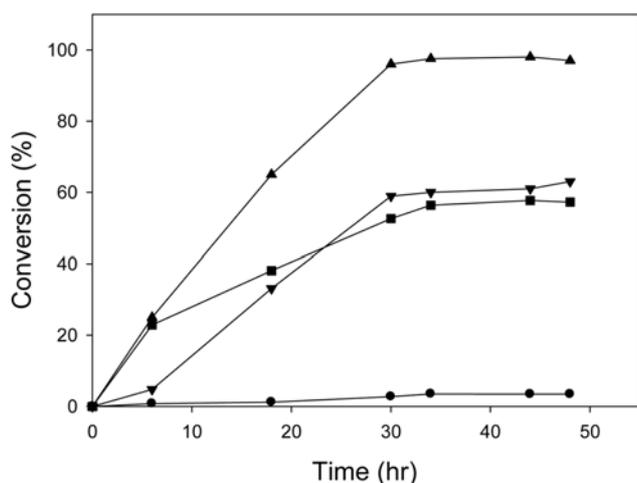
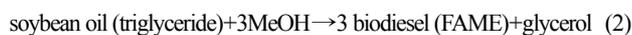


Fig. 3. Effect of aliquot number of methanol on the biodiesel conversion. ●: 1 aliquot, ■: 3 aliquots, ▲: 6 aliquots, ▼: 12 aliquots.

nol was fed during the 36 h of the reaction, regardless of the aliquot number. For example, in the case of the 3 and 12 aliquots, each aliquot was fed every 12 and 3 h, respectively. As shown in Fig. 3, the initial biodiesel conversion rates for the first 6 h were 11.4, 334.4, 365.1, and 70.2 mg/hr for 1, 3, 6, and 12 aliquots, respectively. The final biodiesel conversions after 36 h of reaction were 3.5, 56.4, 97.5, and 60.0% for 1, 3, 6, and 12 aliquots, respectively. These results suggest that the enzyme activity was severely inhibited when the entire methanol was fed at once. Although 3 aliquots showed an initial conversion rate similar to that of 6 aliquots for the first 6 h, this conversion rate decreased afterward, which resulted in a much lower (42%) final conversion rate compared to the case of 6 aliquots. In all cases, the final conversion rate did not noticeably change after 36 h of reaction. For a full explanation of these results, a detailed analysis for Fig. 3 was carried out as follows.

The stoichiometric equation for biodiesel production can be expressed as Eq. (2). The average molecular weight of biodiesel formed is estimated to be 292.14 g/mol for molecular weights of soybean oil, methanol, and glycerol of 872.4, 32.04, and 92.1 g/mol, respectively. Since the rate of molar methanol consumption is theoretically equivalent to the rate of biodiesel formation, the rate of methanol consumption can be suggested as Eq. (3).



$$\text{Methanol consumption rate (mg/h)} = \text{biodiesel formation rate (mg/hr)} \times \frac{32.04}{292.14} \quad (3)$$

Accordingly, the initial rates of methanol consumption were 1.25, 36.7, 43.2, and 7.7 mg/h for 1, 3, 6, and 12 aliquots, respectively. The consumption of methanol in the first 6 h was then 7.5, 220, 259, and 46 mg for 1, 3, 6, and 12 aliquots, respectively. The amount of methanol fed to the reaction for the first 6 h was 1,440, 480, 240, and 240 mg for 1, 3, 6, and 12 aliquots, respectively. Therefore, after 6 h of reaction, the remaining methanol was estimated to be 1,432 mg, 260, 0.0, and 194 mg for 1, 3, 6, and 12 aliquots, respectively. The molar ratios of methanol to soybean oil and biodiesel were es-

Table 2. Mass and molar ratios of methanol to soybean oil and biodiesel after 6 hours' reaction with respect to the methanol aliquot number

Number of aliquots	(Methanol/Soybean oil)		(Methanol/Biodiesel)	
	Mass ratio ($\times 10^2$)	Molar ratio	Mass ratio	Molar ratio
1	16.6	4.51	20.9	190.8
3	3.86	1.05	0.13	1.18
6	0.00	0.00	0.00	0.00
12	2.33	0.64	0.46	4.19

timated and summarized in Table 2. The results show that the molar ratio of methanol to biodiesel was over 1.0 for 1, 3, and 12 aliquots. Since the enzyme was reported to be inhibited when the molar ratio of methanol to soybean oil and biodiesel was over 1.5 and 0.5 respectively, the enzyme was subject to methanol inhibition for 1, 3, and 12 aliquots. Recalling that methanol serves as a substrate as well as an inhibitor of enzyme activity, and that a low concentration of substrate results in a low conversion rate, the low initial conversion rate of the 12 aliquots may be due to too low a concentration of methanol. The reason that 12 aliquots showed a much lower final conversion rate than 3 and 6 aliquots, however, was not determined. On the basis of the results in this section, it could be concluded that 6 aliquots is the optimal split of methanol to obtain a high initial biodiesel conversion rate and a high final conversion.

4-2. Effect of Feeding Interval

The previous results indicate that 6 aliquots of methanol fed every 6 h produced the best performance in terms of initial biodiesel conversion rate and final conversion. To increase the productivity of biodiesel, the mass of biodiesel formed per time per mass of enzyme, the effect of a feeding interval of 6 methanol aliquots are investigated in this section. When each aliquot with a 0.75 molar ratio to soybean oil was fed every 1, 3, and 6 h, the initial conversion rates for the first 6 h were approximately 147, 1,323, and 834 mg/L, respec-

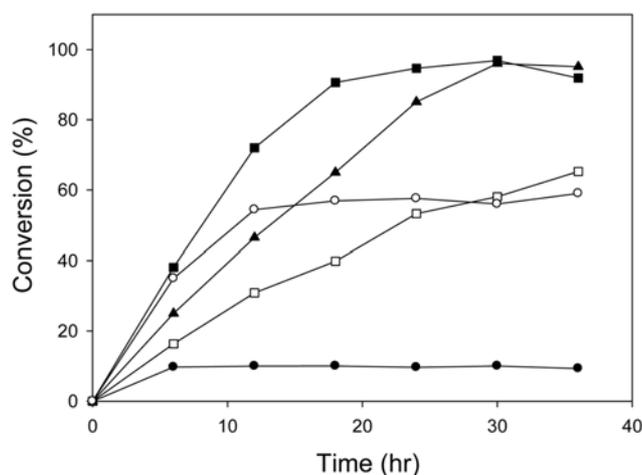


Fig. 4. Effect of feeding interval of methanol on the biodiesel conversion. ●: 6 aliquots every 1 hr, ■: 6 aliquots every 3 hr, ▲: 6 aliquots every 6 hr, ○: 3 aliquots every 1 hr, □: 3 aliquots every 3 hr.

tively, as shown in Fig. 4. The final conversions after 30 h of reaction were 10.0, 96.9, and 80.0%, respectively. In particular, the time required to reach a final conversion greater than 90% was shortened to 20 h when methanol was fed every 3 h instead of every 6 h. The initial conversion rate for 6 aliquots fed every 3 h was also increased by 30% compared to that for every 6 h. Before we concluded that the feeding of 6 aliquots every 3 h was optimal, we investigated two more cases: 3 aliquots (1.5 molar ratio of methanol to soybean oil each) every 3 and 6 h as shown in Fig. 4. In each case, the initial biodiesel conversion rates and final conversions were lower than in the case of 6 aliquots fed every 3 h. It can therefore be said that in terms of the initial biodiesel conversion rate and final conversion, the optimal feeding mode is 6 aliquots fed every 3 h. This feeding mode makes it possible to achieve over 90% conversion within 20 h, which is approximately 50% higher than the results others have achieved in terms of biodiesel productivity [12].

5. Pre-dissolution of Methanol

Since methanol is a hydrophilic compound, it is hardly dissolved in soybean oil. Droplets of pure methanol are believed to cause the irreversible inhibition of enzyme activity [12]. In this study, the pre-dissolution of methanol in compounds with a high solubility for methanol was attempted in order to reduce methanol's inhibitory effect. Water and biodiesel were considered as the compounds not

only because they display a high degree of methanol solubility but also because they themselves cause little inhibition of enzyme activity. As shown in Fig. 5, when 2.88 g of methanol was pre-dissolved in 1 g of water, the enzyme was not protected at all from methanol inhibition, while 20% of the enzyme activity was lost when the methanol was pre-dissolved in 3 g of water. When methanol was pre-dissolved in 5 or 10 g of water, no loss of enzyme activity was observed. It is thus obvious that the pre-dissolution of methanol in water can prevent methanol from inhibiting enzyme activity. In addition, these results suggest that the degree to which the enzyme is protected from methanol inhibition is proportional to the water content, and that there may be a threshold of water content that provides complete protection. Approximately 5 g of water, 173% (w/w) or 310% (mol/mol) of methanol, is required to completely prevent methanol inhibition in this study. In the case of biodiesel, the pre-dissolution of methanol in 1 g of biodiesel, 34.7% (w/w) or 3.9% (mol/mol) of methanol, decreased enzyme deactivation by approximately 80%, and in 3 g or more of biodiesel, enzyme deactivation that was less than 2.5% was ensured. Of the two compounds, biodiesel seems to be more effective than water in protecting methanol inhibition of enzyme activity. Although these results are promising for the prevention of enzyme deactivation, water and biodiesel could also hinder

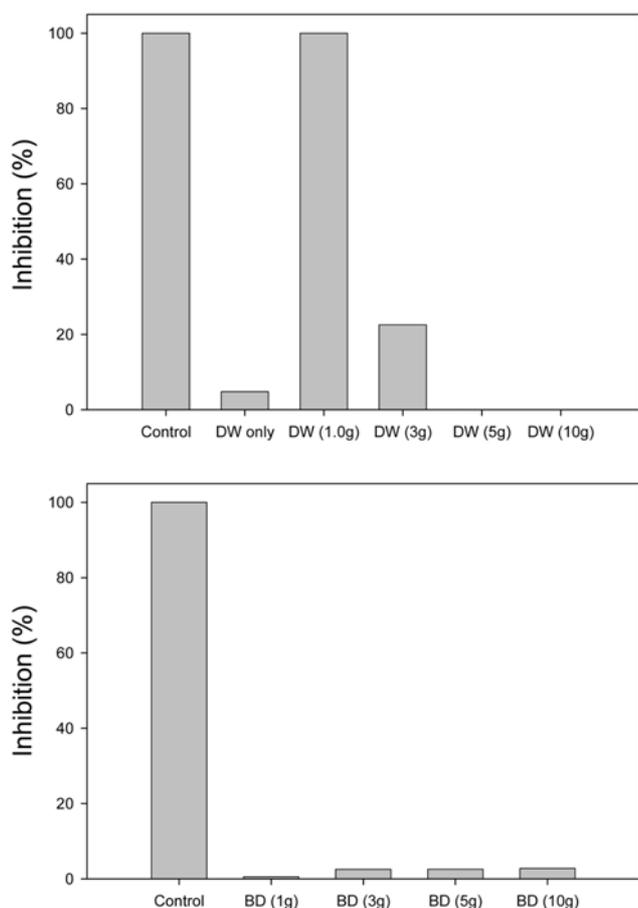


Fig. 5. Effect of methanol pre-dissolution in water or biodiesel on the inhibition of enzyme. DW and BD represent distilled water and biodiesel, respectively. The inhibitions were measured after 24 hours' reaction.

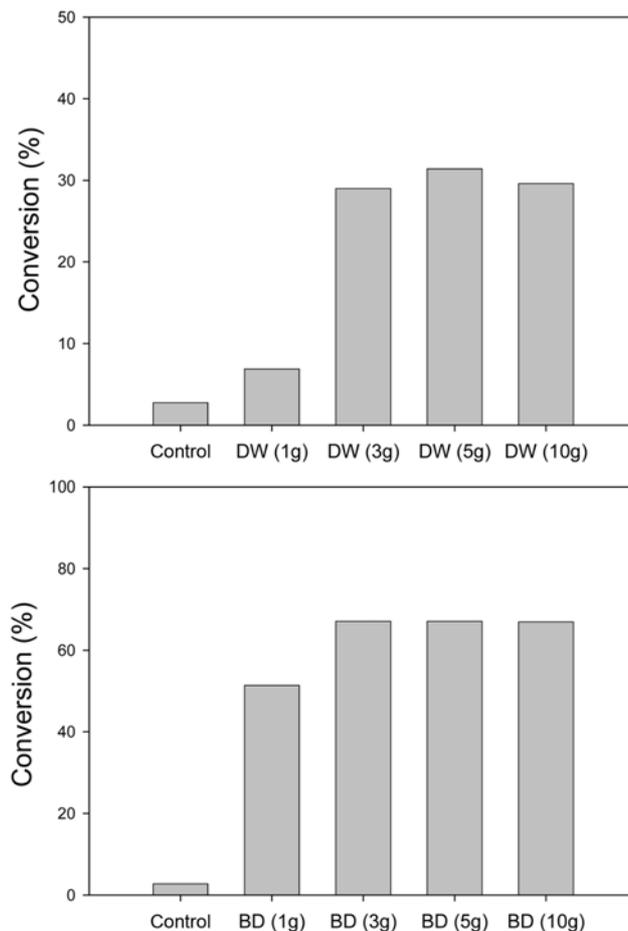


Fig. 6. Effect of methanol pre-dissolution in water or biodiesel on the biodiesel conversion. DW and BD represent distilled water and biodiesel, respectively. The conversions were measured after 24 hour's reaction.

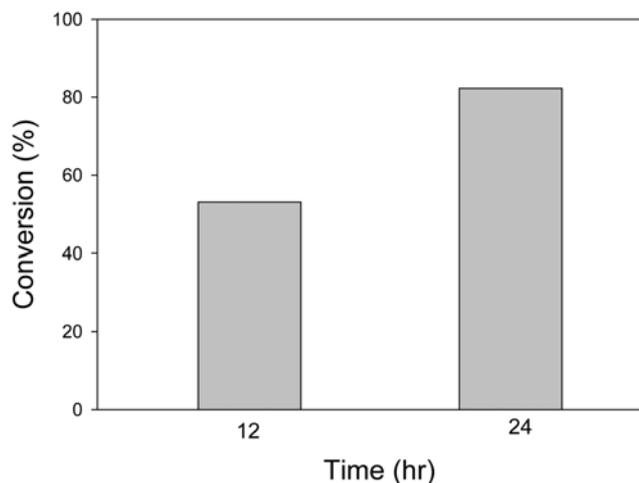


Fig. 7. A simple strategy for the enhancement of biodiesel conversion without the loss of enzyme activity. Half (1.44 g) of methanol was dissolved in 1.5 g of biodiesel and fed to 17.4 g of soybean oil. After 12 hours of reaction, the other half of methanol was fed to the reaction.

the conversion of biodiesel. For the application of this method, then, biodiesel conversion as well as enzyme activity must be investigated. With the pre-dissolution of methanol in water, biodiesel conversion was increased with increasing water content due to the protection from methanol inhibition, but it did not exceed 32%, as shown in Fig. 6. With the pre-dissolution of the same amount of methanol in 1 g and 3 g of biodiesel, the biodiesel conversions were 51.3 and 67.1%, respectively. Biodiesel over 3 g showed no increase in conversion. These results suggest that in terms of biodiesel conversion, biodiesel is much more efficient than water. However, although the pre-dissolution method can provide complete protection against methanol inhibition, the final level of biodiesel conversion is not satisfactory. In particular, despite the negligible enzyme deactivation resulting from the pre-dissolution of methanol in 3 g of biodiesel, the final level of conversion was less than 70%. A simple strategy was employed in an attempt to improve the final biodiesel conversion. That is, half of the methanol was pre-dissolved in 1.5 g of biodiesel and the other half was fed after 12 h of reaction. As shown in Fig. 7, this strategy led to 82.3% biodiesel conversion, which suggests that a more elaborate strategy could markedly improve the final conversion, making this a promising area of future research.

CONCLUSIONS

Although enzymatic biodiesel production has recently attracted a great deal of attention because of its environmentally friendly nature, the ease with which biodiesel can be recovered, the mild operating conditions involved, and easy recovery of high-purity glycerol, the deactivation of enzymes during production remains a large barrier to commercial production. In this study, methanol was found to have a major inhibitory effect on enzyme activity, whereas glycerol had a minor effect during the enzymatic production of biodiesel. Various methods to prevent methanol inhibition were tested and compared. The revitalization of deactivated enzyme and the pre-incubation of enzyme did not prove to be effective. The stepwise feeding of

methanol—the most widely used method and one of proven effectiveness—was optimized in terms of the methanol aliquot number and feeding interval. When 6 aliquots of methanol, each with a 0.375 molar ratio to soybean oil, were fed every 3 h, it took 20 h to attain a final biodiesel conversion of over 90%, and this led to 50% higher productivity than others' results. The pre-dissolution of methanol in water or biodiesel was also tried. Using this method, methanol inhibition of enzyme activity was completely prevented, and final biodiesel conversion of 82.3% was obtained. If a more elaborate strategy is developed based on this pre-dissolution method, a higher final biodiesel conversion can be achieved without enzyme deactivation; such a strategy is expected to remove the barriers to the commercial enzymatic production of biodiesel.

ACKNOWLEDGEMENT

This work was supported by the Korea Research Foundation Grant funded by the Korean Government (KRF-2008-331-D00127) and Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2010-0025572). The authors greatly appreciate these supports.

REFERENCES

1. Y. K. Hong and W. H. Hong, *Korean Chem. Eng. Res.*, **45**(5), 424 (2007).
2. D. Antoni, V. V. Zverlow and W. H. Schwarz, *Appl. Microbiol. Biotechnol.*, **77**, 23 (2007).
3. D. H. Lee, J. M. Kim, H. Y. Shin, S. W. Kang and S. W. Kim, *Biotechnol. Bioprocess Eng.*, **11**(6), 522 (2006).
4. H. Fukuda, S. Hama, S. Tamalampudi and H. Noda, *Trends in Biotechnol.*, **26**(12), 668 (2008).
5. Z. M. Wang, J. S. Lee, J. Y. Park, C. Z. Wu and Z. H. Yuan, *Korean J. Chem. Eng.*, **25**(4), 670 (2008).
6. S. A. Jun, C. H. Kang, S. W. Kong, B. I. Sang and Y. S. Um, *Korean J. Biotechnol. Bioeng.*, **23**(5), 413 (2008).
7. K. Ban, S. Hama, K. Nishizuka, M. Kaieda, T. Matsumoto, A. Kondo, H. Noda and H. Fukuda, *J. Mol. Catal. B: Enzym.*, **17**, 157 (2002).
8. L. Li, W. Du, D. Liu, L. Wang and Z. Li, *J. Mol. Catal. B: Enzym.*, **43**, 58 (2006).
9. D. J. Jeon and S. H. Yeom, *Korean J. Chem. Eng.*, **27**(5), 1555 (2010).
10. Y. Shimada, Y. Waanabe, T. Samukawa, A. Sugihara, H. Noda, H. Fukuda and Y. Tominaga, *JAACS*, **76**, 789 (1999).
11. Y. Shimada, Y. Watanabe, A. Sugihara and Y. Tominaga, *J. Mol. Catal. B: Enzym.*, **17**, 133 (2002).
12. Y. Watanabe, Y. Shimada, A. Sugihara, H. Noda, H. Fukuda and Y. Tominaga, *J. Am. Oil. Chem. Soc.*, **77**, 355 (2000).
13. T. Samukawa, M. Kaieda, T. Matsumoto, K. Ban, A. Kondo, Y. Shimada, H. Noda and H. Fukuda, *J. Biosour: Bioeng.*, **90**, 180 (2000).
14. V. R. Srivathsan, L. N. Srinivasan and M. Karuppan, *Biosour: Technol.*, **99**, 3975 (2008).
15. S. J. Kim, S. M. Jung, Y. C. Park and K. M. Park, *Biotechnol. Bioprocess Eng.*, **12**(4), 441 (2007).
16. D. Royon, M. Daz, G. Ellenrieder and S. Locatelli, *Biosour: Tech-*

- mol.*, **98**, 648 (2007).
17. T. C. Hung, R. Giridgar, S. H. Chiou and W. T. Wu, *J. Mol. Catal. B: Enzym.*, **26**, 69 (2003).
 18. A. W. Schwab, M. O. Bagby and B. Freedman, *Fuel*, **66**, 1372 (1987).
 19. K. B. Bako, F. C. S. Kova, L. Gubicza and J. K. Hansco, *Biocatal. Biotransform.*, **20**, 437 (2002).
 20. M. Iso, B. Chem, M. Eguchi, T. Kudo and S. Shrestha, *J. Mol. Catal. B: Enzym.*, **16**, 53 (2001).
 21. M. K. Modi, J. R. C. Reddy, B. V. S. K. Rao and R. B. N. Prasad, *Biotechnol. Lett.*, **28**, 637 (2006).
 22. J. W. Chen and W. T. Wu, *J. Biosci. Bioeng.*, **95**, 466 (2003).
 23. K. Nie, F. Xie, F. Wang and T. Tan, *J. Mol. Catal. B: Enzym.*, **43**, 142 (2006).