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An Organic Soluble Lipase for Water-Free Synthesis of Biodiesel

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Abstract Lipase AK was modified with short alkyl chains to form a highly organic soluble enzyme and was used to catalyze the synthesis of biodiesel from soybean oil in organic media. The effects of several key factors including water content, temperature, and solvent were examined for the solubilized enzyme in comparison with several other commercially available lipases. Whereas native lipases showed no activity in the absence of water, the organic soluble lipase demonstrated reaction rates of up to 33 g-product/g-enzyme h. The biocatalyst remains soluble in the biodiesel product, and therefore, there is no need to be removed because it is expected to be burned along with the diesel in combustion engines. This provides a promising one-pot mix-and-use strategy for biodiesel production.

Keywords Biodiesel · Transesterification · Nonaqueous biocatalysis · Lipases · Enzymes

Introduction

Biodiesel is pursued not only for the consideration of the future shortage of petroleum supplies but also for the wellbeing of the environment, as it is carbon-neutral. The European Union's Directive published in 2003 estimated that a content of 5.75% of biodiesel would be used for transportation fuels by the end of 2010 [1]. The National Biodiesel Board of the USA estimated that biodiesel industry would reach 650 million gallons annually by 2015

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and would add $24 billion to the US economy between 2005 and 2015 [2]. Although raw vegetable oils can be used directly as fuels, their high viscosity and low volatility make them inefficient for most combustion engines. Biodiesel that has a molecular weight of about one third of their parent oils in form of either methyl or ethyl monoesters of fatty acids is, on the other hand, much easier to handle as a liquid transportation fuel.

All types of vegetable oils and animal fats can be utilized for production of biodiesel. Current commercial production of biodiesel is achieved mainly through alkali-catalyzed transesterification of soybean oil. In spite of the high conversion efficiency achieved, several aspects of the process require further improvements. These include the removal of inorganic salts from the products, the recovery of glycerol, dehydration, and the treatment of alkaline wastewater [3, 4]. The use of lipase-catalyzed transesterification reactions in low-water media has been pursued recently as a promising approach to overcoming these problems [3, 5]. It was expected that the application of lipases could afford high conversion yields of oils and simplify downstream product purification at the same time [6, 7].

As reported so far, however, native or immobilized lipases require the addition of certain amount of water to ensure enzyme activities that are only moderate in most cases. Several recently reported works have examined the influence of water on the transesterification activities of lipases. According to these works, a water content of at least 0.48% (wt) is required to activate the enzymes [5, 8–10]. That is about ten times of the allowed 0.05% water content for biodiesel in the USA [11]. Therefore, additional dehydration steps will be needed for such lipase-catalyzed synthesis of biodiesel.

Anhydrous transesterification reactions are, hence, desirable as for many other synthetic purposes [12]. One way to improve enzyme activity in anhydrous organic media is to solubilize the enzymes and, thus, achieving homogenous reactions. PEG-modified enzymes are probably the most studied enzymes for organic-phase homogeneous reactions [13, 14]. However, pegylated enzymes have usually limited solubilities (mostly much less than 1 mg/ml). Distel et al. [15] reported recently that enzymes modified with short alkyl chains could achieve very high organic solubilities (up to 44 mg/ml) and, thus, afford high enzyme activities in organic solvents. In this work, a one-pot water-free biodiesel synthesis process using alkyl-modified lipases is examined.

Materials and Methods

Materials Several commercial available lipases were kindly provided as gifts from Amano Enzyme Inc. (Nagoya, Japan). The sources and protein contents of these enzymes are tabulated in Table 1. Soybean oil, decanoyl chloride, oleic acid, ethyl oleate, and methyl oleate were obtained from Sigma-Aldrich (St Louis, MO, USA). Iso-octane, methanol, and ethanol was purchased from Burdick & Jackson (Muskegon, MI, USA). Unless specially mentioned, all other reagents used in the experiments are ACS grade.

Preparation of Solubilized Lipase The solubilized lipase AK (S-AK) was prepared according to the previously reported procedure [15]. Typically, 30 mg of lipase was dissolved in 3 ml of phosphate buffer (0.2 M, pH 10) contained in a 20-ml glass scintillation vial. A high pH value is desired, as the modification reaction releases HCl. Our experiments showed that the enzyme was stable with pH values up to 10. To start the modification reaction, 6.0 ml of chloroform containing 15% (*v*/*v*) decanoyl chloride was added, and the mixture was magnetically stirred at room temperature for 8 h. The pH value of the aqueous solution was maintained at 10.0 by adding sodium hydroxide solution

Table 1 Transesterification reaction catalyzed by different lipases.

Lipase	Source	Protein content (wt%)	Observed conversion (*v*/*v*%)	Normalized conversion (*v*/*v*%/mg protein)
AH	*Burkholderia cepacia*	39	0.5	0.26
AK	*Pseudomonas fluorescens*	53	36	13
AY	*Candida rugosa*	43	0.5	0.30
F-AP	*Rhizopus oryzae*	71	1.7	0.48
G	*Penicillium camembertii*	11	0.0	0.04
M-10	*Mucor javanicus*	94	0.3	0.06
R	*Penicillium requeforti*	6.9	0.8	2.4

The conversion was measured for reaction of 1 h with 2 ml of soybean oil and 2 ml methanol in the presence of 1 ml water and 5 mg lipase.

(10 wt%) periodically. The reaction was stopped by centrifugation. The concentration of solubilized lipase in the organic phase was determined by absorbance at 280 nm. A typical concentration of modified lipase in the organic phase was around 4 mg/ml, indicating a modification yield of 80%.

Biodiesel Synthesis Reactions The transesterification reactions were conducted in 20 ml glass scintillation vials under shaking (180 rpm) in water bath (for elevated temperatures) or air (for room temperature reactions). Soybean oil and alcohols were mixed in a molar ratio of 1:3 unless specified otherwise. Because the oil and alcohol are not miscible, iso-octane was used to generate monophase solutions. The ratio between iso-octane and alcohol–soybean oil mixture was 1:1 (*v*/*v*) if methanol was used, and 4:1 (*v*/*v*) for ethanol with a total volume of 5 ml. Both native and solubilized lipases were used with an amount equivalent to 1 mg/ml. The reaction was analyzed by monitoring changes in the concentration of product. Typically, aliquots of 1 ml sample were taken from the well-mixed reaction medium and were centrifuged at 14,000×*g* for 10 min. Five hundred microliters of supernatant was diluted four times with iso-octane and was analyzed by using gas chromatography (Shimadzu GC-17A, Kyoto, Japan) equipped with a RTX-5 column (15 m×0.35 nm×1.0 μm, Restek, MD, USA). The column temperature was kept at 200°C, whereas the injector and detector were kept at 250°C. Ethyl oleate and methyl oleate were chosen as a biodiesel standard for GC analyses. The conversion of soybean oil was calculated in this work by taking the volume ratio between the detected monoester products and the soybean oil added to the reaction.

Results and Discussions

Transesterification Reactions with Different Lipases

It has been well demonstrated that hydrolases such as lipases and proteases catalyze the reversed reactions of their hydrolytic reactions once placed in organic media because of the shift in thermodynamics of the reaction equilibria [16, 17]. Although most of the hydrolases can catalyze the esterification and transesterification reactions of a wide range of substrates in organic media, enzymes may show very different preferences of conditions for a given reaction. Although all lipases are efficient enzymes evolved over time in their biological

origins to hydrolyze lipids, they are expected to show very different efficiency when they are required to function in an artificial reaction medium. In this work, seven commercially available lipases, most of which were developed for use as detergent enzymes, were first tested for the transesterification reaction between soybean oil and methanol at the same reaction conditions (Table 1). The reaction was monitored by detecting the production of biodiesel using gas chromatography (GC). In a typical GC chromatogram using the analytical method described in this work, two product peaks were determined (separated by approximately 2 min) with the larger peak matching that of the methyl oleate standard. The smaller was believed to be corresponding to the monoester product of lighter lipid acid components, as the refined soybean oil contains ~15% of C16 and ~85% of C18 lipids. Both peaks are accounted as products in the subsequent analysis of reaction rates.

Although most of the enzymes showed detectable activities, lipase AK showed the highest activity with over 35% of soybean oil converted within 1 h of reaction (Table 1). The gross amounts of enzymes instead of effective enzyme contents were controlled in these reactions to keep the phase ratio of liquid to solid of the suspension reactions (as enzymes were not soluble in the reaction media) consistent. Lipase AK was the most active enzyme even when the normalized enzyme activities based on protein content were considered. Lipase AK was then selected for further transesterification studies.

Factors that Affect the Activity of Native Lipase AK

To examine the primary factors that control the performance of lipase AK, experiments were conducted according to a factorial design with respect to four factors including temperature, water content, pH, and molar ratio of substrates, with two levels of variations. Changes in all the factors except the substrate molar ratio impacted evidently the production yield of the biodiesel. These sensitive factors were then examined further in the following tests.

Temperature may impact the reaction rate through two different mechanisms. On one hand, it promotes faster reactions through Arrhenius relationship; on the other hand, it may denature and inactivate the enzyme and, thus, significantly slow down the reaction. The effect of temperature on the activity of lipase AK was further examined in the presence of 2% (*v*/*v*) water (Fig. 1). For the reaction with ethanol, the temperature impacted the reaction

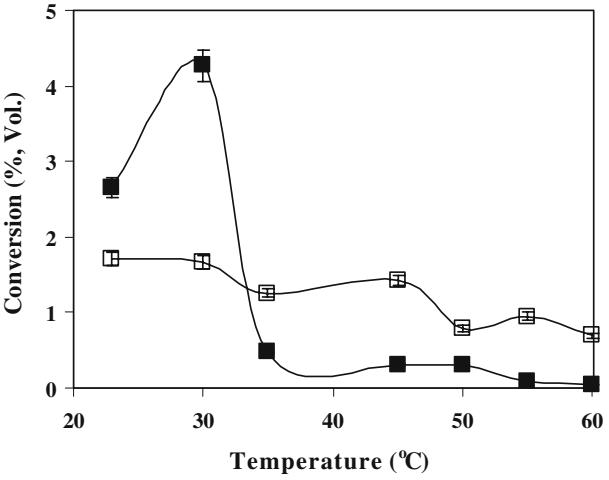

Fig. 1 Effect of temperature on the activity of lipase AK. Reaction time was 1 h in the presence of 2% (*v*/*v*) pH 7.0 aqueous buffer. *Open square* Methanol and *filled square* ethanol

as expected. The reaction rate increased first with increase in temperature, indicating the effect of thermal reaction kinetics, but it dropped sharply after reaching a peak activity at 30°C, implying significant enzyme inactivation at temperatures above that. Interestingly, the activity of the enzyme toward the transesterification reaction with methanol did not change much within the temperature range tested. The mechanism for that was not clear to the authors, although we tend to believe that the effect of substrate inhibition in the case with methanol is more prominent than any other factors, as indicated by its low reaction rates. As to be shown in the following, the transesterification reaction with ethanol also showed greater sensitivities than that with methanol in responding to changes in pH and water content. Overall, these observations indicate that ethanol is a much more efficient substrate than methanol for the enzymatic production of biodiesel with lipase AK.

The effect of pH is shown in Fig. 2. It appeared that the reaction preferred alkaline conditions, and higher conversions were observed when the pH value reached above 7. The effect of water content was then examined with pH fixed at 7.0 (Fig. 3). No product was detected within the reaction time with either ethanol or methanol without addition of water (aqueous buffer) to the reaction media. The reaction rate with ethanol increased sharply with increase in water content and reached a peak high value of 57% at the water content of 10% (*v*/*v*). Subsequent increase in water content did not help to accelerate the reaction, and the conversion at water content of 20% was slightly lower than that at 10%, indicating an adverse effect of excess amount of water to the transesterification reaction. A similar trend was observed for the reaction with methanol, except that the reaction rate did not change as dramatically as that with ethanol. The highest enzyme activity was observed at water content of 5% (*v*/*v*) for methanol, the same optimal water content as reported in a study of methanolysis activities of different lipases for synthesis of biodiesel from soybean oil [9].

As water is not desired in the final biodiesel product and has to be removed to satisfy the quality requirements, it is ideal to achieve the reaction without the use of any additional water. Because the soybean oil and alcohols are not miscible and can limit the achievable reaction velocity, one possibility to achieve nonaqueous synthesis of biodiesel is to dissolve both the oil and alcohol into one common organic solvent to improve their availability for reaction. Accordingly, we further tested the use of organic solvents for lipase AK. Solvents including acetone, dichloromethane, tetrahydrofuran (THF), chloroform, hexane, and iso-

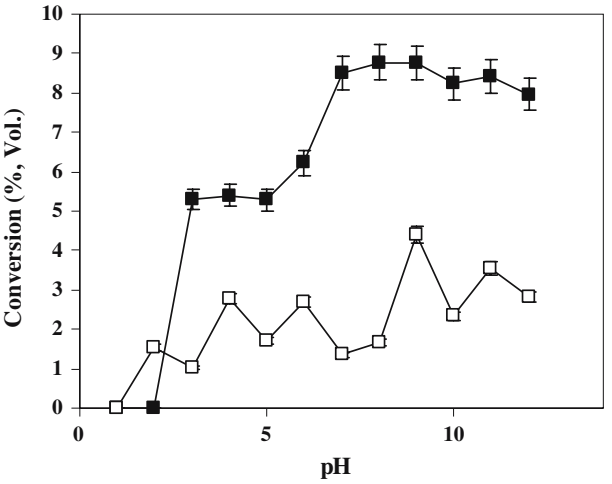

Fig. 2 Effects of pH on the activity of lipase AK. Reactions were conducted at room temperature for 1 h in the presence of 2% (*v*/*v*) aqueous buffer (with pH varied from 1 to 12). *Open square* Methanol, *filled square* ethanol

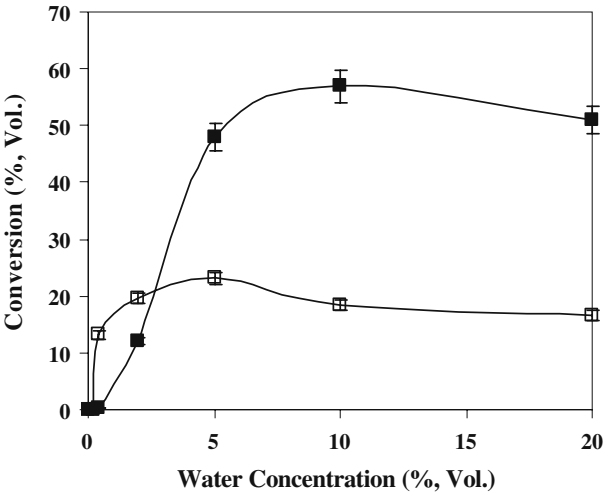

Fig. 3 Effect of water content on the activity of lipases. Reactions were performed at room temperature for 21 h. *Open square* Methanol, *filled square* ethanol

octane were tested for the transesterification reaction between soybean oil and ethanol with a volume ratio of solvent vs substrates as 3:1 with 1% of water added to ensure the activity of the enzyme. No product detected the reactions conducted with acetone, dichloromethane, THF, and chloroform, whereas reactions were observed with hexane and iso-octane over the 24-h screening tests. The results are in agreement with the expectation that solvents of higher log *P* values afford better nonaqueous activities for hydrolyases [18].

Synthesis of Biodiesel with Solubilized Lipase AK

Although the use of organic solvents can help to achieve a monophasic reaction medium, native enzymes are not soluble in the organic phase, and thus, the reaction velocity is still limited by the heterogeneous nature of the reaction. Our previous study has shown that enzymes modified with short alklane chains can significantly improve the organic compatibility of the enzymes, thus, achieving high nonaqueous solubility and activity [15]. Because alklane molecules should present no adverse contamination to biodiesel

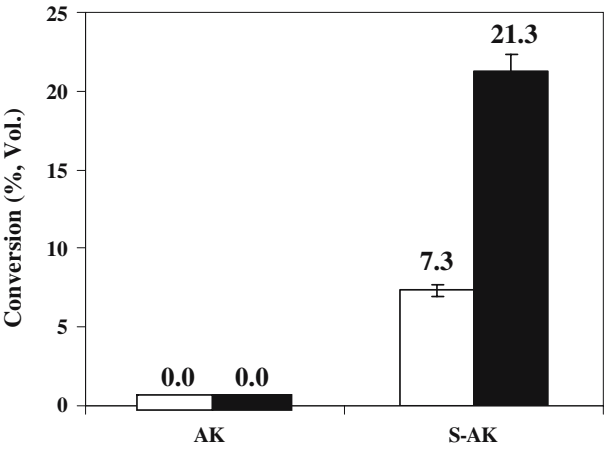

Fig. 4 Transesterification reaction catalyzed by native and solubilized lipase (S-AK) in water-free reaction media. Reactions were conducted at room temperature for 21 h. *Open square* Methanol, *filled square* ethanol

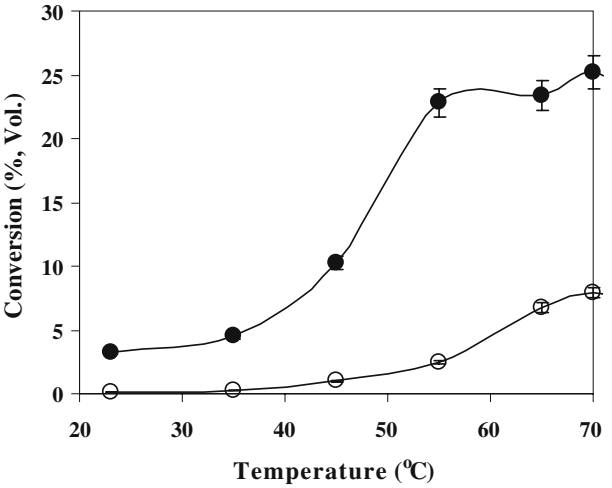

Fig. 5 Effects of temperature on the activity of solubilized lipase (S-AK) in water-free reaction media. Reactions were conducted for 1 h. *Open circle* Methanol, *filled circle* ethanol

product, such a modification for lipase AK was explored in this work. Figure 4 compares the performances of native and solubilized lipase AK (S-AK) for the transesterification of soybean oil with ethanol in iso-octane in the absence of water. Within the tested reaction time of 21 h, the native enzyme did not produce any detectable products with either methanol or ethanol. However, the solubilized enzyme catalyzed the conversion of the added soybean oil up to 7.3 and 21% with methanol and ethanol, respectively.

To further improve the reaction rate, the temperature effect on the catalysis of S-AK was also studied (Fig. 5). Interestingly, the solubilized enzyme in the homogeneous nonaqueous solution showed much better thermal stability than the native enzyme tested in the soybean oil–alcohol–water heterogeneous system (Fig. 2). The activity of S-AK generally increased with increase in temperature within the temperature range from room temperature up to 70°C. The highest conversions of the reaction were observed at 70°C with the maximum conversions of 9.8 and 25% for methanol and ethanol, respectively.

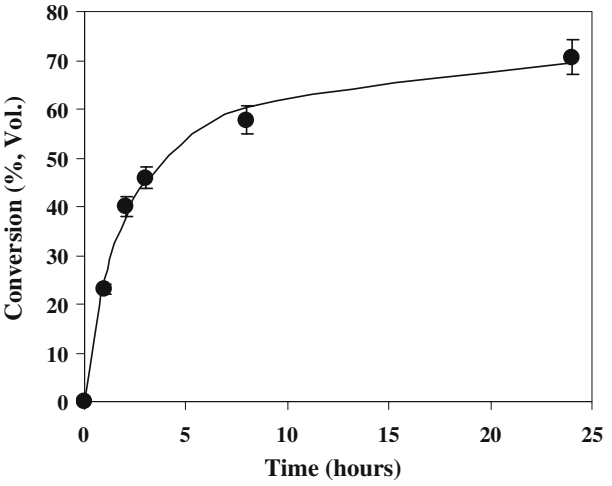

Fig. 6 Time course of transesterification reaction for solubilized lipase (S-AK) in water-free reaction media. Temperature was controlled at 70°C, with initial molar ratio of ethanol to soybean oil as 3:1

Figure 6 shows the time course of the transesterification with ethanol using S-AK at optimization condition. After 24 h of reaction, the conversion of soybean oil reached 71%. Accounting the amount of enzyme applied (1 mg/ml), the initial reaction rate was calculated as 33 g-product/g-enzyme h, which is much higher than those reported for Novozym 435 [19] and Lipozyme TL IM (0.25 g-product /g-enzyme h) [20].

Conclusions

This work demonstrated that the solubilization of lipase significantly increased its activity for synthesis of biodiesel. This provides the potential to develop a one-pot and "mix-and-use" synthetic strategy of biodiesel, as all the components including the solubilized enzyme, substrates, and products, which remain as soluble organic matters, may not need to be removed from the final biodiesel product. The feasibility of such a concept, however, is subject to environmental and combustion evaluations. Interestingly, although the current industrial biodiesel products from chemical synthetic routes are predominantly methyl esters, it appeared in our work that ethanol was actually a much more efficient substrate than methanol for the biosynthesis of biodiesel.

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